Biophysical studies of membrane interacting peptides derived from viral and Prion proteins

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ABSTRACT

Peptide-membrane interactions can trigger different physiological mechanisms, e.g. perturbation of phospholipid bilayers by pore-formation or carpeting, cell toxicity, lysis of phospholipid vesicles and peptide internalization or aggregation. The so-called cell-penetrating peptides (CPPs) are a class of peptides that have been proven to translocate across membranes of live cells, even when complexed with large cargoes, while exhibiting low levels of cell toxicity. The translocation property is not only important for the development of new, non-invasive drug-delivery systems, but can also explain disease-related mechanisms. This thesis focuses on the properties of peptides derived from the Prion, Doppel and Influenza haemagglutinin proteins in the context of bilayer interactions with model membranes and live cells. The studies involve mainly spectroscopic techniques like fluorescence, fluorescence correlation spectroscopy (FCS), circular and linear dichroism (CD and LD), confocal fluorescence microscopy and NMR.

The N-terminal signal sequence (residues ~ 1-22) of the prion protein (PrP) is usually cleaved off during maturation, but under certain conditions it remains in place. Together with the subsequent nuclear localization-like sequence (residues 23-28 in mouse and 25-30 in cow PrP), the peptide sequence resembles a CPP. mPrPp(1-28), corresponding to the first 28 amino acids of the mouse PrP, was shown to translocate across cell membranes, but concomitantly caused cell toxicity.

The membrane perturbing effects of mPrPp(1-28) were compared to the effects of its bovine counterpart bPrPp(1-30), the strongly perturbing bee venom melittin and the non cell toxic CPP penetratin in membrane model systems comprised of large unilamellar vesicles (LUVs) with entrapped fluorescent dyes. The leakage induction potency was found to be melittin >> mPrPp(1-28) > bPrPp(1-30) >> penetratin. The potency was in most cases enhanced by interactions with partly acidic membranes. FCS confirmed that the vesicles were generally intact during this process. bPrPp(1-30) was shown by confocal fluorescence microscopy to enter live cells, with and without complexed gold particles, mainly via macropinocytosis.

The mPrPp(23-50) peptide sequence overlaps with mPrPp(1-28) by six amino acids. The shared basic sequence (KKRPKP) was
believed to encompass the driving force behind translocation, but mPrPp(23-50) was found to be unable to cross over cell membranes and had virtually no perturbing effect on membranes at all.

The Doppel protein (Dpl), being a homologue and possibly an antagonist to PrP, also possesses a signal peptide at its N-terminus, that is followed by a basic sequence. The corresponding peptide, mDplp(1-30), was hence studied in terms of bilayer perturbation and changes in secondary structure induced by membrane interactions. NMR spectroscopy showed a high propensity for α-helix formation in micelles. ²H₂O exchange experiments in bicelles implied a transmembrane configuration of the helix. Membrane perturbation studies on calcein-entrapping LUVs showed that mDplp(1-30) is almost as leakage-inducing as melittin and preliminary results indicate cell toxicity as well, thus suggesting a possible neurotoxic effect for the unprocessed Dpl. Subsequent studies of mDplp(1-30) associated with shear-deformed LUVs using LD corroborated its transmembrane orientation in the bilayer. The positioning of the mDplp(1-30) was compared to the orientation of the well-known CPP transportan, for which the induced α-helix was found to be more parallel to the bilayer surface.

The influence on secondary structure and membrane positioning of two Influenza derived fusion peptides imposed by environmental changes was investigated with polarized light spectroscopic techniques. The native role of the fusion peptide derived from the Influenza haemagglutinin protein is to promote endosomal escape of the viral material via membrane merger. The native sequence did not exhibit significant pH sensitivity with respect to membrane interactions. Its α-helix was found to be obliquely inserted (60-65° relative to the membrane normal) at both physiological and endosomal pH, while the α-helicity increased from 39% to 44% as a result of the decreased pH. The glutamic acid enriched variant exhibited a heightened pH-sensitivity by changing its insertion angle from 70° to a magic angle alignment relative the membrane normal upon a drop in pH from 7.4 to 5.0. Concomitantly, the α-helical content dramatically rose from 18% to 52% in partly anionic membranes.

This thesis will present the concept of membranes, give some background information about the three disease-related proteins from which the studied peptides are derived and discuss translocation and endosomal escape strategies in hope of shedding some light on effects induced by peptide-membrane interactions.
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Paper II


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Paper V

Elin K. Esbjörner*, Kamila Oglęcka*, Per Lincoln, Astrid Gräslund and Bengt Nordén, Membrane binding of pH-sensitive Influenza fusion peptides. Positioning, configuration and induced leakage in lipid vesicles models, (accepted for publication in Biochemistry in September 2007; *both authors contributed equally to this work).

Paper VI

Kamila Oglęcka*, Elin Esbjörner*, Per Linclon, Bengt Nordén and Astrid Gräslund, Linear dichroism studies of geometry and positioning of two α-helical peptides in large unilamellar phospholipid vesicles, (manuscript; *both authors contributed equally to this work).
ABBREVIATIONS

3MI  3-methyindole
ANTS  8-aminonaphtalene-1,3,6-trisulphonic acid
BBB  Blood Brain Barrier
bPrPp(1-30)  peptide corresponding to the N-terminus of the bovine PrP, residues 1-30
CD  Circular Dichroism
CHO  Chinese hamster ovary
CNS  Central Nervous System
COSY  Correlation Spectroscopy
CPP  Cell-Penetrating Peptide
CSF  Cerebrospinal Fluid
DHPC  1,2-dihexanoyl-sn-glycero-3-phosphatidylcholine
DHPC  1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DPH  1,6-diphenylhexa-1,3,5-triene, a membrane bound fluorescence probe
Dpi  Downstream of Prion protein-Like protein (Doppel)
DPPC  1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPX  p-xylene-bis-pyridinium bromide
DRM  Detergent-Resistant Microdomains
ER  Endoplasmic Reticulum
FID  Free Induction Decay
FCS  Fluorescence Correlation Spectroscopy
FRET  Fluorescence Resonance Energy Transfer
GPI  Glycosylphosphatidylinositol
HA  Haemagglutinin
HeLa  Henrietta Lacks
hRBCs  Human Red Blood Cells
LD  Linear Dichroism
LUVs  Large Unilamellar Vesicles
mDplp(1-30)  peptide corresponding to the N-terminus of the mouse Dpl, residues 1-30
mPrPp(1-28)  peptide corresponding to the N-terminus of the mouse PrP, residues 1-28
mPrPp(23-50)  peptide corresponding to the N-terminus of the mouse PrP, residues 23-50
MTD  Membrane Transduction Domain
MTS  Membrane Translocating Sequence
NA  Neuroamidase
NLS  Nuclear Localization Sequence
NMR  Nuclear Magnetic Resonance
NOESY  Nuclear Overhauser Effect Spectroscopy
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol
pAntp  Antennapedia homeodomain-derived peptide (penetratin)
PDB  Protein Data Base
PrP  Prion Protein
PrPc  cellular isoform of PrP
PrPSc  scrapie isoform of PrP
Rh  Rhodamine
REVs  Rh-Entrapping LUVs
RLVs  Rh-Labeled LUVs
SDS  Sodium Dodecylsulphate
SM  Sphingomyelin
SRCD  Synchrotron Radiation Circular Dichroism
TMD  Transmembrane Domain
TOCSY  Total Correlation Spectroscopy
TSE  Transmissible Spongiform Encephalopathy (Prion disease)
UV/Vis  Ultraviolet/Visible
**INTRODUCTION**

**Phospholipid Membranes**

Most biological membranes contain lipids as major constituents. Lipids all have polar headgroups and in most cases, two hydrocarbon tails. A double tail yields a cylindrical molecule that can easily be packed in parallel to form extended sheets of bilayers. *Glycerophospholipids* are the major class of naturally occurring phospholipids and lipids with phosphate-containing headgroups (shown in figure 1) can all be considered to be derivatives of glycerol-3-phosphate.

The second carbon in glycerol-3-phosphate is a chiral (asymmetric) centre and naturally occurring glycerol-phospholipids are derivatives of the L-enantiomer. Usually the $R_1$ and $R_2$ substituents are acyl chains derived from fatty acids. One of them is often saturated, while the other is not. The $R_3$ group varies greatly and is hence responsible for creating the largest variation in properties among the glycerophospholipids [1].

Another class of membrane lipids is built on the long-chain amino alcohol sphingosine. When a fatty acid is linked via an amide bond to the $\text{–NH}_2$ group to sphingosine, the class of *sphingolipids* (referred to as *ceramides*) is obtained. Further modification by addition of groups to the hydroxyls, creates a variety of other membrane lipids. Among these, *sphingomyelin* (SM) is an important example. One hydroxyl group of SM is phosphorylated and a cationic group – *choline* (top of figure 1) is attached.

Some lipids built on sphingosine contain carbohydrates and belong to the group *glycosphingolipids*, including molecules such as *cerebrocides* and *gangliosides*. Generally, lipids containing carbohydrate groups go under the name of *glycolipids*. *Glycoproteins* and glycolipids in the outer leaflet of a plasma membrane contribute, via their oligosaccharide chains, to the identification of cells [1].

![Figure 1. POPC (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), an example of a glycerophospholipid.](image)
Cholesterol (figure 2A) is the most abundant steroid in the body. It is weakly amphipathic due to the hydroxyl group attached at the end of the molecule and resides in the hydrophobic interiors of membranes (figure 2B).

Cholesterol is quite bulky due to its three cyclohexane rings that are all in the chair stereochemical conformation [2]. The rigidity of the molecule tends to disrupt membrane regularity, but cholesterol also has a specific and complex effect on membrane fluidity. It does not influence the transition temperature of the membrane (going from a solid gel state to a liquid crystalline state), but rather broadens the transition interval of the membrane. This is most likely due to the fact that cholesterol has the ability to both stiffen the membrane above the transition temperature and inhibit the structure regularity below the transition temperature. There is evidence that variations in cholesterol content are used to regulate membrane behaviour in some organisms [1].

Figure 2. A) Structure of cholesterol. B) Cholesterol in a phospholipid bilayer.

Altogether, membranes of mammalian cells are comprised of more than 2000 species of lipid molecules, including sphingolipids and sterols [3]. Since 1972 up until a few years ago, the lipid bilayer of eukaryotic plasma membranes had been regarded as a two-dimensional "fluid mosaic" [4]. In the liquid-crystal state of this fluid mosaic, packing was thought to be loose and lateral diffusion relatively rapid [5]. This implied that all plasma membrane proteins should be subjected to random mixing and thus be uniformly dispersed in the liquid-crystal solvent. However, for several years evidence has accumulated for the existence of more than one lipid bilayer phase, namely a liquid-ordered (L_0) and a liquid-disordered (L_d) phase [6]. This presents an alternative to random mixing as postulated by the fluid mosaic-model. In addition to lateral movement, the “flip-flop” mechanism causes lipids to switch sides from one leaflet to the other. This motion is however much slower than lateral diffusion. Integral membrane proteins on the other hand, almost never flip in the plasma membrane [1].

Sphingolipids (containing mostly saturated aliphatic hydrocarbon chains), phospholipids with relatively long, saturated acyl chains and cholesterol form the L_0-phase. The bulk phospholipids on the other hand, composed of glycerophospholipids with polyunsaturated fatty acids, form the L_d-phase. The difference in phase propensity is thought to drive the lateral separation of L_0 and L_d in the plane of the membrane [7].
Cholesterol plays a key role in supporting and enhancing the tendency of saturated phospholipids and sphingolipids to segregate from unsaturated phospholipids in membranes [8] by forming lipid rafts.

Rafts are highly dynamic membrane domains whose concept is still under debate. The term "lipid raft" [9] describes specialized, detergent-resistant microdomains (DRM), isolated by their insolubility in non-ionic detergents at 4 °C, that are enriched in cholesterol, sphingomyelin and glycolipids [10]. The bilayer asymmetry, determined by the unique composition of the outer leaflet in the lipid rafts which leads to an increased intrinsic bilayer curvature, is believed to be the basis of processes such as budding, fission, plasma membrane invagination, etc. [11]. Studies indicate that raft microdomains are the sites of budding of several viruses, such as the Influenza virus [12] and together with actin filaments, raft-rich areas can form thread-like filopodial projections, called cytonemes [13].

Presumably, rafts have the ability to move laterally on cell surfaces and may coalesce into large structures called super rafts, that can become 500 nm wide [13]. This fusion process enables new protein-protein interactions between proteins originally located on separate rafts [7]. The most common protein-raft associations take place via transmembrane domains (TMDs) or hydrophobic tails (such as GPI-anchors, N-myristolation or S-palmitoylation).

Glycosylphosphatidylinositol (GPI)-anchoring directs proteins to bind at the cell surface or at the lumen side (internal space) of the Endoplasmic Reticulum (ER). Acylation, protein-protein and protein-lipid interactions on the other hand, allow cytoplasmic proteins to interact with the cell membrane from the cytoplasmic side [7].

In studies regarding peptide-membrane interactions, either live cells or membrane mimetic systems in the form of micelles, bicelles and Large Unilamellar Vesicles (LUVs) (figure 3) were used. Micelles and bicelles are useful in NMR experiments since LUVs cause broadening of the spectral lines due to their slow diffusion in solution. There are however many experimental advantages with LUVs in other techniques. Not only can the chosen composition of lipids reflect different properties (such as surface charge and membrane rigidity), but the chemical environment of the LUVs’ interior and exterior can be varied. Peptides do not experience the same sharp curvature when interacting with LUV surfaces, as they do when in contact with micelles. The interaction thus results in more natural binding configurations whose details may reflect the governing properties behind ongoing peptide-membrane interactions in vivo.

Bicelles are coin-shaped structures that are smaller than LUVs, but still provide the peptides with a flat surface for interaction. The size and shape of a bicle is largely determined by the ratio of short acylchained detergents and long acylchained phospholipids. The ratio is given as a q-value for a given bicle. The detergents are believed to place themselves
predominantly at the circumference while the phospholipids arrange themselves in a bilayer. The bicelles used in our NMR experiments were composed of deuterated DMPC and DHPC at a q-value of 0.25.

During the course of experiments described in this thesis, LUVs were formed by the hydration of dried lipid films of desired compositions. After extensive vortexing, freezing and toughing, the suspensions were pushed through a membrane of fixed pore size (100 nm, 21 times) using an extruder. In some experiments the LUVs were filled with either calcein, rhodamine or ANTS/DPX to study leakage induced by membrane perturbing peptides. Also, membrane probes such as DPH and retinoic acid were used to observe changes in membrane anizotropy upon peptide binding.

Figure 3. Schematic cross-sections of a LUV, a micelle, a bicelle and a phospholipid bilayer. A micelle's diameter is the same as the thickness of the bilayer, while LUVs usually are ~ 100 nm in diameter and the disc shaped bicelles ~ 40 nm, depending on the ratio between detergents and lipids.
The Prion Protein (PrP)

Transmissible Spongiform Encephalopathies (TSEs), also known as Prion diseases, are fatal disorders that can appear in most mammals causing a spongiform degeneration of the central nervous system (CNS). These neurodegenerative disorders can be hereditary, caused by infection or appear sporadically. The cause of the disease is believed to be a conformational change of the cellular, benign form of the Prion protein (PrP\textsuperscript{C}) into the, infectious scrapie isoform (PrP\textsuperscript{Sc}) that may be neurotoxic [14, 15].

The theory stating that PrP\textsuperscript{Sc} might be the only agent responsible for the TSE infectivity is supported by evidence from studies on transgenic knockout mice that are totally resistant to the disease when lacking PrP [16]. Furthermore, the amounts of PrP mRNA transcripts in the brain do not rise as disease progresses [17]. As yet, researchers have however not been able to convincingly prove the “protein-only” theory and induce a transmissible neurological disease with PrP\textsuperscript{Sc} generated by in-vitro conversion [18].

PrP\textsuperscript{Sc} is resistant to protein K digestion and forms insoluble aggregates, whereas PrP\textsuperscript{C} is monomeric and highly susceptible to proteolytic digestion [19, 20]. The PrP\textsuperscript{C} adopts a predominantly \(\alpha\)-helical conformation. It has been shown by x-ray crystallography and NMR to have three \(\alpha\)-helices at the C-terminal part (two of which are interlinked by a disulphide bridge) and an unstructured N-terminus up to residue 120 [21, 22].

The structure of PrP\textsuperscript{Sc}, having a high \(\beta\)-sheet content (> 30%), has not yet been solved due to its innate amyloid nature and strong tendency for self-aggregation and fibril formation [23]. However, crystallographic studies conducted on the human PrP dimer, showed the formation of a two-stranded, antiparallel \(\beta\)-sheet formed out of the second helix in both subunits, suggesting an initial step for oligomerization [21] (figure 4).

**Figure 4.** Top) The bovine PrP determined by NMR [24] (PDB structure 1dx0) (N-terminus not shown). Bottom) Hypothetical structure of PrP\textsuperscript{Sc} monomer. Illustration reprinted with permission from Dr. Cohen.

Once PrP\textsuperscript{Sc} is formed, it appears to accumulate in late endosomes, lysosomes and on the cell surface [25-27]. When found in extracellular spaces, it forms amorphous deposits, diffuse fibrils or dense amyloid plaques [28]. While PrP\textsuperscript{C} is harmless to neurons, the extracellular PrP\textsuperscript{Sc} aggregates are neurotoxic [29].
Neither the mechanism behind the neurotoxicity of PrP\textsuperscript{Sc}, nor the cellular role of the native PrP\textsuperscript{C} is yet fully understood. However, for the native protein several roles have been proposed involving cell adhesion [30, 31], signal transduction [32, 33], copper metabolism [34-37], neuron-protection [38-43], neurotransmitter metabolism [44-48] and antioxidant activity [35, 49-52].

PrP is encoded by \textit{PRNP} – a small single-copy gene with three exons located on chromosome 20. It is expressed at highest levels on the surfaces of neurons [17] and glia cells of the CNS [14, 53, 54], possibly residing presynaptically [55].

The PrP\textsuperscript{C} is synthesized as a ~253 amino acid polypeptide chain (depending on source) from which the first ~22 amino acids (the signal peptide) are cleaved off shortly after translation commences. At the C-terminus, we find another signal peptide corresponding to the last ~22 residues. The role of the N-terminal signal peptide is to direct PrP to the ER where simple N-linked oligosaccharides and a C-terminal GPI-anchor (residue ~230) are added during posttranslational processing [56].

Most of the PrP then travels through the Golgi apparatus (where further oligosaccharide modifications take place) and finally ends up at the outer leaflet of the cell membrane. Here, it is held in place by the GPI-anchor [57], which is also believed to attach the protein to the ER at an early step of PrP\textsuperscript{C} formation. Like other GPI-anchored proteins, PrP\textsuperscript{C} is predominantly attached to rafts [25, 58-60].

PrP\textsuperscript{C} can adopt multiple membrane topologies [61-65] (figure 5). Besides the GPI-anchored forms of PrP\textsuperscript{C}, a soluble, anchorless variant has been found in the ER. Two different transmembrane forms CtmPrP and NtmPrP (with either the C- or the N-terminus at the lumen side) are first associated with the ER membrane and then become transported to the cell surface. The CtmPrP form has been postulated to cause neurodegeneration since it has been found in damaged mice brains where PrP\textsuperscript{Sc} was absent [62]. The two transmembrane forms span the lipid bilayer via a common transmembrane segment composed of residues 113-135 [66, 67] (figure 7).

It is still unclear how GPI-anchoring correlates to infectivity. A anchorless, soluble form of PrP shows higher propensity for PrP\textsuperscript{Sc} conversion \textit{in vitro} than does the GPI-anchored form [68], but at the same time the anchor seems vital for the development of a symptomatic disease [69].

In order for the infective agent (presumably PrP\textsuperscript{Sc}) to cause neuronal degeneration it must first reach the CNS and the brain. This requires a passage through the blood brain barrier (BBB) and perhaps also through the epithelial cells separating the digestive tracts from the blood stream.
Figure 5. Bovine PrP (PDB code 1dwy) attached to a schematic, copper binding N-terminus. Two of the PrP forms span the ER membrane with either the N- or C-terminus facing the cytosolic side. Some forms lack the N-terminal signal sequence, while some retain it. Not all forms are attached to the membrane via the C-terminal GPI-anchor.

A study using radioactively labelled, highly purified murine PrP\textsuperscript{Sc}, explored the possibility of PrP passing through the BBB directly [70]. A rapid uptake of PrP\textsuperscript{Sc} from the vascular space into both brain and cerebrospinal fluid (CSF) was found. Uptake rates were at least 10 times faster than those of other toxic glycoproteins [71-73] and even that of morphine [74]. Therefore, PrP\textsuperscript{Sc} is well within the range of uptake rates measured for substances known to affect the CNS. In comparison, albumin was not taken up by the brain during the course of experiments, showing that PrP\textsuperscript{Sc} did not disrupt the BBB [74].

Experiments involving model membranes show that PrP\textsuperscript{C} and PrP\textsuperscript{Sc} have different binding affinities to lipid bilayers and exhibit different pH-dependent binding behaviour [20, 75]. The binding of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} to negatively charged lipid membranes result in increased $\beta$-sheet content, which destabilizes the membrane and leads to amorphous aggregates of PrP. Binding of PrP\textsuperscript{C} to raft membranes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol and sphingomyelin, increases its $\alpha$-helical structure, which does not destabilize the lipid membrane or lead to protein aggregation. Binding of PrP\textsuperscript{Sc} to rafts has been suggested to partially unfold the protein and induce fibrillization, but without destabilizing the lipid membrane either [20, 75].
The Doppel Protein (Dpl)

16 kb downstream of PRNP, a gene (PRND) coding for a PrP homologue called the Doppel protein (German for "double") or Dpl (downstream Prion protein-like protein) is found. Dpl was the first PrP homologue to be described in mammals and it is controlled by the PrP promoter [76]. Just like PrP it contains a GPI-anchor and three α-helices that are similar to those of PrP (compare figures 4 and 6).

The function of neither Dpl nor PrP is well understood, but the two proteins seem to possess an antagonistic effect (possibly through competitive ligand binding) [77]. Doppel has the ability to bind copper ions in a selective manner in vitro [33] and displays ~25% primary sequence identity with the C-terminal two-thirds of PrP, besides exhibiting a similar topography (figure 7).

The Dpl locus produces two major transcripts of 1.7 and 2.7 kb, as well as unusual chimeric transcripts generated by intergenic splicing with PRNP. Like PrP, Dpl mRNA is expressed during embryogenesis, but in contrast to PrP, it is expressed at low levels in the adult CNS and at high levels in the testis. Absence of Dpl has been shown to cause sterility in male mice [78].

Dpl is upregulated in the CNS in Prnp<sup>0/0</sup> lines of transgenic mice that develop late-onset ataxia (symptom of incoordination due to neuronal damage) and Purkinje cell degeneration. The Prnp<sup>0/0</sup> lines that do not develop ataxia show no signs of upregulation. These findings suggest that Dpl may provoke neurodegeneration in PrP-deficient mice and could also explain why some lines of Prnp<sup>0/0</sup> mice develop cerebral dysfunctions and Purkinje cell death, while others do not [79].

Dpl seems therefore to be neurotoxic, causing apoptotic death in cerebral neurons of transgenic mice, whereas PrP<sup>C</sup> plays a neuroprotective role [41, 77, 80]. Deletion of large portions of the N-terminus of PrP (PrPΔ32-121) makes the protein resemble Dpl and this truncated form of PrP induces the same pro-apoptotic mechanism in transgenic mice as Dpl [77]. Even though PrP and Dpl have so far not been proven to interact directly [81], their reaction pathways may very well be interlinked. The truncated form of PrP (122-235 or 135-235) is a likely candidate for being a Dpl competitor [33].
Overexpression of PrP has been shown to abrogate ataxia correlated with Dpl-expression in the brain [82], but it has also been shown that Dpl has no influence on TSE induced in transgenic mice [83, 84]. Therefore, the functional relationship between the two proteins seems very complex.

In spite of the homology between PrP and Dpl, the latter is not believed to have the ability to convert into a protease-resistant form analogous to PrP$^{\text{Sc}}$. Neither is it believed to support the Prion replication mechanism [84, 85], even when one of the Dpl disulfide bridges has been removed [81].
The Haemagglutinin Protein (HA)

The name haemagglutinin, (HA) (or hemagglutinin), is derived from this protein's ability to agglutinate (glue together) erythrocytes in vitro. It is a glycoprotein found on the surface of the Influenza virus, as well as on other viruses and even bacteria. Its role is to bind to the N-acetylneuraminic acid (generically called sialic acid) of the target host cell's receptors. After binding, the virus particle becomes incorporated into the host cell via receptor-mediated endocytosis. When the pH inside the endosome drops to ~ 5-6, haemagglutinin alters conformation and becomes a membrane fusion facilitator allowing a merger between the viral membrane and the endosomal membrane of the host cell [86]. The membrane enveloped Influenza virus thus looses its membrane during the release of viral material into the cytosol, but regains it again during the budding process that concludes its life cycle [87].

The Influenza virus belongs to the Orthomyxoviridae family which has three subtypes; A, B and C [88]. In 1892 Richard Pfeiffer isolated a bacterium, Hemophilus Influenzae, which he believed to be the cause of the flu and that later gave Influenza its name. Influenza type A is divided into subgroups which differ in their content of haemagglutinin and neuraminidase subtypes. Currently 16 haemagglutinin and 9 neuraminidase (NA) subunits are known [89] out of which H1 – H3 have the ability to infect human cells.

The haemagglutinin precursor HA0 is further divided into two parts by proteolytic cleavage; HA1 and HA2 [90]. The separation is believed to be necessary for activation of HA since it is followed by a conformational change that separates the earlier linked N-terminus of HA1 and C-terminus of HA2 by approximately 20 Å [91, 92].

Figure 8. The haemagglutinin monomer (left) is composed of a HA1 (gray) and HA2 (white) subunit. On the right, the homotrimer (PDB file 1RUZ) of the 1918 Influenza virus [93].

The C-terminal part of HA2 spans the viral membrane once anchoring the whole HA protein [94], while the HA1 subunit carries the recognition part for the sialic acid and is located entirely outside the viral membrane. Haemagglutinin forms homotrimers [95] (shown in figure 8) on the surface of the virus that are stabilized by hydrophobic interactions between the
major α-helices found in the HA2 subunit. It has been proposed that a minimum of 3-4 trimers are necessary to induce membrane fusion [96].

The so-called fusion peptide is comprised of the N-terminal part of the HA2 subunit. It is positioned inside the HA complex, ~ 30 Å above the viral membrane attachment site, at neutral pH [91]. When the pH drops to ~ 5-6, HA undergoes a conformational change that enables the fusion peptide to interact with the endosomal membrane of the host (figure 9).

**Figure 9.** The conformational change of the HA2 subunit. *Left*) The x-ray structure at pH 7.4 (PDB file 1mql) [97]. *Right*) The x-ray structure at pH 5.0 (PDB file 1htm) [98]. The α-helix formed after the conformational change is one of the longest discovered so far.
Cell-Penetrating Peptides (CPPs)

Cell-penetrating peptides (CPPs) are short, cationic (basic), water-soluble molecules with the ability to translocate through various types of cell membranes [99-109]. In contrast to many pore-creating peptides, e.g., the European honeybee toxin melittin from Apis mellifera [110], they generally have a low lytic activity.

CPPs contain segments similar to nuclear localization sequences (NLSs), which typically consist of a few consecutive lysines and arginines. The role of a NLS is to guide a protein through the nuclear pore complex into its rightful place in the nucleus. A commonly used NLS in research is PPKKKRKV. This sequence is derived from the Simian virus 40 and is the first NLS to be discovered [111]. The mode of action of a CPP differs from the one of a NLS however. The latter acts inside a cell and binds strongly to importin (which incorporates the NLS-bearing protein through the nuclear pore), while a CPP can cross a membrane without the help of a receptor. The translocation mechanisms of CPPs are not fully understood, but seemingly, chirality of the peptide is not relevant, the process is non-celltype specific and endocytosis only explains part of the mechanism for some of the known CPPs.

The field of CPP research begun in the late 1980s with the discovery of a translocative ability found in the human immunodeficiency virus type 1 (HIV-1) Tat regulatory protein [99, 100]. A few years later, the translocative ability of the homeodomain of the Drosophila Antennapedia transcription factor was discovered. It was shown that the third helix (residues 43-58) was responsible for this ability and that the corresponding peptide, named penetratin or Antp, could carry large cargoes across cell membranes [103, 112]. Over the years, a variety of cargoes (often weighing many times the mass of the CPP) have been covalently linked or complexed with CPPs and successfully incorporated into cells. Among these we find PNA, nanoparticles, liposomes, oligonucleotides, polypeptides, etc. [113-117].

More and more CPPs have been both discovered and constructed [109]. One example of a designed CPP is the chimeric sequence of transportan. It is comprised of the 12 N-terminal residues of galanin (a neuropeptide) interlinked with a lysine to mastoparan (a wasp venom from Paravespula lewisi) [105, 118]. The lysine residue replaced an original proline to make labelling and cargo attachment convenient, but turned out to make translocaction more efficient in the process. Transportan has been show to efficiently transport cargoes such as peptides, PNA and proteins into live cells [106] without exhibiting high levels of cell toxicity. The internalization
of transportan is assumed to mainly take place via endocytic pathways [119].

In the early years of CPP research, it was believed that endocytosis had been excluded as a possible route of entry since studies on fixed cells showed peptide translocation at 4°C when endocytosis is know to takes place at very slow rates. However, it was later shown that harsh cell fixation methods could generate false positive results. Before this discovery, it was believed that secondary structure and translocation ability were correlated and that an amphipathic helix was required for efficient translocation, but this model turned out to be too simplistic. Hence, the field shifted towards including the distribution of hydrophobic and hydrophilic residues of the primary sequence as well.

Amphipathicity in itself was however shown early on to be insufficient for translocation since replacement of penetratin’s two tryptophans with phenylalanines, drastically decreased the translocation efficiency [102]. Similarly, simply a helical structure was also found to be insufficient since a penetratin variant containing three prolines has been shown to cross cell membranes [103] and opioid dynorphin peptides are believed to translocate in a random coil conformation [120]. Neither is a positive charge sufficient in itself to induce translocation, since while polyarginines translocate efficiently, polymers of Lys and His do not [121]. Moreover, branched polyarginines seem less efficient than linear ones [122].

Endocytosis is today considered to be the main internalization mechanism for many CPPs, but some less understood processes still take place [123]. Most importantly, even though endocytosis can explain how peptides are able to enter cells, it does not explain how they translocate across the endosomal membranes. During endocytosis, the external environment of the cell becomes the internal environment of the formed endosome, hence leaving the peptides with the task of translocating across the exact same membrane. One immediate difference between the two environments however, is that the pH inside an endosome is known to decrease during its maturation. Hence, it is vital to investigate the peptides’ secondary structure conversions, changes in membrane interaction properties, differences in self-association etc. that may occur as a result of changes in pH or alterations of membrane compositions.
Peptides Derived from PrP, Dpl and HA

Since the signal peptide of PrP is usually cleaved off by a signal peptidase acting in the lumen of the ER, it is common to study PrP without its first ~ 22 amino acids. It has however been shown that in some cases the Prion protein retains its N-terminal signal peptide [64] and that this particular sequence is associated with functions such as topogenesis and targeting [124-126]. It has also been proposed that it could induce the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, hence making it neurotoxic [124, 127].

As this signal peptide is highly hydrophobic in itself, it cannot readily be studied in aqueous solutions. The combination of the signal sequences with the subsequent KKRPKP however, not only makes it water soluble, but also turns the new peptide construct into a possible CPP. We have therefore constructed this water soluble peptide corresponding to residues 1-30 of the bovine PrP, (abbreviated bPrP\textsubscript{p}(1-30)) and 1-28 of the mouse PrP (mPrP\textsubscript{p}(1-28), see table 1). Their hypothetical translocation ability was tested on both live cells and cell-mimetic systems. Both mPrP\textsubscript{p}(1-28) and its bovine counterpart bPrP\textsubscript{p}(1-30) turned out to be able to cross cell membranes of mammalian cells together with covalently linked cargoes, but concurrently proved to be rather cell toxic [127-129]. However, this suggests that these peptides could cross the BBB while dragging the rest of the Prion protein along as cargo, in spite of their toxic side effects.

In order to find out how important the NLS-like sequence is for translocation ability, a peptide corresponding to amino acids 23-50 of the mouse Prion protein, denoted mPrP\textsubscript{p}(23-50), was synthesized. This sequence preserves the KKRPKP part, but places it on the N-terminal side of the new peptide instead. Previous studies have shown that CPPs can be equally efficient when their linear sequences are inverted or when they are constructed out of D-enantiomers [130]. This peptide turned out to have virtually no translocation ability and its toxicity levels were lower than that of penetratin [paper IV]. We thus concluded that the NLS-like part itself cannot be responsible for the translocation capability. The apparently needed hydrophobic counterpart might not necessary have to be directly linked the KKRPKP-sequence, but could in theory be located somewhere else in the protein.

The N-terminus of the PrP homologue Dpl, constitutes a signal sequence (figure 7) out of which another signal sequence-associated peptide was constructed and investigated. The new mDpl\textsubscript{p}(1-30) peptide corresponds to the first 30 N-terminal amino acids from mouse Dpl. It does not so obviously possess an NLS-like sequence, but the net charge of the last six C-terminal residues (KARGIK) is still positive. Surprisingly, this peptide proved to be more potent than any of the other PrP-derived peptides studied so far with regards to causing leakage from calcein entrapping vesicles. In fact, it proved to be almost as potent as melittin in this respect [131]. Preliminary studies involving live cells have so far only been conducted on human red blood cells (hRBCs) (see Results and
Discussion, paper III), but we expect that toxicity levels on cultured cells will correlate well with the leakage experiments.

Curiously, the net charge of the fusion peptide derived from haemagglutinin (denoted HA2(1-23)) is -3, whereas all the other sequences mentioned so far bare a net positive charge (see table 1). Nevertheless, this fact does not seem to hinder the peptide-membrane interactions even when the membranes are negatively charged. HA2(1-23) induces substantial leakage in calcein entrapping LUVs at both pH 5.0 and 7.4, as does the glutamic acid enriched variant Inf7(1-23) (Oglęcka et al. unpublished results). The latter bears two additional negative charges and has been shown to be more pH-sensitive than HA2(1-23) [132] [paper V]. The two extra negative charges are hypothesized to stabilize an α-helical conformation upon a pH drop according to predictions based on the crystallographic data of HA2(1-23) [132].

The secondary structures of some of the peptides in table 1 have been determined either by NMR or x-ray crystallography. Although the experimental conditions varied during structure determination, a rough comparison of their secondary structures is possible by studying figure 10. It is however important to note that the fusion peptide derived from HA2, is three amino acids shorter than the one studied in this thesis.

Table 1. Comparison of some physical properties of the peptides discussed in this thesis. Aromatic residues are written in bold and charged ones in bold italic. The NLS-like sequence are underlined. * One-letter code corresponding to figure 10. † The average hydrophobicity calculated according to values devised by Kyte and Doolittle [133]. ‡ Net charge of the peptide. # Total number of residues.
Figure 10. The secondary structures of
A) Transportan (25 NMR structures, PDB code 1smz) [134],
B) bPrPp(1-30) (22 NMR structures, PDB code 1skh) [135],
C) mDplp(1-30) (22 NMR structures, PDB code 1z65) [131],
D) HA2(1-20), pH 5 (20 NMR structures, PDB code 1ibn [136],
E) HA2(1-20), pH 7.4 (20 NMR structures, PDB code 1ibo) [136],
F) Penetratin, pH 5.5 (20 NMR structures, PDB code 1omq) [137],
G) Melittin (x-ray, PDB code 2mlt) - here shown as a monomer.

An estimation of the three-dimensional shapes of the peptides lacking NMR or x-ray structures can be deduced from Circular Dichroism (CD) spectra. According to these, the mPrPp(1-28) and mPrPp(23-50) both have the ability to form mixtures of $\alpha$-helices and $\beta$-sheets when salt and peptide concentrations are favourable (Ogłecka et al., unpublished observations). They also show sensitivity towards prolonged exposure to room temperature with aggregation as a consequence. However, with freshly made solutions, mPrPp(23-50) does not show any significant structure induction in the presence of 20% negatively charged LUVs, while mPrPp(1-28) undergoes a conversion from random coil to a mixture of $\alpha$ and $\beta$ structure [paper IV].

In the case of HA2(1-23) and Inf7(1-23), the CD spectra show a moderate to high degree of $\alpha$-helix induction in the presence of both neutral and 20% negatively charged LUVs, at pH 7.4 as well as pH 5.0 [paper V]. We have attempts to resolve NMR structures in bicelles for these
fusion peptides have been made, but proved difficult due to signal broadening.

The compiled experimental data suggests a general predisposition towards α-helix formation for the majority of the peptides in table 1, although their functions differ in the context of membrane interactions. Membrane perturbation potency and translocation efficiency can therefore not entirely depend on secondary structure.

The distribution of hydrophobic and hydrophilic residues of all nine peptides is shown in figure 11. The two different views present two conformational extremes, showing primary and secondary amphipathicities.
Figure 11. Top) Helical wheel projections showing the distribution of hydrophobic and hydrophilic residues. Bottom) Primary sequences showing the corresponding distribution.
Internalization Mechanisms

As mentioned earlier, translocation of a CPP across a lipid bilayer is receptor-independent, non cell-type specific [108] and cannot be fully explained by endocytosis because the CPP and its cargo have to escape the endosomal compartment in order to reach the cytosol. Alongside endocytosis, the most common translocation models proposed thus have to do with perturbation and thinning of the membrane bilayer itself.

In the inverted micelle model (figure 12a), a CPP is first associated to the membrane mainly through electrostatic interactions. The lipids are then believed to encapsulate the CPP in an inverted micelle [138]. Because the interactions between the hydrophilic tails of the lipids and hydrophilic environment are unfavourable, the membrane would still maintain a bilayer arrangement according to this model. A subsequent lipid rearrangement would, via the same mechanism, cause the CPP to enter the cytosolic side.

The carpet model [139-141] (figure 12b), which has previously been suggested as a mechanism of action for antimicrobial peptides, also includes an initial association of the peptide with the membrane surface. Interactions between basic residues and negative lipid headgroups cause a redistribution of the lipid packing – that is further enhanced by hydrophobic interactions – leading to membrane thinning.

Pore formation can occur in two ways according to the barrelstave and the toroidal models (figure 12c). The former describes a circular, transmembrane assembly of amphipathic helices that penetrate the lipid bilayer by creating a hydrophilic pore [142]. The pores described by the toroidal model instead place the headgroups of lipids alongside the transmembrane helices [143]. In general, it is believed that the modes of action differ for different classes of CPPs. For example, it has been shown that a heptaarginine CPP can translocate across the membranes of live, unfixed cells even when the endocytotic mechanism is shut down in an energy-independent fashion [144]. Moreover, comparison between different mechanisms is not always straightforward because translocation studies were earlier conducted on fixed cells and should thus be interpreted with care, since it has been shown that they can produce false positive results [145].
Endocytosis is a term that incorporates several processes by which the cell internalizes particles and macromolecules. Phagocytosis and pinocytosis (Greek for "cell eating" and "cell drinking", respectively) are the two major subcategories of endocytosis. Phagocytosis is a receptor-mediated mechanism used for example by the immune system to engulf pathogens after antibody recognition. Pinocytosis on the other hand, is a non-selective process that occurs in all cell types. There are four subcategories of pinocytosis: macropinocytosis, clathrin-mediated pinocytosis, caveolae-mediated pinocytosis and caveolae-independent pinocytosis [146] (figure 13). The endocytotic routes generally lead to degradation of encapsulated material via lysosomes and recycling via various pathways.

Macropinosomes are fairly large structures that range over 1-5 µM in diameter, making good candidates for being transport vesicles for CPPs and their cargoes [129]. It has been suggested that the macropinosomal domain starts taking form at rafts and that their formation is initiated by external stimuli. Initially, polymerization of actin filaments gives shape to membrane protrusions that project into the external medium. They then collapse back onto the membrane while capturing membrane bound materials and extracellular fluid.

Figure 13. A schematic cross-section of a cell membrane showing pinocytotic pathways. A) Caveolae-mediated and Clathrin-independent macropinocytosis. B) Clathrin-mediated entry via "coated pits". C) Formation of macropinosomes mediated by projection of actin filaments.
Fluorescence Spectroscopy

Photoluminescence occurs when photons are emitted by excited electrons as they relax back to their ground energy state. In the UV/Vis (ultra violet/visible) region we can see either fluorescence or phosphorescence, depending on the electron configuration of the fluorophore and the subsequent photon emission pathway. A fluorophore is a functional group containing valence electrons of low excitation energies. When a photon is absorbed by an electron, the energy needed to go from the ground state to an excited state is provided. From the excited, lowest singlet vibrational state ($S_1$), an electron can relax to the ground state ($S_0$) via fluorescence, vibrational relaxation, quenching, internal conversion or intersystem crossing. The last four examples are non-radiative processes and occur without the emission of photons. Fluorescence is defined as the spontaneous emission of a photon from the lowest excited electronic state.

An electron, in a high vibrational state of an excited electronic state (figure 15), will quickly lose energy via intermolecular collisions (i.e. vibrational relaxation) and by partitioning the excess energy to other possible modes of vibrations and rotations (internal conversion). Electronic transitions occur on the order of $\sim >10,000 \text{ cm}^{-1}$ (which corresponds to UV/VIS spectroscopy), vibrational transitions occur around $\sim 1000 \text{ cm}^{-1}$ (utilized by Infra red (IR) and Raman spectroscopy) and rotational transitions occur between $\sim 0.1 - 100 \text{ cm}^{-1}$ (observable by microwave spectroscopy).

Internal conversion takes place when an excited electron de-excites to a lower electronic state of the same spin multiplicity, e.g. $S_2 \rightarrow S_1$. Intersystem crossing on the other hand, is a transition to a lower electronic state between two different spin multiplicities, e.g. $S_1 \rightarrow T_1$ (figure 14).

Figure 14. The spin states of two electrons in different electronic states of single and triple multiplicity.
The energy levels of vibronic (electronic and vibrational) singlet (S₀, S₁, S₂) and triplet (T₁) states. The processes by which an electron can gain and loose energy are illustrated by different types of arrows.

The possible electronic transitions of n, π and σ electrons, are n→π*, n→σ*, π→π* and σ→σ*, where n designates a non-binding orbital, π and π* the π-bonding and π-anti-bonding orbitals respectively and σ and σ* the σ-bonding and σ-anti-bonding orbitals respectively (figure 16).
The different energy levels of atomic and molecular orbitals. An orbital is an area described by a wave function where a given electron can be found with the highest probability. Two constructive wave functions form a bond, while two deconstructive ones result in an antibond.

When an electron absorbs a photon of frequency $\nu$, it increases its energy by:

$$\Delta E = h\nu = \frac{h\omega}{2\pi} = h\omega$$  \hspace{1cm} (1)$$

where $h$ is Plank’s constant and $\omega$ is the angular frequency.

Compounds having lone pair (non-bonding) electrons are capable of $n\rightarrow\sigma^*$ transitions, but not many organic functional groups with this capability absorb in the UV region. For this excitation to takes place light with a wavelength between 150-250 nm is necessary. The energy required to induce a $\sigma\rightarrow\sigma^*$ transition is generally higher, having an absorption max around 125 nm. Typically these transitions are not seen in UV/Vis spectra ($\sim$ 190-700 nm). Instead, most spectroscopic techniques of organic compounds utilize the remaining two transitions ($n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$) during spectrum generation, that conveniently have their emission maxima in the UV/Vis region.

Only light with the correct energy can cause a transition from one energy level to the next. However, even if the energy is right, not much will happen if certain selection rules are broken. These rules are however not absolute, thus so-called “forbidden transitions” may still occur, but are
inefficient. $n \rightarrow \pi^*$ transitions are actually forbidden, but in reality we see them as weak signals and refer to them as weakly allowed. $\pi \rightarrow \pi^*$ transitions are allowed and therefore much more intense.

Fluorescence intensity is an arbitrary unit that depends among other things on the sensitivity of the fluorimeter. The fluorescence quantum yield $\Phi$ is a measurement of the efficiency with which absorbed light yields fluorescence. Quenching for example competes with fluorescence, thus decreasing $\Phi$. Quenching can occur via collisions (the overlapping of van der Waal radii is sufficient) that transform the excess energy into thermal energy or via FRET.

FRET (Förster Resonance Energy Transfer or Fluorescence Resonance Energy Transfer) is non-radiative process that involves long-range dipole-dipole couplings between two chromophores, the donor and the acceptor. (The term chromophore is derived from the Greek word χρώμα (chroma), meaning colour). The donor and acceptor have to be close in space (within the so-called Förster distance, typically 15-60 Å) for this process to take place and their emission and absorption spectra must overlap (figure 17).

Figure 17. Example of a donor emission spectrum overlapping with the absorption spectrum of an acceptor. The difference between the lowest energy of the absorbance and the highest energy of emission, is called a Stokes shift.

Equation 2 makes it possible to calculate inter or intra molecular distances between donors and acceptors, as well as solvent accessibility, based on the energy transferred;

$$E = \frac{R_0^6}{R_0^6 + r^6}$$  \hspace{1cm} (2)

where $r$ is the distance between the chromophores and $R_0$ is the Förster distance, i.e. the distance at which the energy transfer efficiency between the donor and acceptor is on average 50%.
In nature, we can for example find naturally occurring fluorophores amongst the amino acids; tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr) (figure 18). Tyr and Trp have a similar quantum yield [147], but although Tyr is more abundant in proteins as compared to Trp, the total fluorescence seen from a protein is usually dominated by Trp because it quenches the Tyr fluorescence via FRET. Phe can usually only be seen in proteins when both Trp and Tyr are absent.

Figure 18. Absorption and emission spectra of three fluorescent amino acids; Trp, Tyr and Phe in water at neutral pH. The figure is based on ref [148].

Polar fluorophores (for example Trp) are effected by their environment (solvent polarity and pH), which can be observed as spectral shifts. The energy of the excited state decreases as solvent polarity increases, since a polar solvent has the ability to stabilize the excited state more efficiently. Absorption spectra are not as sensitive to solvent polarity as emission spectra since stabilization of the excited states occurs after excitation. The probable reason why stabilization takes place, is that the charge separation in the fluorophore is greater in the excited state, hence resulting in a greater dipole moment [148].

In our leakage experiments often we used a fluorescein analogue called calcein as a reporter molecule (figure 19). Calcein quenches its own fluorescence in its dimeric form, but becomes monomeric when diluted and subsequently fluorescent.

Figure 19. The structure of calcein, a fluorescein analogue.
By entrapping calcein (55 or 70 mM) inside large unilamellar vesicles (LUVs) and getting rid of the external calcein by gel filtration, we observed a very low background fluorescence. The volume inside a LUV (~100 nm in diameter), as compared to the volume surrounding it, was usually in the range of 0.3% in our experimental setups. This ensured a sufficient dilution factor that would make escaped calcein dimers monomeric. By adding anti-microbial and cell-penetrating peptides to the calcein entrapping LUVs, we could directly assess the damage being done to the lipid bilayer by monitoring the increase in calcein fluorescence. The peptides’ leakage induction potencies were shown to correlate well with cell toxicity tests conducted on different cell types. Complete lysis of the vesicles was achieved by the addition of Triton-X 100 (a detergent) in order to establish the maximum fluorescence intensity.

We used the entrapped fluorescent dye/quencher pair ANTS/DPX in LUVs in the same way as calcein when studying the Influenza fusion peptides. The concentration of ANTS was 12.5 mM and that of DPX 45 mM. When leakage occurred, the pair became separated due to dilution and DPX no longer could quench the fluorescence of ANTS.

We compared the membrane perturbing potency of PrP and Dpl derived peptides with the effects caused by melittin and penetratin in LUV membrane systems. The fusiogenic peptides HA2(1-23) and Inf7(1-23) were studied the same way, but the objective was to investigate the influence of membrane charge and pH on leakage-induction efficiency.

The translocation of fluorescein-labelled PrP-derived peptides across membranes of live cells was monitored by confocal microscopy. A rough estimation of translocation efficiency could be made based on observations of the amounts of fluorescent material incorporated into the cells. The observed intensities were compensated for differences observed in the fluorescence quantum yield of fluorescein when attached to different peptides. Co-localization experiments were performed with the mentioned peptides and fluorescently-labelled endocytotic markers in order to establish the main route of cell entry.

In the case of the fusion peptides, the changes in tryptophan fluorescence were studied during their interaction with membranes in different environments. Tryptophan quenching experiments using acrylamide were conducted in order to assess the depth of the tryptophans’ position in phospholipid bilayers.

The extent of membrane polarization caused by the Influenza and PrP-derived peptides was monitored by observations of the changes in fluorescence intensity of the membrane-incorporated polarization probe DPH (1,6-diphenyl-1,3,5-hexatriene). By attaching a Shimadzu polarizer to the fluorescence spectrophotometer, we could determine the steady-state polarization before and after incubation with peptides by the use of the following equation:
were $P$ is the polarization, $G$ is an instrumental factor ($G = I_{HH} - I_{VH}$) and $I$ is the fluorescence emission intensity. The subscripts $h$ and $v$ stand for the horizontal and vertical plane, respectively; the first letter describing the excitation plane and the second the emission plane. Subsequently, the term $I_{VH}$ gives the intensity of emitted light, coming from the parallel plane to the vertical excitation plane.

Any increase in polarization is a sign of lowered mobility of the acyl chains in the phospholipid membrane, indicating a higher order in the bilayer and thus possibly pore formation. A decrease in polarization therefore equals increased membrane fluidity, consistent with the carpet model and membrane thinning discussed in the previous chapter.

Polarization ($P$) and anisotropy ($r$) are commonly known to describe the same phenomenon, but from different points of view. They can easily be interconverted by the use of the following equations:

$$P = \frac{3r}{2 + r}$$ \hspace{1cm} (4)

$$r = \frac{2P}{3 - P}$$ \hspace{1cm} (5)

where $P$ is polarization and $r$ anisotropy.

According to a new study however [149], a scaling relationship is present between $P$ and $r$, hence after normalization they are directly comparable. Anisotropy takes into consideration all degrees of rotational freedom in a system and is therefore viewed as a more accurate representation of the underlying physical processes in question. We however used polarization in most of our experiments out of conventional reasons.
Fluorescence Correlation Spectroscopy (FCS)

Fluorescence Correlation Spectroscopy (FCS) is based on the observations of fluorescence fluctuations. It enables counting of fluorescent molecules under observation, measurement of the relative fluorescence quantum yield of molecules in inhomogeneous solutions, determination of translational diffusion coefficients of macromolecules and also discrimination between amounts of different molecular species. The diffusion time of a molecule is directly correlated to its mass and shape. Consequently, any increase in mass of a biomolecule, e.g. as a result of interaction with a second molecule, is readily detected as an increase in the particle's diffusion time.

Observed fluorescence fluctuations are caused by the diffusion of a fluorophore through the excitation volume or by changes of the fluorescence quantum yield due to chemical reactions (figure 20). The excitation volume is tiny (in the range of femtoliters) and should contain around 10 fluorophores in optimal conditions.

Excitation is achieved by means of a focused laser beam. The excitation volume is determined by the beam’s diameter at the objective. The volume is assumed to be of prolate (ellipsoidal sphere) Gaussian shape and fits in the diamantions displayed in figure 23.

A fluorophore will emit photons as long as it moves through the laser spot. The number of emitted photons depends on the number of fluorescent molecules (concentration), their diffusion time (mass of molecule), the size of the excitation volume (instrumental parameter) and the quantum yield of the fluorophore. During the course of an experiment all emitted photons are detected during the time interval ($\delta t$). The total light intensity observed during $\delta t$ can therefore be described as a constant mean intensity $<I>$ with a fluctuating contribution $\delta I(t)$. Information regarding molecular movements and interactions is reflected in these fluctuations (figure 21).
Examination of the autocorrelation function (6), gives information about changes in fluctuations, i.e. it shows how fast the fluorophores move in and out of the excitation volume (figure 22).

\[
G(\tau) = \frac{\langle s(t) s(t+\tau) \rangle}{\langle I(t) \rangle^2} \tag{6}
\]

where \( <> \) stands for temporal average, \( t \) is the time and \( \tau \) is the absolute time separation.

If the system is controlled only by the diffusion of non-interacting molecules, the fluctuation can be characterized by the diffusion time and the average number of molecules in the volume. The amplitude of the correlation function increases as the number of molecules decreases. According to Rigler et al. [103], the normalized autocorrelation function for a molecule diffusing three-dimensionally through a volume element is defined as:

\[
G(t) = 1 + \frac{1}{N} \left( \frac{1}{1 + \frac{4Dt}{\omega_1^2}} \right) \left( \frac{1}{1 + \frac{4Dt}{\omega_2^2}} \right)^{1/2} \tag{7}
\]

where \( G(t) \) is the distribution of diffusion times, \( N \) the number of fluorophores, \( D \) the translational diffusion coefficient, \( \omega_1 \) the radius of the volume element and \( \omega_2 \) half of the length of the volume element (figure 23). It is assumed that \( \omega_1 << \omega_2 \).

FCS was used to study peptide-vesicle interactions. The peptides involved in these experiments were melittin, mPrPp(1-28), bPrPp(1-30) and penetratin. The vesicles were composed of POPC:POPG (7:3) and were either rhodamine labelled (RLVs) or rhodamine entrapping (REVs). The diffusion times of free rhodamine (Rh) and RLVs, were used as references and controls of vesicle integrity. The content of Rh inside the REVs was either 200 or 2 nM and the total peptide-to-phospholipid molar ratio varied from 0.05 to 0.10. All experiments were performed at 20°C.
In *Circular Dichroism* (CD) spectroscopy circularly polarized light is used to study chiral chromophores. "Chiral" is derived from the word Greek χειρ, meaning hand, which is why the term “handedness” is sometimes used instead of *chirality*. Chiral substances are asymmetric, *i.e.* they lack elements such as mirror planes (σ) (figure 24), inversion centres (i) or rotation-reflection axes (Sn). The two possible mirror images are referred to as the R- and S-enantiomer (*R* standing for rexus and *S* for sinister).

![Figure 24](image.png) Illustration of the effect of a mirror plane on enantiomers.

Due to the lack of symmetry in a chiral molecule, any rearrangement of its electrons will also lack a symmetry plane. This will cause the electrons to travel in helical patterns that interact differently with right and left circularly polarized light.

Linearly/plane polarized light is created by letting light pass through a polarizer that transmits electromagnetic waves with only a single plane of polarization, *i.e.* only those components of the E-vector (the electric field component) that are parallel to the axis of the polarizer, are allowed to pass through (figure 25a).

Circularly polarized light is created by means of two perpendicular, plane polarized light waves with a phase shift of ¼ *λ* (wavelength) with respect to each other. The resulting E-vector of the two interfering waves does not oscillate in amplitude, but instead traces out a helical pattern along the direction of propagation (figure 25). In other words, in plane polarized light the direction of the E-vector is constant while the magnitude varies and in circularly polarized light, the magnitude remains constant, while the direction varies (see E-vector projections at the bottom of figure 25).

The CD spectropolarimeter creates two superimposed, circularly polarized waves of equal amplitude and phase, but with opposite handedness. The resulting wave thus actually becomes plane polarized. When passing through an optically active sample, the right and left circularly polarized light become absorbed to different extents generating a CD-signal that is elliptical in shape (figure 26). Its magnitude is calculated according to (10).
Figure 25. A) The oscillating electric field component $E$ of a light wave in linearly polarized light.
B) The electric field component $E$ of a light wave in circularly polarized light. A designates the wavelength.
Absorbance: \[ A = \log\left(\frac{I_0}{I}\right) \]  

The Beer-Lambert's law: \[ A = \varepsilon \cdot l \cdot c \]  

\[ CD = (A_L - A_R) = \Delta A = (\varepsilon_L - \varepsilon_R) \cdot l \cdot c = \Delta \varepsilon \cdot l \cdot c \]

where \( I_0 \) is the original light intensity, \( I \) the intensity after the passage through the sample, \( \varepsilon \) the molar extinction coefficient in \((M^{-1}cm^{-1})\), \( \lambda \) the wavelength (nm), \( l \) the optical path length (cm), \( c \) the concentration of the sample in \((M)\) and \( \Delta \varepsilon \) the molar circular dichroism \((M^{-1}cm^{-1})\).

**Figure 26.** Left) The plane polarized \( E \)-vector, seen along the z-axis, as a result of two circularly polarized light waves with equal amplitude and phase, but different handedness. Right) The resulting \( E \)-vector of the elliptical signal, seen along the z-axis, after passage through a chiral sample. The resultant amplitude of the more strongly absorbed component (here, \( E_L \)) will be smaller than that of the less absorbed component (here, \( E_R \)). Rotation of the major axis of the ellipse by \( \alpha \), when plotted against wavelength, is referred to as optical rotatory dispersion (ORD).

**Ellipticity**, \( \theta \), is the arc tangent \((\tan^{-1})\) of the ratio between the major and minor axes (figure 27) of an ellipse.

**Figure 27.** An ellipse and its major \((a)\) and minor \((b)\) axis.
CD is related to ellipticity according to:

\[ CD = (A_L - A_R)_\lambda = \frac{4\pi \theta}{180\ln 10} = \frac{\theta}{32.982} \Rightarrow \]

\[ \theta = \frac{180\ln 10(A_L - A_R)_\lambda}{4\pi} \]  

(11)

In the biochemical sciences, CD is commonly expressed in terms of molar ellipticity, \([\theta]\) that is defined as:

\[ [\theta] = \frac{100\theta}{c \cdot l} \]  

(12)

By combining (10), (11) and (12) we obtain a relationship between the molar ellipticity and the molar extinction coefficient:

\[ [\theta] \approx 3298.2 \cdot \Delta \varepsilon \]  

(13)

[\theta] is often given as the mean residual molar ellipticity (i.e. it is given as [\theta] per chiral residue – in our case the peptide bond). The corrected [\theta] is given in (mdeg cm\(^{-2}\) dmole\(^{-1}\)).

The chiral centre of an amino acid is situated at the asymmetric \(\alpha\)-carbon. The \(n\) and \(\pi\)-electrons in the peptide bond linking two amino acids are capable of \(n\rightarrow\pi^*\) and \(\pi\rightarrow\pi^*\) transitions, respectively. Due to the difference in induced electron flow in different conformations of a peptide backbone, it is possible to discriminate between several secondary structures.

In nature, almost all amino acids are left handed. Consequently, \(\alpha\)-helices become right-handed with 3.6 residues per turn. They are stabilized by hydrogen bonds connecting the \(n\):th amino acid’s –CO group to the –NH of the \((n+4)\):th amino acid and the \(n\):th –NH group to the –CO of the \((n-4)\):th amino acid (figure 28).
In antiparallel β-sheets strong H-bonds are formed between the –NH groups and –CO groups situated on different strands, while weak H-bonds arise between the \( C_\alpha H \) and the –CO groups situated on opposite strands. If the two strands are connected, a β-turn is formed by a hydrogen bond between the \( n \):th amino acid and the \((n+3)\):rd or 4:th one.

Figure 28. The hydrogen bonds in an α-helix and the resonance structure of the peptide bond.

Parallel β-sheets form strong H-bonds that connect the –NH group of the first strand with the –CO groups on the subsequent residue (figure 29).

Figure 29. The H-bonds of antiparallel and parallel β-sheets.

Antiparallel β-sheet spectra show a characteristic negative band around 216 nm (\( \pi \rightarrow \pi^* \)) and a positive one around 196 nm (\( n \rightarrow \pi^* \)) of similar size. Random coil spectra have their minimum around 197 nm (\( n \rightarrow \pi^* \)) and look very similar to spectra of lefthanded 3_1 helices. Due to exciton couplings, the \( \pi \rightarrow \pi^* \) transition in an α-helix splits into two parts – one perpendicular and one parallel to the helix. A maximum is found \( \sim 195 \) nm due to \((\pi \rightarrow \pi^*)_\perp\) and a minimum at \( \sim 208 \) nm due to \((\pi \rightarrow \pi^*)_\parallel\) [150]. The minimum at 222 nm corresponds to a red shifted \( n \rightarrow \pi^* \) transition (figure 30).
The spectrum of the disulfide bond has a broad band between ~250-260 nm. Its stretch depends on the $\chi$ angle (~90º [151]) around of the S-S bond and it can extend beyond 300 nm. The two $n\to\sigma^*$ transitions responsible for this absorption band can complicate interpretations of CD spectra even though its extinction coefficient only ~120 M$^{-1}$cm$^{-1}$ [152].

Figure 30. Example of CD-spectra of three secondary structures measured by synchrotron radiation circular dichroism spectroscopy (SRCD). Data in the gray area < 190 nm is generally only accessible using SRCD, while spectra > 190 nm are readily acquired using conventional CD instruments. SRCD reveals more transitions and can therefore distinguish between different secondary structures with greater accuracy [153].

The aromatic residues Trp, Tyr and Phe also give rise to CD signals in the near UV region (figure 31). Their transitions will be discussed in detail within the next chapter.

Figure 31. Vacuum ultraviolet CD spectra of the models of aromatic side chain residues. Glutamyl tyrosine (Y); lysyl-phenylalanine (F); glutamyl-tryptophan (W).
Linear Dichroism Spectroscopy (LD)

Linear Dichroism (LD) is a method that can only be used on systems that are either intrinsically oriented or on ones that orient during the course of the experiment. Orientation can be achieved e.g. by stretching polymer films containing the molecules of interest or by rotating a viscous sample solution in a cylindrical quartz couette flow cell (figure 32).

When LUVs are subjected to the shear forces that arise during rotation, they become somewhat elliptical in shape [154]. By orienting along the direction of rotation, they provide us with the means necessary to distinguish between parallel and orthogonal orientations in the sample. Peptides that bind to the flattened vesicles do so at different angles, but the overall average insertion angle can be determined using LD.

In LD, linearly polarized light is used to determine the difference between the absorption parallel to and orthogonal to the main orientation direction of the system. The LD signal is thus given by:

\[ LD = A_y - A_\perp \]  

(14)

Reduced LD (LD') is a function of only the geometric arrangement of the sample. Concentration, light path length and dipole strength (\( \mu^2 \)) of the sample have all been compensated for [155]. It is calculated according to:

\[ LD' = \frac{LD}{A_{tot}} = \frac{A_y - A_\perp}{A_{tot}} \]  

(15)

where \( A_{tot} = \frac{1}{3}(A_x + A_y + A_z) \)  

(16)

\[ LD' = 3 \frac{A_y - A_\perp}{A_x + A_y + A_z} = 3 \left( \bar{\mu}^z_x - \bar{\mu}^z_y \right) \]  

(17)

When a chromophore absorbs a photon during a LD experiment, an electronic transition takes place (described on page 30). However, in this technique the direction of the transition is of great importance. This feature is described by the transition moment, which is a quantum-mechanical parameter. A specific direction is present both during absorption and
emission. There is an electric ($\mu$) and a magnetic ($m$) dipole transition moment to consider. When a transition is purely electric ($\mu \neq 0, m = 0$), the electric charge distribution is pushed along the axis of the transition moment axis. A purely magnetic transition ($\mu = 0, m \neq 0$) on the other hand, sets the charge distribution in a circular motion around the transition moment axis. Chiral molecules all have ($\mu \neq 0, m \neq 0$), which results in a helical motion of charge along the direction of the transition moment [155].

Chromophores have their transition moments directed internally, but are at the same time arranged in space themselves. An LD$^{r}$ spectrum describes an average over all chromophores and all transition moments, thus including many superimposed uncertainties. It might therefore sometimes be useful to factorize the LD$^{r}$ into an optical factor $O$ and an orientation factor $S$. The optical factor depends on the internal orientation of the transition moments inside a chromophore, whereas the orientation factor depends on the macroscopic arrangement of the sample molecules. If either $O$ or $S$ is known, the other one can be derived based on (18). The internal transition moment here lies at an angle $\alpha$ relative to the direction of the molecular orientation axis $z$ [155].

\[
LD^{r} = 3\left\{\frac{1}{2}(\cos^{2} \alpha - 1)\right\} \times \left\{\frac{1}{2}(3\left\{\cos^{2} \theta\right\} - 1)\right\} = 3O(\alpha)S(\theta) \tag{18}
\]

where $S$ is an orientation factor (or scaling factor) ranging between 0 and 1 and $\theta$ the angle between the macroscopic orientation direction and the molecular orientation. $O$ is the optical factor and $\alpha$ the angle between the molecular direction and the direction of the transition moment responsible for absorption of light at a specific wavelength.

The value $S$ for uniaxial, rodlike molecules can be calculated according to:

\[
S = \frac{1}{3}\left\{3\left\{\cos^{2} \theta\right\} - 1\right\} \tag{19}
\]

When $S = 1$, the sample is perfectly aligned along the direction of the macroscopic orientation axis. When $S = 0$, the orientation is random (i.e. the sample is isotropic) or at an inclination of $54.7^\circ$, known as the magic angle. When peptides are added to an oriented LUV solution, we predominantly detect signals from oriented aromatic residues and peptide bonds involved in $\alpha$-helical segments. These segments can be considered rodlike and thus uniaxial.

*Sreerama and Woody write “The electronic transitions in an achiral molecule have either the electric (e.g. $n \rightarrow \pi^*$ transitions) or the magnetic (e.g. $\pi \rightarrow \pi^*$ transitions) dipole transition moment equal to zero or the two kinds of transition moments are perpendicular to each other (e.g. $n \rightarrow \sigma^*$ transitions), which results in zero rotational strength” [151].
The peptide backbone predominantly absorbs light in the far UV region (< 250 nm), whereas the side chains of all aromatic amino acids absorb light in the near UV/aromatic region (250-300 nm). The latter applies to the cysteine S-S bond as well (tables 2) [155].

Table 2. The physical parameters for the optically active elements in peptides. ε values for the aromatics are from [156] and the Φ values from [147]. Cysteine has a broad band that can stretch beyond 300 nm. The absorption maximum of the peptide bond depends on secondary structure.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Trp</th>
<th>Tyr</th>
<th>Phe</th>
<th>Cys S-S</th>
<th>Peptide bond n→π*</th>
<th>Peptide bond π→π*</th>
<th>Peptide bond π→π*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε (M⁻¹cm⁻¹) at Absₘₚ</td>
<td>5600</td>
<td>1420</td>
<td>197</td>
<td>~120</td>
<td>~100</td>
<td>~7000</td>
<td>100-200</td>
</tr>
<tr>
<td>Abs λₘₚ (nm)</td>
<td>279.8</td>
<td>274.6</td>
<td>257.4</td>
<td>250-260</td>
<td>210-220</td>
<td>192-198</td>
<td>~208</td>
</tr>
<tr>
<td>Em λₘₚ (nm)</td>
<td>348</td>
<td>303</td>
<td>282</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Φ</td>
<td>0.20</td>
<td>0.21</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In a peptide bond, the transition lowest in energy occurs in the carbonyl, going from the non-bonding (n) orbital, to the π antibonding one (π*) (figure 16). The transition energy is in the order of ε ≈ 100 M⁻¹cm⁻¹ and it occurs around ~ 210-220 nm (depending mainly on the amount of hydrogen bonding to the oxygen lone pairs). The polarization of this transition lies more or less in the direction of the carbonyl [157].

The next transition in the carbonyl is π→π* (ε ≈ 7000 M⁻¹cm⁻¹) which is centred ~ 208 nm [157]. Its polarization roughly follows the direction of a line between oxygen and nitrogen. Although other amino acid side chains may absorb in the peptide-region with even stronger intensity then the π→π* peptide transition, the carbonyls are in excess which makes the other signals almost impossible to detect with CD. However, they become visible in LD measurements where the signal form Trp is particularly strong.

π→π* transitions of aromatic rings are of L and B type in the Platt notation [158]. They denote quantum states of electrons reallocating from filled orbitals to empty ones. In most cases, these states are double degenerate since the direction of momentum can be in either way. In highly symmetric aromatics however (such as triphenylene and benzene), the degeneracy is removed and Bₐ, Bₜ, Lₐ and Lₜ become distinguishable. When an electron is excited, it may take on a singlet or a triplet state depending on weather its spin is parallel or antiparallel to its ground state. This divides the L and B states further giving them superscripts of 1 or 3 respectively (e.g. 1²LA). The energy of the ground state (1A) is lower than for L, which is lower than B. A triplet state always has lower energy than a singlet and B has lower energy than L [158]. Polarization of the Lₐ and Bₐ transitions runs along the short axis of the molecule, while Lₜ and Bₜ follow the long axis [152]. Table 3 shows the optical parameters for the aromatic residues.
Table 3. The molar absorptivities of transitions occurring in aromatic amino acids. Values given for Trp are determined for 3-methyindole (3MI) [159]. The extinction coefficients for the L transitions in Trp are measured by LD in polyethylene (PE) sheets at -50ºC. The corresponding value for the 1Bb transition is estimated from an absorption spectrum for Trp in aqueous solution [Paper V]. 1La(Trp) has a characteristic feature with two peaks around 290 nm. The transition moments for Tyr and Phe are derived from [151]. The corresponding B transitions are practically degenerate in these amino acids.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Trp (3MI)</th>
<th>Tyr</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition</td>
<td>1La</td>
<td>1Lb</td>
<td>1B</td>
</tr>
<tr>
<td>Em (\lambda_{\text{max}}) (nm)</td>
<td>279</td>
<td>290.5</td>
<td>&lt;200</td>
</tr>
<tr>
<td>(\varepsilon) (M⁻¹cm⁻¹)</td>
<td>3800</td>
<td>1900</td>
<td>-</td>
</tr>
</tbody>
</table>

The \(1L_b\) and \(1L_a\) transitions in benzene are actually electrically forbidden, but when benzene is substituted (as when forming Tyr and Phe) they become weakly allowed. Forbidden and weakly allowed L transition in Tyr and Phe derive part of their energy from the allowed B transitions by vibronic mixing [150, 152]. L \(\pi\rightarrow\pi^*\) transitions are directed in the plane of the \(\pi\)-bonding system and are positioned at a \(\cong 90^\circ\) angle to each other (figure 33). Phe has a small extinction coefficient (see table 2) because of its high symmetry. It is also the least sensitive aromatic amino acid with respect to alterations of its environment. Tyr, being slightly less symmetric then Phe, has a more intense absorption (maximum \(\sim 276\) nm and a shoulder around 283 nm). A redshift up to 4 nm can be observed due to hydrogen-bonding to the \(-\text{OH}\) group. Because the B transitions of the aromatics overlap with the \(\pi\rightarrow\pi^*\) transitions of the peptide bond, the interpretation of spectra in this region can be complicated.

The complex spectral features of tryptophan, with a double peak centred at 290 nm arising from the \(1L_a\) transitions and the \(1B_b\) overlapping with the peptide transition, can become shifted due to H-bonding to a \(-\text{NH}\) group, which is usually the case when the Trp is involved in formation of a secondary structure. The \(1L_a\) band can move by as much as 12 nm depending on the environment, hence either one of the L bands may have the lower energy. What is normally observed in a peptide spectrum is \(\sim 270\) nm for \(1L_a\), \(\sim 289\) for \(1L_b\) and \(\sim 225\) nm for the \(1B_b\) transition [160] [papers V and VI].

In order to determine how certain peptides align themselves with respect to membranes, we again used LUVs as membrane mimetic systems. Peptides incapable of forming well-behaved \(\alpha\)-helices and lacking Trp, are incapable of generating nice LD spectra. The PrP-derived peptides were therefore not studied using this technique. Penetratin has been investigated earlier with LD and was therefore not investigated either [161].
The influence of a pH-drop on the membrane association mode and orientation of the α-helical fusion peptides Inf7(1-23) and HA2(1-23) in a bilayer was assessed using LD [paper V]. The impact of membranes surface charge on the inclination angles of these peptides was investigated in the same way and compared to existing NMR studies on HA2(1-20).

The membrane surface charge during the course of experiments was either 20% negatively charged or zwitterionic. Experiments were conducted at room temperature, with a total lipid concentration of 5 mM and a peptide concentration of 50 µM. The 50 mM potassium phosphate buffer, pH 7.4, contained 50% (v/w) sucrose in order to increase the viscous drag during rotation and to better match the refractive index of the quartz couette.

Transportan and mDplp(1-30) had previously been studied in bicelles and micelles using NMR [131, 134]. The implied orientations form these peptides in bilayers derived from these studies, were corroborated by the LD data. In addition, orientation geometries for the two Trps in mDplp(1-30) at the membrane interface were proposed. The LUVs used were composed of POPC:POPG (4:1) [paper VI].
Nuclear Magnetic Resonance (NMR)

*Nuclear Magnetic Resonance* (NMR) is a method used for structural and dynamic studies of molecules generally smaller than 30 kDa. It uses a strong magnetic field \((\vec{B})\) to align the *spin* component of magnetic nuclei, either with or against the external field. Spin \((I)\) is a quantum mechanical property describing an intrinsic angular momentum possessed by isotopes with nonzero spin. The value which \(I\) takes on for a given nucleus is largely determined by the number of its unpaired protons and neutrons, which are themselves fermions with spin \(\frac{1}{2}\). Isotopes with \(I = 0\) lack net spin and are therefore incapable of generating NMR-signals. The number of spin states for a nucleus is given by \((2I + 1)\).

In the case of \(^1\text{H}\) (spin \(\frac{1}{2}\)) there are two spin states (denoted \(\alpha\) and \(\beta\)) with slightly different energies. \(\beta\) has the higher energy of the two since it is oriented against the direction of \(\vec{B}\). By irradiation of the nucleus with the frequency \(\nu\) corresponding to the resonance frequency (known as the Larmor frequency), the spin increases its energy. During the relaxation process that follows, the spin rotates around the direction \(\vec{z}\) of the magnetic field, while successively loosing its magnetization in the \(xy\)-plane. The rotation induces a current in a detector coil that is displayed as a *Free Induction Decay* (FID) signal. The FID is Fourier transformed, generating the familiar features of an NMR spectrum.

The frequency with which the spin rotates depends not only on the nuclear species, but also on its local environment. Since charges in motion induce magnetism, they can either *shield* or *deshield* a certain nucleus from \(\vec{B}\). Field modulations are thus caused by the local electron distributions and neighbouring nuclei, which slightly shift the resonance frequency for particular nuclei species. This so-called *chemical shift* \((\delta)\) is small (measured in ppm) and relative; shifts in resonance frequency are compared to a reference value. The integrated peak areas provide information on the distribution of nuclei present in different environments.

Besides specific chemical shift, the NMR spectrum shows a fine structure introduced by interactions between magnetic nuclei. These so-called *spin-spin couplings*, *scalar couplings* or *J-couplings* occur between covalently linked nuclei and split peaks into *multiplets*. This phenomenon arises because nuclei “pair up” according to the Pauli principle forming pairs of spins. Since the energies of \(\alpha\) and \(\beta\) differ, the interactions (or couplings) between them and a neighbouring spin state will also differ. This causes peaks to split according to their energy levels. The multiplicity of a peak and its intrinsic intensities, depend on the number of possible states for the involved nuclei, the number of coupling spins involved and their relative distances to one and other. The most common solution-NMR techniques focus on \(^1\text{H}, ^{13}\text{C}, ^{15}\text{N}\) and \(^{31}\text{P}\) nuclei.
NMR is a far too complex and variable technique to be described in great detail here, hence only the relevant methods for this thesis will be mentioned (which all exclusively deal with $^1$H-NMR of peptides in lipid environments i.e. micelles and bicelles).

Most importantly, NMR signals can be acquired in more than one dimension which provides a means for resolving how magnetic nuclei interact in space or along covalent bonds. The most common types of 2D experiments are COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy) and NOESY (nuclear Overhauser effect spectroscopy).

In a COSY experiment, magnetization is transferred through scalar couplings. Protons that are more than three covalent bonds apart give no cross-peaks in the second dimension since the $^3J$ coupling constants are close to negligible. Consequently, only signals derived from protons separated by two or three bonds ($^2J$ and $^3J$) are visible in a COSY spectrum. The cross-peaks between HN and H$^\alpha$ protons are of special importance since they provide information on the phi torsion angle of the protein backbone based on $^3J$ coupling constants, which in turn provides secondary structure information.

The magnetization in a TOCSY experiment on the other hand, is dispersed over the complete spin system of an amino acid by successive scalar couplings. Therefore, a TOCSY spectrum has all the features of a COSY experiment, but also involves additional signals originating from the interactions of all protons involved in a particular spin system.

The NOESY experiment is crucial for the determination of the 3D structure of macromolecules such as peptide backbone arrangements since it reveals the dipolar interactions (the nuclear Overhauser effect, NOE) that occur through space instead of bonds. The intensity of the NOE is to a first approximation proportional to $1/r^6$, with $r$ being the distance between two interacting nuclei. Under normal conditions a signal is only observable if the distance is less than 5 Å. By analyzing the cross-peaks in a NOESY spectrum, a list of NOE-derived distances can be obtained that underlies the solution structure calculations.

Amide protons located on molecules that are exposed to a hydrophilic phase, exchange with protons originating from the aqueous solvent, provided that they are not involved in stable hydrogen bonds. If deuterium is present in the solvent, the $^1$H signal will disappear from the part of the molecule that is exposed to the isotope. A so-called amide proton exchange experiment can thus be used to determine which protons are accessible to solvent, and which ones are shielded by a hydrophobic environment.

In our $^1$H NMR experiments we added mDplp(1-30) to 100 mM DHPC micelles, pH 3.5 (with 10% $^2$H$_2$O for field/frequency lock stabilization), or bicelles with $q = 0.25$ composed of 25 mM DMPC and 100 mM DHPC ($10\%$ $^2$H$_2$O). Water suppression was achieved with the WATERGATE pulse.
sequence. For amide proton exchange experiments, a series of 2D TOCSY spectra were acquired. Distance constraints were calculated based on 2D NOESY spectra and used for structure calculations. All 2D experiments were conducted at 25 or 45°C.
RESULTS AND DISCUSSION

**Paper I - Membrane perturbation effects of peptides derived from the N-termini of unprocessed Prion proteins**

It had been established in 2002 that mPrPP(1-28) (derived from the mouse Prion protein) could cross membranes of neuroblastoma N2A and Bowes melanoma N1e fixed cells [127]. Not only could it translocate on its own, but also as Nα-biotinyl-mPrPP(1-28) coupled to avidin-FITC, at both 37°C and 4°C [127]. The same study showed that mPrPP(1-28) was toxic to cells, leaving only ~ 50% of the N2A cells alive after 20 h exposure to 5 µM peptide. At the time, the positive results at 4°C were considered proof of translocation via a non-endocytotic mechanism, but are now believed to be at least partly an artefact caused by cell fixation.

A continuation of this work focused on the membrane perturbing effects induced by mPrPP(1-28) using biophysical methods and was compared to the potency of its bovine counterpart bPrPP(1-30), melittin and penetratin [paper I]. LUVs composed of either POPC or POPC:POPG (7:3) with encapsulated calcein were incubated with peptides of increasing concentration and the consequent calcein release monitored by steady-state fluorescence spectroscopy. We found that the potency of mPrPP(1-28), bPrPP(1-30) and penetratin increased in the 30% negatively charged membranes, which is consistent with the fact that they all bare net positive charges and thus should interact stronger with acidic membranes (see table 1). The increase in leakage induction due to negative membrane charge correlated the strongest for penetratin (see figure 1, paper I).

Interestingly, a leakage plateau was reached for both PrP-derived peptides, both in kinetic experiments as well as in concentration-dependent ones, implying a complex membrane perturbing mechanism that can reach saturation. Even though addition of peptides has been shown to slightly quench the fluorescence of calcein (Oglięcka et al. unpublished observations) and that variations in fluorescence quantum yield of fluorescent labels depend on the peptide species, these effects do not fully compensate for the observed system saturation. The implication would instead be that the PrP-derived peptides may form aggregates, in solution or bound to membranes, that would prevent e.g. transient pore-formation to take place involving a non-aggregated peptide form.

Fluorescence correlation spectroscopy (FCS) experiments involving rhodamine entrapping LUVs (POPC:POPG (7:3)) and the four above mentioned peptides, confirmed their order of potency. FCS measurements also revealed that POPG LUVs are very prone to aggregate upon addition of peptides (melittin was not examined). Based on additions of penetratin, the extent of aggregation was peptide-concentration dependent. This effect can
be explained by the existence of strong electrostatic interactions between penetratin (with a total charge of +7) and the acidic bilayer.

The FCS experiments also showed that addition of peptide to partly acidic RLVs, caused no alternation in diffusion time at peptide-to-lipid ratios of 0.05 for melittin and 0.10 for the other three peptides. The PrP-derived peptides are at their fully bound state at this ratio and the diffusion data thus indicated that the vesicles were intact during the measurements. Addition of peptides to REVs, caused Rh leakage in a manner consistent with the calcein leakage experiments. Melittin caused approximately 80% leakage, penetratin ~ 20% and the mouse and bovine PrP-derived peptides ~ 70 and 65% respectively.

The correlation between membrane polarization and insertion mode of a given peptide into a bilayer was examined. The probe DPH (1,6-diphenylhexa-1,3,5-triene) was used in order to establish whether the lipid order in LUVs of different compositions increased or decreased upon addition of peptide. DPH is a commonly used membrane probe whose fluorescence is detectable only in its membrane bound state. Any increase in polarization is a sign of decreased mobility of the acyl chains of the phospholipids, implying formation of a structure of higher order in the bilayer. Consequently, a decrease in polarization indicates increased membrane fluidity. By the attachment of a polarizer to the spectrophotometer, the changes in fluorescence emission of vertically and horizontally polarized light could be calculated according to (4) on page 35.

Both PrP-derived peptides induced a concentration dependent increase in lipid order [figure 2, paper I]. Again, the partly acidic membranes had an enhancing effect on peptide potency in comparison to the zwitterionic ones. Penetratin showed no significant change in polarization, neither when added to 30% negatively charged membranes, nor with increasing peptide concentration. Melittin however, increased the lipid side chain order in partly acidic LUVs, but decreased it in zwitterionic ones. This implies two charge-dependent modes of action that are comparable with melittin’s different binding geometries depending experimental conditions [162-165]. These modes of action (presumably pore-formation and carpeting) do however not differ significantly with regard to perturbation potency, which corroborates the fact that melittin is a non-celltype specific venom [166].

Circular Dichroism (CD) spectra showed that the PrP-derived peptides have a propensity towards β-sheet formation in both 50 mM potassium phosphate buffer, pH 7.4, and in contact with both types of membranes. Interactions with partly acidic LUVs induced β-sheet formation in penetratin as well, while both types of LUVs resulted in the induction of an α-helical conformation in melittin.
Since bPrPp(1-30) showed similar behaviour in membrane mimetic systems as did mPrPp(1-28), its cell-penetrating ability was investigated with and without cargo. As certain cell-fixation techniques had since the mPrPp(1-28)-study been proven to potentially generate false positive results, translocation experiments were conducted on live cells [167, 168]. In spite of the differences in experimental procedures, the results of the mPrPp(1-28) studies proved to correlate well with the findings of the present study. bPrPp(1-30) was shown to translocate across membranes of CHO (Chinese hamster ovary) and HeLa (Henrietta Lacks) cells both with and without cargo. bPrPp(1-30)-DNA-gold complexes were internalized into CHO cells approximately 100 times more efficiently as compared to controls lacking the peptide. The internalized fraction was predominantly found inside vesicles, suggesting incorporation via endocytosis.

Just as in the case of mPrPp(1-28), bPrPp(1-30) itself predominantly associated with the plasma membrane and the corresponding peptide-cargo-complexes were mostly found in aggregated form on cell surfaces. However, a considerable portion of the complexes was also found in the cytosol, but nuclear localization was not observed for either peptide. Morphological distortions of the cells were observed in both studies, suggesting a similar mechanism of action underlying cell toxicity.

Dextran (an macropinocytosis marker) and transferrin (a specific marker for clathrin-mediated endocytosis) were used in co-localization studies with bPrPp(1-30) in CHO cells in order to determine whether bPrPp(1-30) internalization follows an endocytic pathway. While little co-localization was observed with transferrin, bPrPp(1-30) showed strong co-localization with dextran, indicating fluid-phase endocytosis as the major incorporation pathway. Inhibitors of specific steps of endocytosis, i.e. cytochalasin D and wortmannin, corroborated that bPrPp(1-30) utilizes macropinocytosis as a major route of cell entry.

Cationic peptides are known to interact strongly with negatively charged lipid headgroups during a membrane-association step presiding internalization. Moreover, the HIV-1 derived Tat peptide has been shown to enter cells in a cell surface proteoglycan (PG) dependent manner [169]. (PG's encompass polysulfated, highly acidic glycosaminoglycan polymers). The internalization efficiency of bPrPp(1-30) was therefore investigated with mutant CHO cells lacking PG (strain pgsA-745). Confocal microscopy images showed that the peptide was bound to the cell surface to a lower extent than when incubated with native CHO cells and that consequently much less bPrPp(1-30) had been internalized. We therefore concluded that bPrPp(1-30) enters cells through macropinocytosis in a PG-dependent manner.
Paper III: NMR solution structure of the peptide fragment 1-30, derived from mouse Doppel protein, in DHPC micelles

It has been hypothesized that since the PrP can sometimes retain its N-terminal signal sequence, this might also hold true for Dpl. The mDplp(1-30) peptide was tested for membrane perturbing activity using calcein entrapping LUVs (POPC:POPG (4:1)) in order to examine a possible correlation with Dpl’s neurotoxicity. Calcein release experiments proved mDplp(1-30) to be almost as potent as melittin. In addition, CD measurements showed that mDplp(1-30) had a very high propensity for formation of stable α-helices (see figure 1b and 1d, paper III). Since melittin is a highly α-helical peptide that presumably perturbs membranes via pore-formation in some cases, the mechanism of actions could be similar for mDplp(1-30). The positioning of the Dpl-derived peptide in lipid/detergent environments, i.e. SDS micelles, DMPC/DHPC bicelles, q = 0.25, and DHPC micelles was therefore investigated using NMR.

The structure calculations of the DHPC micelle-embedded peptide were based on sequence specific chemical shift assignments for the backbone and most of the side chains. Peptide backbone torsion angles form COSY experiments and distance constraints in the same micelles acquired from NOESY cross-peaks (mixing time 50, 150 and 300 ms), indicated a helical structure between residues 8 and 19 (figure 5, paper III). Based on secondary chemical shifts for Hα protons, a 43% helical content was estimated which is in good agreement with the CD data that yielded ~ 40% helicity under similar conditions.

H2O exchange experiments (1D TOCSY with mixing times of 30 and 80 ms) showed a solvent-protected structure between residues 7 and 19 embedded inside the DHPC micelle. This implied that the peptide would adopt a transmembrane configuration in a bilayer. A 2D TOCSY experiment (mixing time 80 ms) in bicellar medium corroborated the preferred orthogonal positioning. Only signals from the unstructured parts of the peptide (located outside the bilayer) turned out to be distinguishable. Due to immobilization of the peptide core by the slowly diffusing bicelles, the remaining signals were broaden beyond recognition.

Figure 34. Illustration of the NMR structures of mDplp(1-30) in DHPC micelles showing its two Trps (PDB code 1z65). Trp8 (left) seems better defined than Trp9 (right) for which two preferred conformations can be distinguished.
A hypothetical model of mDplp(1-30) positioned inside a DHPC micelle is shown in figure 2, paper III.

Another distinguishable feature is the positioning of Trp8 and Trp9 whose ring planes seem to be close to parallel to the transmembrane α-helix (see figure 34). Trp 9 is however much more mobile than Trp8. They are both located at the DHPC/water interface and since both Trp positions are well conserved between species, a vital role of the protein may be linked to their membrane anchoring properties. A DHPC micelle has however a much sharper curvature than a membrane, why the positioning of the tryptophans might not be entirely accurate.

The translocation ability of mDplp(1-30) has not yet been fully tested on live cells, but preliminary results obtained from haemoglobin leakage studies predict that it will prove to be more cell toxic than the PrP-derived peptides (figure 35). The neurotoxic effects of Dpl could therefore possibly be linked to the strong membrane perturbing activity of the N-terminus of the unprocessed protein.

Figure 35. Preliminary results on haemoglobin leakage induced by mDplp(1-30) and mPrPp(23-50) in hRBC.

mDplp(1-30)’s potency of inducing calcein-leakage from LUVs (POPC:POPG (4:1)) was compared to the effects of mPrPp(1-28), mPrPp(23-50) and melittin. Again, the effects of mDplp(1-30) were close to those of melittin, but the virtual lack of leakage induction caused by
mPrPp(23-50) was unexpected. Since mPrPp(1-28) had been shown earlier to be membrane perturbing, the same was expected to hold true for mPrPp(23-50) due to the fact that both contain the NLS-like sequence (KKRPKP) that was believed to harbour a major element responsible for translocation ability (see table 1). The basic residues were thought to play an important role at least during association with negatively charged lipid headgroups or proteoglycans, which could in turn mediate internalization. Questions raised concerning the NLS-like sequence were to be the focus of the subsequent study (see paper IV).

**Paper IV: Relevance of the N-terminal NLS-like sequence for membrane interactions of the Prion protein**

A series of experiments were conducted involving mPrPp(1-28) and mPrPp(23-50) in order to establish the importance of the NLS-like sequence for peptide internalization. Calcein leakage from LUVs (POPC:POPG (4:1)) and haemoglobin leakage from hRBC induced by the mPrP-derived peptides were compared to the effects of penetratin – a CPP known to give rise to low cell toxicity. Both methods revealed that mPrPp(23-50) was virtually inert (figure 2 and 3, paper IV), which correlates well with CD measurement showing that mPrPp(23-50) remains in a random coil conformation even when in the presence of phospholipid membranes, which implies lack of membrane interaction. mPrPp(1-28) on the other hand, exhibits membrane perturbing features and adopts different conformations depending on the environment (figure 4, paper IV).

Co-localization studies of the fluorescein labelled PrP-derived peptides with dextran were conducted on live HeLa cells. While the location of internalized mPrPp(1-28) correlated well with that of dextran, mPrPp(23-50) was virtually not internalized at all (see figure 36). In order to confirm that the endocytotic marker was not inducing peptide translocation through complexation, control experiments were performed in the absence of dextran. The results of the control experiment confirmed the original findings. It also became evident from confocal images that mPrPp(23-50) does not exhibit any preference for residing at the membrane surface (figure 5, paper IV).

Since membrane perturbation and leakage experiments are not evidence of cell toxicity per se, lactase dehydrogenase (LDH) release studies were performed on live HeLa cells. After 30 min of incubation with peptides at 37°C, the amount of LDH activity was assessed and compared to the total LDH activity measured upon cell lysis. Melittin and penetratin were used for comparison and the order of cell toxicity established was melittin >> mPrPp(1-28) > penetratin > mPrPp(23-50).

The fact that the mPrPp(23-50) peptide does not interact with membranes to any significant degree, could be explained by its hydrophilic nature (see table 1). The NLS-like part of the peptide sequences is most probably still an important factor in the translocation process, but
apparently a complementary hydrophobic counterpart is necessary. However, we cannot exclude the possibility that this criterion could be fulfilled by a non-adjacent sequence. In addition, a predisposition for aggregation in solution would hinder a peptide from gaining access to the membrane. As it turns out, all PrP-derived peptides discussed in this thesis show sensitivity towards heightened salt concentrations and prolonged room temperature exposure, resulting in an increase of β-structure.

**Figure 36.** Confocal microscopy images showing uptake of fluoresceinyl-labelled PrP-derived peptides and their co-localization with rhodamine-B-labelled dextran in live HeLa cells. (a) mPrP (1-28) is effectively internalized into HeLa cells, whereas (d) mPrP(23-50) shows negligible internalization. (b) and (c) show the internalization of dextran. mPrP(1-28) exhibits a substantial co-localization with dextran (overlay image c), while mPrP(23-50) did not co-localize with dextran as a result of its poor cellular uptake (overlay image f). The incubation time was 30 min at 37 °C and the final peptide concentration 2 µM.

mDplp(1-30) and mPrPp(23-50) both virtually lack secondary amphipathicity according to their helical wheel projections (see figure 11). Nevertheless they have completely different impacts on membranes, proving the importance of not solely comparing features like amphipathic helicity or sequence similarity when predicting biophysical properties of new peptides.
The fusion peptide HA2(1-23) derived from the Influenza virus (strain H3N2) and its variant Inf7(1-23) (see table 1) interact strongly with zwitterionic and partly acidic membranes although they both carry net negative charges. The variant was designed [132] with the objective to exhibit enhanced pH-sensitivity, mediated through the two additional Glu’s at positions 4 and 7. They would have a disruptive effect on helix formation at neutral pH, but after charge neutralization at pH 5.0, formation of a stable α-helix would become possible. In order to evaluate the influence of pH and membrane surface charge on positioning in a phospholipid bilayer, experiments were performed at pH 7.4 and 5.0 in LUVs composed either of POPC or POPC:POPG (4:1). The main focus was put on LD measurements, but complementary methods such as CD, quenching of tryptophan fluorescence by acrylamide and leakage induction of entrapped ANTS/DPX (a dye/quencher pair) [170] from LUVs, were also utilized.

CD measurements show that HA2(1-23) has a high propensity towards α-helix formation (~ 40%) that is fairly insensitive towards changes in solvent composition and pH (see figure 4, paper V). The Inf7(1-23) variant was according to expectations much more sensitive towards changes of the environment. In buffer solution (50 mM potassium phosphate) at pH 7.4 the overall structure induction in Inf7(1-23) was very low, while at pH 5.0 α-helix formation was observed. The highest α-helical content for the variant at pH 7.4 was observed in zwitterionic LUVs (33%) and at pH 5.0 in partly acidic LUVs (52%).

Having established that the Influenza derived fusion peptides could form α-helices (see figure 6, paper V), LD experiments could be interpreted based on the assumption that we studied the alignment of rodlike, uniaxial molecules in phospholipid bilayers. Resolved binding geometries of the peptides thus represent ensemble averages of these rodlike helixes. Estimations of the orientation factor $S$ were based on data gathered on an inner standard, i.e. the lipid order probe retinoic acid [171], which in turn made it possible to determine the LD' spectra according to equation 3, paper V.

The time-dependent membrane insertion process of the peptides was monitored via observed changes in LD signals from retinoic acid embedded in LUV membranes (figure 8, paper V). An intensity change of the probe signal indicates an overall orientational change of the sample that generally can be a result of vesicle deformation, change in vesicle alignment or a change of lipid chain order. An increased LD signal reflects a higher order of the system, hence destruction of vesicles result in signal loss. Control experiments however proved that the changes in intensity correlated with lipid chain order in our experimental setup.
The polarities of aromatic side chain and peptide bond transitions are illustrated in figure 1, paper V. As mentioned previously, the $\pi \rightarrow \pi^*$ transition becomes split into two signals due to the exciton couplings that occur in an $\alpha$-helix. The transition parallel to the helix axis is observed at $\sim 208$ nm, while the perpendicular one is undetectable in our experimental setup as it arises where the sample exhibits high absorptivity i.e. below 200 nm. The $n \rightarrow \pi^*$ transition of the peptide bond is seen at $\sim 220$-230 nm and has an orthogonal orientation to the helix axis. Transitions from Tyr and Trp can be observed in the 220-300 nm region.

Figure 6, paper V, shows the LD$^*$ spectra of both peptides at pH 7.4 and 5.0, in both neutral and 20% negatively charged LUVs. The positive spectral peaks of the $\pi \rightarrow \pi^*$ transition indicate a preferred orientation in parallel to the membrane surface. Since the intensity is however 5-10 times weaker than observed for penetratin-like peptides known to reside on bilayer surfaces, it was assumed that the peptides had an oblique (inclined) orientation, which is in agreement with NMR studies conducted on HA2(1-20) [136].

Peptide alignment in zwitterionic LUVs is in general poorer than in partly acidic ones. Also, the spectral features of the aromatics in POPC LUVs are only distinguishable in the case of Inf7(1-23), pH 7.4, indicating randomized positioning of Trp and Tyr side chains in the remaining cases.

According to calculations based on the $\pi \rightarrow \pi^*$ transitions at 210 nm, the native fusion peptide in 20% negatively charged LUVs at pH 7.4 is oriented at an inclination of 60-65º to the membrane normal – a position that was only slightly affected by a drop in pH. All three aromatics in HA2(1-23) (Trp14, Trp21, Tyr22) were resolved well enough to indicate that they should be at least partially oriented in the membrane. Their contributions to the LD spectrum were estimated via least-square analysis fitting of concentration-scaled reference spectra for the $^1L_a$(Trp), $^1L_b$(Trp) and $^1L_a$(Tyr) transitions onto the experimental spectrum.

The $^1B_b$ transition moments of HA2(1-23)'s both tryptophans were assumed to be close to the magic angle (54.7º) at pH 7.4 and around 60-65º at pH 5.0. Tyr, having its $^1L_b$ transition moment at an inclination of 35º to the membrane normal, was seemingly unaffected by changes in pH. Since the geometry of Tyr's transitions prohibit that both transitions would simultaneously generate a positive signal in our case, the $^1L_a$(Tyr) was assumed to contribute to a negative LD signal in the 220-230 nm-region.

The influence of pH and membrane surface charge had a far greater impact on the variant peptide compared to HA2(1-23). In partly acidic LUVs at pH 7.4, the inclination angle to the membrane normal was estimated to $\sim 70º$, signifying an orientation almost in parallel to the membrane surface. Signals from $^1L_a$(Trp) and $^1L_b$(Tyr) were undetectable, indicating flexibility.
of the residues. A positive band was however observed for $^1L_e$(Trp) in the aromatic region, implying an orientation parallel to the membrane surface.

At pH 5.0 the variant displayed a dramatically different insertion geometry. The LD signal intensity however decreased with a factor of four. The inclination of the peptide axis is hence estimated to be close to the magic angle, since randomization would contribute to some positive LD. However, the previously observed $^1L_e$(Trp) signal vanishes completely at pH 5.0, suggesting increased flexibility of the Trp side chains.

Both insertion modes of the variant generate high perturbing effects that can be compared to those of melittin that is known to change membrane bound configuration depending on its environment although retaining high membrane perturbation potency.

Leakage experiments conducted on LUVs entrapping ANTS/DPX (with 100 mM sucrose on the outside to match osmolarity), showed that both fusion peptides are very potent in all experimental setups (see figure 2, paper V). Again, Inf7(1-23) proved to be very pH-sensitive, particularly in zwitterionic membranes, while HA2(1-23) exhibited more uniform behaviour in all four cases. The kinetics observed in different membrane types, depending on the environment for both peptides. 100% leakage is achieved much faster in the partly acidic bilayers (note the logarithmic time scale). While the variant is more potent at pH 5.0, the native sequence surprisingly induces leakage faster at pH 7.4 in acidic LUVs. Even when the lipid ratio is lowered from 1:100 to 1:1600, the same pattern is observed. This is in accordance with the pH-sensitive design of Inf7(1-23), but not what we first expected regarding the native fusion peptide sequence. The virtual pH-insensitivity of HA2(1-23) could however be explained by its native role, since it is shielded from solvent by the rest of the protein until endosomal pH is reached, thus rendering an efficient perturbing potency at physiological pH insignificant.

Observations of Trp emission during the incorporation process of peptides into LUV membranes showed a blue-shift and a concomitant increase of emission intensity. This is in agreement with what is expected when Trp is moved to a more hydrophobic environment. The emission maxima also give an indication of how deeply buried the Trps are in the bilayer [148, 161, 172]. Based on this data, we can conclude that the Trps are membrane bound, but do not reside in a completely hydrophobic environment. The Trps of Inf7(1-23) in acidic LUVs at pH 7.4 are an exception however, as the emission maximum at 341 nm indicates a surface bound arrangement.

Figure 9, paper V, illustrates how the Trp emission generated from the two peptides changes with time in zwitterionic and partly acidic LUVs, at pH 7.4 and 5.0. The plots are not linear, suggesting at least a two-step mechanism of insertion. The first and fastest phase is completed within a few minutes, which corresponds to terminated leakage of ANTS/DPX shown
in figure 2, paper V. The following rearrangement process gradually shields the Trps from water, except in the case of Inf7(1-23) at pH 5.0 that exhibits the opposite behaviour after an initial membrane-association step.

Tryptophan quenching experiments were performed on HA2(1-23) in both types of vesicles at pH 7.4. The extent of Trp fluorescence quenching by acrylamide indicated the Trps level of exposure to aqueous solvent. As a reference, Stern-Volmer plots were obtained for the quenching efficiency of free Trp in 50 mM potassium phosphate buffer (see equation 4, paper V). Plots illustrating the solvent accessibility (figure 10 A, paper V) are linear, indicating that both Trp14 and Trp21 are equally exposed to the water-soluble quencher.

The Trps in both peptides are in general more protected at pH 5.0 than in 7.4 in both types of membrane composition (figure 10 B, paper V). This trend is again more evident for the variant than for the native sequence, reflecting its stronger pH-sensitivity. In buffer solution, both fusion peptides show a significant protection of the Trps, which implies partial peptide self-association. The trend in pH-dependence however remains the same and can be explained by the neutralization of negative charges. Protonation of the negative charges not only stabilizes an α-helix but also makes the peptides more hydrophobic, hence facilitating membrane insertion.

**Paper VI:** Linear dichroism studies of geometry and positioning of two α-helical peptides in large unilamellar phospholipid vesicles

Two α-helical peptides, transportan and mDplp(1-30) were studied with respect to their inclination angles in acidic LUV membranes. NMR experiments on bicelles and micelles have shown that transportan tends to insert into membranes in a surface-oriented manner, while mDplp(1-30) is most likely a transmembrane peptide [131, 134]. LUVs are better membrane modelling systems than micelles and bicelles but cannot easily be studied with NMR due to their slow tumbling movements that make signals broaden.

Our LD data corroborated the preceding NMR studies in POPC:POPG (4:1) LUVs, pH 7.4 (see figure 2, paper VI). The Trp signals from mDplp(1-30) were well resolved (see figure 3 paper VI) and fitting of Trp reference spectra suggested a positioning of the ring planes at the membrane interface, close to parallel with the membrane surface. In the NMR experiments however, the Trps were observed in a position placing the ring planes more in parallel to the transmembrane helix axis (see figure 34). Since these experiments were conducted on DHPC micelles, the curvature and diameter of the pseudo-membrane are different form the ones in LUV membranes. Moreover, since LD is a more sensitive technique as compared to NMR, we believe that the data acquired from the LD experiments is more likely to reflect a true biological system.
The LD\textsuperscript{r} values for the almost perpendicular transitions \(^1\text{L}_a(\text{Trp})\) and \(^1\text{L}_b(\text{Trp})\) in mDplp(1-30), were derived from anisotropy measurements conducted on the membrane probe retinoic acid incorporated in LUV membranes [171]. The derived values (-0.84 for \(^1\text{L}_a(\text{Trp})\) and +0.54 for \(^1\text{L}_b(\text{Trp})\)) correspond to angles of 32° and 72°, respectively, relative to the membrane normal and indicate that the ring planes are somewhat tilted with respect to the surface. The inclination of the \(^1\text{B}_b\) transition in mDplp(1-30) was estimated to be \(~75°\) relative the membrane normal and its contribution to the LD signal around 225 nm was assessed to be rather strong.

The LD\textsuperscript{r} value derived for this transition is >0.75 which indicates a high order in the sample around this area. This contributes to the assumption that mDplp(1-30) adopts a transmembrane configuration. The LD is zero < 210 nm where a negative peak emanating from the \(\pi\rightarrow\pi^*\) transition would be expected for a transmembrane peptide configuration. However, it is probable that the \(^1\text{B}_b\) signal masks that spectral feature. It is important to instead note the absence of a positive peak, which would have implied a surface-oriented position of mDplp(1-30). Also, since the band \(~225\ nm is strong, we conclude that the \(n\rightarrow\pi^*\) transitions must contribute to the signals intensity, which again points to a transmembrane orientation.

The aromatic side chains in transportan did not generate an equally well resolved spectra as did mDplp(1-30) (see figure 4, paper VI). Transportan has been shown by NMR to possess a flexible hinge linking its galanin and mastoparan parts together that by allowing movement, also would attenuate LD signals (PDB structure 1smz). The major peak around 225 nm is negative which implies a surface-oriented position as the \(n\rightarrow\pi^*\) transition is concerned, but since this is only a weakly allowed transition, the \(^1\text{B}_b\) and/ the \(^1\text{L}_a(\text{Tyr})\) transition must therefore contribute strongly in this region. This peak is however rather narrow in comparison to the one observed for mDplp(1-30), implying that the \(^1\text{B}_a\) transition can either be relatively weak because it is positioned close to the magic angle, or be partly cancelled out by a positive signal generated by the \(^1\text{L}_a(\text{Tyr})\) transition.
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Membrane perturbation effects of peptides derived from the N-termi

N of unprocessed prion proteins

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Abstract

Peptides derived from the unprocessed N-termini of mouse and bovine prion proteins (mPrP and bPrP, respectively), comprising hydrophobic signal sequences followed by charged domains (KKRPKIP), function as cell-penetrating peptides (CPPs) with live cells, concomitantly causing toxicity. Using steady-state fluorescence techniques, including calcein leakage and polarization of a membrane probe (diphenylhexatriene, DPH), as well as circular dichroism, we studied the membrane interactions of the peptides with large unilamellar phospholipid vesicles (LUVs), generally with a 30% negative surface charged density, comparing the effects with those of the CPP penetratin (pAntp) and the pore-forming peptide melittin. The prion peptides caused significant calcein leakage from LUVs concomitant with increased membrane ordering. Fluorescence correlation spectroscopy (FCS) studies of either rhodamine-entrapping (REVs) or rhodamine-labeled (RLVs) vesicles, showed that addition of the prion peptides resulted in significant release of rhodamine from the REVs without affecting the overall integrity of the RLVs. The membrane leakage effects due to the peptides had the following order of potency: melittin->mPrP->bPrP->pAntp. The membrane perturbation effects of the N-terminal prion peptides suggest that they form transient pores (similar to melittin) causing toxicity in parallel with their cellular trafficking.

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Keywords: Prion protein; Phospholipid vesicle; Membrane perturbation; Fluorescence; FCS; CD

1. Introduction

Prion diseases, also known as transmissible spongiform encephalopathies, are fatal neurological disorders of humans and animals that appear in sporadic, familial and infectiously acquired forms. These disorders are caused by conversion of a normal neuronal glycoprotein (PrP\(^{\text{C}}\)) into an infectious, conformationally altered isoform (PrP\(^{\text{Sc}}\)) [1–3].

The conversion of PrP\(^{\text{C}}\) into PrP\(^{\text{Sc}}\) occurs via a post-translational process [4]. PrP\(^{\text{Sc}}\) is monomeric and readily digested by proteinase K, whereas PrP\(^{\text{Sc}}\) forms insoluble aggregates and shows a high resistance to proteolytic digestion [5]. The characteristics of the two forms of the prion protein (PrP) can be related to their differences in secondary structure. PrP\(^{\text{C}}\) adopts predominantly a-helical structure in its globular C-terminal half, and its N-terminus is largely unstructured [6], whereas PrP\(^{\text{Sc}}\) has a large content of \(\beta\)-sheet secondary structure [7].

Abbreviations: PrP, prion protein; PrP\(^{\text{Sc}}\), cellular isoform of PrP; PrP\(^{\text{C}}\), scrapie isoform of PrP; mPrP, bPrP, peptide with sequence corresponding to the N-terminus of the mouse prion protein, residues 1–28; bPrP, peptide with sequence corresponding to the N-terminus of the bovine prion protein, residues 1–30; NLS, nuclear localization sequence; CPP, cell-penetrating peptide; pAntp, penetratin, Antennapedia homeodomain-derived CPP; ER, endoplasmic reticulum; DHPC, 1,2-dihexadecanoyl-sn-glycero-3-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; 10:70 notation refers to vesicles with 30 mol% POPG content; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; Rh, rhodamine; RLVs, Rh-entrapping LUVs; RLVs, Rh-labeled LUVs; FCS, fluorescence correlation spectroscopy; CD, circular dichroism; DPH, diphenylhexatriene, membrane bound fluorescence probe; P/L, total peptide-to-phospholipid molar ratio

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Prβ is unusual in that it can adopt multiple membrane topologies during biogenesis at the ER membrane [8–12]. Most Prβ molecules are fully translocated into the lumen of the ER; this form, denoted Prγ, is eventually attached to the outer leaflet of the plasma membrane through a C-terminal glycosylphosphatidylinositol (GPI) anchor. Some Prβ molecules assume transmembrane orientations. These forms, designated Prγ and Prγ, span the ER lipid bilayer once, either with the C- or N-terminus, respectively, on the luminal side of the ER.

The signal sequence of Prβ is normally cleaved by a signal peptidase that acts in the lumen of the ER. However, in a recent study, it has been shown that in neuronal cells, the Prβ form retains its N-terminal signal sequence following its exit from the ER and during its trafficking to the cell surface [11]. It has also been reported that Prγ is unprocessed and contains an uncleaved N-terminal signal peptide [13]. The signal sequence appears to have an unusual role in prion proteins in that it has two separate functions, both targeting and topogenesis [13–15]. It has also been suggested that Prγ is associated with the neurodegeneration observed in prion disease [9]. Another variant of Prβ discussed as a neurotoxic intermediate is cytosolic PrP, Prγ, which also sometimes retains the signal peptide [16–18]. Hence, even if the situations where unprocessed prion proteins appear may be relatively rare, these situations may be important for a pathological process.

Previously, we have investigated certain properties of the N-termini of the unprocessed mouse (residues 1–28) and bovine (residues 1–30) PrPs, denoted mPrP [19] and bPrP [20], respectively. These sequences (Table 1) comprise the signal peptide (residues 1–22 for mPrP, residues 1–24 for bPrP) and an identical and highly positively charged, NLS-like, sequence (residues 23–28 for mPrPs, residues 25–30 for bPrPs). The mPrP and bPrP sequences are similar to those of certain chimeric cell-penetrating peptides (CPPs), and we found that mPrP and bPrP can indeed function as CPPs [21], and Magzoub et al., unpublished results]. CPPs are able to translocate into various cells, carrying a conjugated hydrophilic macromolecular ‘cargo’ [21,22]. Recent observations on CPP entry into cells emphasize the role of heparan sulphate as a mediator of raft-dependent macropinocytosis, a particular form of endocytosis [23,24]. Cellular heparan sulphate has also been shown to interact with prion proteins [25], and Prβ incorporation into CHO cells requires glycosaminoglycan expression [26]. The interaction of various forms of PrP with model membranes have also been investigated [27,28].

In the present study, we have studied the interactions of mPrP and bPrP with large unilamellar phospholipid vesicles (LUVs) of varying surface charge densities, using fluorescence and CD spectroscopic methods. The membrane interaction effects of the two prion peptides were compared with those of two well-characterized peptides: the CPP penetratin (pAntp), and the pore-forming peptide melittin (Table 1). The steady-state fluorescence studies were complemented by fluorescence correlation spectroscopy (FCS) studies. FCS gives information on the translational diffusion of fluorescent particles with high specificity [29].

2. Materials and methods

2.1. Materials

The prion peptides and pAntp were produced by Neosystem Laboratoire, Strasbourg. Peptides were used as purchased. The identity and purity were controlled by amino acid, mass spectral and HPLC analyses. Peptides were of Immuno grade quality (purity estimated at ~95%). In each case, peptides from more than one batch were used. Melittin from the venom of honeybee (Apis mellifera) was obtained from Sigma; 1-palmityl-2-oleoyl-phosphatidylcholine (POPC), and 1-palmityl-2-oleoyl-phosphatidylglycerol (POPG), were purchased from Avanti Polar Lipids, Alabaster, of the best quality, and were used without further purification. Diphytanylphosphatidylcholine (DPPC) was obtained from Sigma. Rh-6-glycidyl-1,2-dihexadecanoyl-3-phosphoethanolamine) were purchased from Molecular Probes, The Netherlands. Calcein, a fluorescent derivative, was also purchased from Molecular Probes (product no. C-481).

2.2. Determination of peptide concentrations

After weighing on a microbalance, the peptide concentrations in the stock solutions were determined by light absorption on a Cary 4 Spectrophotometer, using quartz cuvettes with a 1-cm light path. All spectra were baseline corrected. Molar absorptivities of 5690 and 1280 M−1 cm−1, at 280 nm, for tryptophan and tyrosine, respectively, were applied.

2.3. Sample preparations

2.3.1. Preparation of LUVs

Large unilamellar vesicles were prepared by initially dissolving the phospholipids at the desired concentration (with the chosen POPG/POPC molar ratio) in a chloroform/methanol mixture, to ensure complete mixing of the components, and then removing the solvent by placing the sample in a high vacuum for 3 h. The dried lipids were dispersed in 50 mM potassium phosphate buffer (pH 7.4). The dispersion was run through a freeze–thaw cycle five times and then passed through two polycarbonate filters (0.1 μm pore size) 20 times in an Avanti manual extruder.

2.3.2. Preparation of REVs and BLVs

To prepare the Pr-entrapping LUVs (REVs), prior to extrusion, the dried lipid film (prepared as described above) was dispersed in 50 mM potassium

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>Sequence</th>
<th>Net charge</th>
<th>Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAntp</td>
<td>Hormedomain (Drosophila)</td>
<td>RQKTFONRBMWKKK</td>
<td>+7</td>
<td>1.7</td>
</tr>
<tr>
<td>mPrP</td>
<td>N-terminus of mouse prion protein (residues 1 to 28)</td>
<td>MANLGWYLLAVFIVMTDWGGLC KKRPKP</td>
<td>+3</td>
<td>0.3</td>
</tr>
<tr>
<td>bPrP</td>
<td>N-terminus of bovine prion protein (residues 1 to 30)</td>
<td>MVSEXGSGWLVFVAHWGKLCKRPPK</td>
<td>+5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a Given are also the net charges of the peptides, as well as their average hydrophobicities. The charged residues are underlined.

b The average hydrophobicity of the peptides is calculated using the scale devised by Kyte and Doolittle [60].
phosphate buffer containing 200 mM Rh. In this case, the LUVs entrapped the dissolved Rh, which can be released if pores/ channels are formed into the vesicles, or if the vesicles are broken. A Rh concentration gradient was created by diluting the DPH samples 100- to 200-fold in the buffer, resulting in a less than 1 mM background Rh concentration outside the vesicles.

To prepare the Rh-labeled LUVs (RLVs), the phospholipids, including 0.1 mol% Rh-labeled lipids, were dissolved in the chloroform/ethanol mixture prior to making the lipid film. Rh is covaletly bound to the head-groups of the phosphatidylthanolamin, and only the disintegration of the RLVs can lead to the appearance of faster diffusing Rh in solution.

2.4. Steady-state fluorescence spectroscopy

Fluorescence was measured on a Perkin Elmer LS 50B Luminescence Spectrometer with FluWinLab software. Measurements were made in 4 x 10 mm quartz cuvettes at 20 °C. For DPH experiments the excitation was at 340 nm and the emission wavelength scanned from 400 to 550 nm. Calcium was excited at 400 nm and the emission scanned from 510 to 600 nm. Scans were usually recorded with 4 nm excitation and emission bandwidths and a scan speed of 250 nm/min. 3 scans were recorded and averaged for each sample.

2.4.1. Peptide-induced calcium release

LUVs with entrapped calcium were prepared by hydrating a lipid film of desired composition with 70 mM calcium present in the buffer (the final pH was adjusted to 7.4 by the addition of NaOH from a 10-M stock solution). The fluorescence from calcium at 70 mM was low due to self-quenching, but increased considerably upon dilution. Free calcium was separated from the LUVs on a Sephadex G-25 column. Increasing concentrations of peptides were added (from a 1-nM stock solution) to LUVs composed of 250 μM phospholipid mixtures of different POPG/POPC content. After 5 min incubation, release of calcium from the LUVs was monitored by an increase in the fluorescence intensity. The maximum fluorescence intensity corresponding to 100% leakage was determined by lysing the vesicles with 10% (w/v) Triton X-100. The % leakage was then calculated according to the following equation:

\[ \% \text{leakage} = 100 \times \frac{F - F_0}{F_{\text{max}} - F_0} \]  

where \( F \) represents the fluorescence intensity of the intact vesicles, \( F_0 \) and \( F_{\text{max}} \) the intensity before and after the addition of the detergent, respectively.

2.4.2. Fluorescence polarization

LUVs were labeled with the membrane-bound probe diphenylhexatriene (DPH). 2 μM DPH (from a 1 mM ethanolic stock solution) was added to LUVs composed of 200 μM phospholipid mixtures of different POPG/POPC content. The samples were allowed to equilibrate for 15 min before measurement. Increasing concentrations of peptides, from a 1-nM stock solution, were added to the labeled LUV samples, and allowed to stand for 10 min. A polarization attachment (Shimadzu) was adapted to the spectrometer. The steady-state polarization was determined using the following equation [30]:

\[ P = \frac{I_{\text{ex}} - G I_{\text{em}}}{G I_{\text{em}}} \]  

where \( I_{\text{ex}} \) is the emission intensity of vertically polarized light parallel to the plane of excitation and \( I_{\text{em}} \) is the emission intensity of horizontally polarized light perpendicular to the plane of excitation. The instrumental factor \( G \) (\( G = I_{\text{em}} / I_{\text{exc}} \)) was determined by measuring the polarized components of fluorescence of the probe with horizontally polarized excitation.

2.5. Fluorescence correlation spectroscopy (FCS)

2.5.1. FCS instrumentation

FCS was performed with confocal illumination of a volume element of 0.2 fl in an instrument as described previously [31,32]. Focusing optics were a Zeiss Neofluar 63 × NA 1.2. A 1.2 was used in an epifluorescence setup. For separating exciting from emitted radiation a dichroic filter (Omega 540 DRL P02) and a bandpass filter (Omega 565 SR 550) were used. RLVs or RLVs were excited with the 514.5-nm line of an Argon laser. The fluorescence intensity fluctuations were detected by an avalanche photo diode (EG and SPCM 200) and were correlated with a digital correlator (ALV 5000, ALV, Langen, Germany).

2.5.2. FCS data evaluation

The observed fluorescence intensity fluctuations (\( S(t) \)) when correlated with fluorescence intensity fluctuations at time \( t + \tau \) yield the normalized intensity autocorrelation function \( G(\tau) \):\n
\[ G(\tau) = 1 + \frac{S(\tau) S(\tau+\tau)}{\langle S^2 \rangle} \]  

(3)

where the brackets describe the time average and \( \langle \rangle \) the mean fluorescence intensity [33,34].

The intensity autocorrelation function \( G(\tau) \) for Brownian motion of molecules/particles in a 3D Gaussian volume element is described [35] as follows:

\[ G(\tau) = 1 + \frac{1}{N} \left( \frac{1}{1 + \frac{\tau}{\tau_D}} \right)^2 \left( \frac{1}{1 + \frac{\tau}{\tau_0}} \right)^2 \]  

(4a)

or \( G(\tau) = 1 + \frac{1}{N} \left( \frac{1}{1 + \frac{\tau}{\tau_D}} \right)^2 \left( \frac{1}{1 + \frac{\tau}{\tau_0}} \right)^2 \]  

(4b)

where \( \tau_D \) is the characteristic translational diffusion time related to the diffusion coefficient \( D : \tau_D = \frac{\sigma^2}{4D} \) and \( N \) is the total number of detected fluorescent molecules, \( \sigma (0.5 \mu m) \) is the radius, and \( z (2 \mu m) \) the length of the volume element of the laser beam.

The expressions for the intensity autocorrelation function \( G(\tau) \) (Eqs. (3) (4a) (4b)) are valid when the particle size is smaller than the volume element. With a volume element of a diameter of 1 μm and a vesicle size of 0.1 μm this condition is fulfilled. To calculate the average number of fluorophores per volume element (\( N \)), and diffusion coefficients of free Rh (\( \tau_D \)) and labeled vesicles (\( \tau_D \)), the intensity autocorrelation function \( G(\tau) \) is analyzed as follows:

\[ G(\tau) = 1 + \frac{1}{N} \left( \frac{1}{1 + \frac{\tau}{\tau_D}} \right)^2 \left( \frac{1}{1 + \frac{\tau}{\tau_0}} \right)^2 + \gamma \left( \frac{1}{1 + \frac{\tau}{\tau_0}} \right)^2 \]  

(5)

where \( N \) is the total number of fluorophores, \( \tau_0 = \sigma^2/4D_A \) is the diffusion time for vesicles, and \( \tau_0 = \sigma^2/4D \) is the diffusion time for free Rh, \( \gamma \) is the fraction of Rh diffusing with the vesicles having the characteristic diffusion time \( \tau_0 \) and \( \gamma = \gamma_0 \) is the fraction of free Rh diffusing with the vesicle. For parametrization and fitting of the autocorrelation function \( G(\tau) \), a non-linear least squares minimization procedure according to the Marquardt algorithm [36] was used.

2.5.3. FCS experiments

Free Rh was used as a reference to calibrate the instrument and to estimate diffusion times of the RLVs, as well as to observe whether the vesicles are intact or destroyed. The diffusion times of free Rh and RLVs were determined separately. The effect of the peptides on vesicles was investigated by incubating the vesicles together with the peptides. The total peptide-to-phospholipid molar ratio (PL) ratio varied from 0.05 to 0.1. 20 μl of the samples were analyzed for 30 s up to 10 min in the FCS instrument, at several time points of incubation, ranging from minutes to hours. All experiments were performed at 20 °C.

2.6. Circular dichroism spectroscopy (CD)

CD measurements were made on a Jasco J-720 CD spectropolarimeter with 0.5 and 1 mm quartz cuvettes. Spectra were measured from 190 to 250 nm, with a 0.2-nm step resolution at 50 nm/min speed. The response time was 2 s.
with 50 mdeg sensitivity and a 1-mm bandwidth. The temperature was regulated by a PTC-343 controller, set at 20 °C. Spectra were collected and averaged over 20 scans. Contributions from background signals were subtracted from the CD spectra acquired for the peptide. Computer fittings using the VARSELEC program [37], with 3 reference component spectra (α-helix, β-sheet and random coil, etc.) were performed to estimate the contributions of spectral components from different secondary structures. The percentages describing the contributions are precise within 5–10%.

3. Results

Table 1 shows the sequences of the mouse and bovine prion peptides (mPrPrp and bPrPrP, respectively), as well as the two archetypal peptides (pAntp and melittin). The pAntp sequence corresponds to the N residues of the third α-helix of the Antennapedia homeodomain protein from *Drosophila* and is a well-known CPP [21]. Melittin is a 26-residue peptide derived from honeybee venom, and is a well-known pore-forming peptide [38].

3.1. Peptide-induced calcein release from LUVs

The membrane-perturbing effects of the peptides were investigated through calcein-release experiments in LUVs. The calcein-release assay is based on a high concentration (up to 70 mM) of entrapped calcein, resulting in self-quenching of its fluorescence. The degree of leakage of the vesicles induced by the peptides is assessed by observing increased fluorescence from the released calcein.

Fig. 1a shows the % leakage induced by the peptides with neutral POPC LUVs, after 5 min exposure, at 20 °C. A study of the time-dependence of calcein leakage from LUVs (Fig. 1b, inset) indicated that the process is a relaxation towards an equilibrium, and has a duration of approximately 5 min, after which no further leakage takes place. Thus, the levels of leakage presented in Fig. 1 should represent the final stage at each peptide concentration. The results are summarized in Table 2.

pAntp, which does not interact with neutral membranes [39,40], induced no leakage. Addition of bPrPrP or mPrPrP to the neutral LUVs results in substantial degrees of leakage, 40% and 66%, respectively. The leakage by the prion peptides occurs at low peptide concentrations, where maximum leakage is achieved at P/L ≈ 0.1, corresponding to 25 μM peptide. Melittin was found to be far more potent: approximately 100% leakage was induced already at P/L ≈ 0.02.

The leakage from charged POPG/POPC [30/70] LUVs induced by the peptides is shown in Fig. 1b (results are summarized in Table 2). Although pAntp does not interact with the charged vesicles [40], there is relatively little leakage effect. bPrPrP and mPrPrP cause higher degrees of leakage, 54% and 74%, respectively, which are higher degrees of leakage than with the neutral LUVs. Melittin gives 100% leakage, at a somewhat higher P/L ≈ 0.03, as compared with the POPC LUVs.

3.2. DPH fluorescence polarization

The perturbation effects of the peptides on the membrane properties of LUVs were studied by steady-state fluorescence polarization of the DPH probe. When added to a membrane suspension, DPH dissolves completely into the bilayer core, with no significant emission in the aqueous phase. Since DPH is buried deeply within the acyl chain region of the bilayer, any increase in polarization reports on decreased acyl chain mobility [41,42].

Fig. 2a shows the effect of addition of the peptides to neutral POPC LUVs on the DPH polarization. Due to lack of binding [39], pAntp does not significantly affect the DPH polarization of the neutral vesicles. mPrPrP and bPrPrP, which are hydrophobic enough to bind to neutral membranes, increase the DPH polarization, with mPrPrP inducing a greater increase in membrane ordering than bPrPrP. Melittin, causes a slight decrease in the polarization, indicating that melittin decreases the membrane ordering, leading to greater fluidity.
### Table 2
Summary of membrane activities and secondary structures of the peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Medium</th>
<th>Secondary structure component (%) (P:L=0.04)</th>
<th>Polarization ∆(P) (P:L=0.11)</th>
<th>% calcin release (P:L=0.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>s</td>
<td>β</td>
<td>r.e.</td>
</tr>
<tr>
<td>pAnpt</td>
<td>Water</td>
<td>13</td>
<td>24</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>17</td>
<td>31</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>POPC</td>
<td>12</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>[30/70]</td>
<td>13</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>bPnPp</td>
<td>Water</td>
<td>15</td>
<td>25</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>24</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>POPC</td>
<td>28</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>[30/70]</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>mPnPp</td>
<td>Water</td>
<td>16</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Buffer</td>
<td>24</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>POPC</td>
<td>16</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>[30/70]</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

*Given are the changes in polarization (∆P) and the % calcin release in the presence of LUVs with different compositions, at P/L ~0.1. Also included are the secondary structures induced in the peptides in the presence of distilled water and 50 mM phosphate buffer, pH 7.4, as well as the LUVs under conditions where the two prion peptides are fully bound.

within the acyl-chain region of the bilayer. The result is in agreement with a published study in which melittin was found to induce lipid flip-flop in neutral, but not charged, LUVs [43].

The effect of the peptides on charged LUVs from POPG/POPC [30/70] is shown in Fig. 2b. pAnpt induces no significant change in the DPH fluorescence polarization. This is probably due to the location of pAnpt at the membrane surface, within the headgroup region [40,44]. The prion peptides cause a considerable increase in the DPH polarization, with mPnPp inducing a slightly higher increase than bPnPp. The interaction of melittin with the charged LUVs, unlike with the neutral ones, resulted in a small increase in the DPH polarization.

Table 2 includes estimated changes (±/-) in the DPH polarization (∆P) values induced by the four peptides at an arbitrary P/L=0.1, with POPC and POPC/POPG [30/70] LUVs. The leakage effects for mPnPp, bPnPp and pAnpt mirror their polarization influences: an increased LUV surface charge density results in a greater degree of polarization and a higher degree of leakage induced by the peptides. The effects are more pronounced for melittin, followed by the prion peptides.

### 3.3. FCS

In FCS measurements, the fluorescence intensity fluctuations are recorded only from the molecules that diffuse through the confocal laser volume element. The time required for the passage of molecules through the volume element is determined by the diffusion coefficient, related to the size and shape of molecules. From the autocorrelation functions of fluorescence intensity fluctuations, the average number (N) and the diffusion time (τD) of fluorescent molecules or particles crossing the confocal volume are determined.

#### 3.3.1. FCS parameters

Fluorescence intensity autocorrelation functions, G(τ), of free Rh, POPG/POPC [30/70] REVs and RLVs (i.e. LUVs with 30% POPG content) are shown in Fig. 3a–c, respectively. In the REV preparation, the Rh concentrations inside and outside the vesicles were 200 nM and 2 nM, respectively. Table 3 summarizes the evaluated values of τD in each case. A two-component analysis of the autocorrelation curves was used to yield a slowly diffusing bound component (Rh diffusing with the LUVs) and a fast moving non-bound one (freely diffusing Rh). The diffusion time of free Rh in solution is 0.070 ± 0.002 ms. The major component of the diffusion time of the REVs (where the entrapped Rh diffuses with the vesicles) is 7.4 ± 1 ms; a minor component (15%) has the τD of free Rh (interpreted as the contribution from the free fraction of Rh).

The τD calculated for the RLVs is 8.4 ± 1 ms. Due to the large difference in diffusion times between free Rh and vesicles, the fractions of Rh free in solution and diffusing with the vesicles (either entrapped or covalently-bound), can be seen separately and comparative observations can be made. However, absolute quantifications of Rh release are not reliable due to the large difference in fluorescence intensities between Rh-entrapping vesicles and free Rh—the so-called ‘brightness ratio’—which distorts the quantification (cf. [45]). In addition, the tendency of Rh to bind to the surface of vesicles (Rh+LVs, Table 3) further contributes to the uncertainty concerning the precise quantification of release of entrapped Rh.

#### 3.3.2. Effect of peptides on RLVs

Addition of melittin to POPG/POPC [30/70] RLVs at P/L=0.05, and mPnPp, bPnPp or pAnpt at P/L=0.1, results in no change in the RLV diffusion time (8.4 ± 1 ms, Table 3), showing that there is no free Rh in solution and that the LUVs are intact. At the chosen P/L (0.1) for these studies both mPnPp...
and bPPp are still in a fully bound state, as observed by changes in fluorescence intensity of the intrinsic tryptophan due to addition of vesicles (data not shown). This particular P/L was also chosen since it allows a near maximum effect of these peptides on the vesicles to be observed, while avoiding artifacts such as LUV aggregation (data not shown).

3.3.3. Peptide-induced Rh release from REVs

FCS was used to monitor the release of entrapped Rh (200 nM) from REVs due to the peptides. Upon the addition of melittin to the REVs (Fig. 3d), at a total peptide-to-phospholipid molar ratio (P/L) = 0.05, the evaluated fraction of free Rh increases from 15% to approx. 80% (Table 3), indicating that addition of melittin results in release of Rh from the REVs, in agreement with previous studies [46,47].

Addition of the two prion peptides, mPrPp and bPrPp (Fig. 3e and f, respectively), at P/L = 0.10, results in an increase in the total fraction of free Rh to 70% and 65%, respectively (Table 3), illustrating also for these peptides the release of Rh from the REVs. Exposure of the REVs to pAntp (P/L=0.1) results in a small increase in the total free fraction to ~20%, indicating a weak effect of the peptide, possibly due to release of peptide associated with the outside of the vesicle (Table 3). Values of the number of diffusing particles N are included in Table 3. The value of N increases from ~0.3 for REVs alone to 4.4 upon addition of melittin, indicating an increase in the number of fluorophores in solution. With the prion peptides the value of N again increases, but to a lesser extent than with melittin (N ~ 1.3 and 1.1 for mPrPp and bPrPp, respectively). No change in the value of N is observed upon addition of pAntp. This confirms the increase in apparent fraction of free Rh upon addition of melittin and the prion peptides to the REVs inferred from the T2D observations.

Lysing the LUVs with Triton X-100 (10% w/v) results in a ~65% fraction of freely diffusing Rh, with T2D ~0.07 ms (data not shown). The remainder has a T2D of 0.5 ms, indicating complex formation between Rh and phospholipids (micelles). Incubation of non-labeled LUVs with free Rh results in a fraction of the Rh (~30%) diffusing with the LUVs (T2D=7.4 ms), while the value of N decreases from 3.2 to 2.4, which confirms the affinity of Rh for lipids (Table 3). Taken together, the FCS results with melittin and the prion peptides indicate that under conditions where leakage of vesicle content is observed, the overall integrity of the vesicle is intact.

3.4. Peptide secondary structures

The secondary structures induced in the peptides in the presence of water, buffer and LUVs of different lipid compositions, were studied by CD spectroscopy (Fig. 4). In all experiments the peptide concentration was 4 μM, and the phospholipid concentration was 100 μ (P/L=0.04). Under these conditions, the prion peptides were found to be in the fully membrane bound state, verified by fluorescence experiments (data not shown). Table 2 summarizes the secondary structure contributions of the peptides evaluated for the various solvents. Although structure analysis of peptides by CD is at best approximate, significant trends are seen. In distilled water, all the peptides are largely in random coil secondary structure (data not shown). The presence of buffer ions (Fig. 4a) induces a somewhat higher degree of secondary structure in melittin (α-helix), mPrPp and bPrPp (β-sheet), but not in pAntp.

In the presence of neutral LUVs (Fig. 4b), the peptides, except for pAntp, become more structured. With its low hydrophobicity (Table 1), pAntp has a low binding affinity to an electrically neutral membrane [39,48], and hence remains unstructured. Introduction of charges (30%) to the LUV surface induces some α-helical structure in pAntp, whereas mPrPp and bPrPp increase their β-structure contributions to some extent (Fig. 4c; Table 2). Melittin, on the other hand, maintains the same high degree of helicity (Fig. 4c).

3.5. Vesicle aggregation due to pAntp

In the presence of partially charged vesicles, pAntp adopts a helical structure, and is generally ‘benign’, with
negligible membrane perturbation effects. However, we have previously observed that in the presence of fully charged vesicles, the peptide adopts a dominating β-structure, particularly at high P/L, and has pronounced membrane perturbation effects [40,48]. Here we use FCS, in tandem with steady-state fluorescence techniques, to characterize the interaction of pAntp with the fully charged POPG LUVs, in comparison with the partially charged POPG/POPC [30/70] vesicles.

Fig. 5a shows evaluated diffusion times, $\tau_{\text{diff}}$, as a function of P/L for the RLVs with the two surface charges. A large increase in the RLV diffusion time for fully charged POPG vesicles is observed in the presence of pAntp, even at low peptide concentrations. This indicates a severe case of vesicle
Table 3
Summary of the FCS parameters derived from the intensity autocorrelation functions G(2) of rhodamine (Rh)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bound/entrapped Rh fraction</th>
<th>Free Rh fraction</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \tau_0 ) (ms)</td>
<td>%</td>
<td>( \tau_0 ) (ms)</td>
</tr>
<tr>
<td>Rh only</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>+LUVs</td>
<td>7.4</td>
<td>30</td>
<td>0.07</td>
</tr>
<tr>
<td>RLVs only</td>
<td>8.4</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>+melitin</td>
<td>8.4</td>
<td>98</td>
<td>0.07</td>
</tr>
<tr>
<td>+nPP</td>
<td>8.4</td>
<td>98</td>
<td>0.07</td>
</tr>
<tr>
<td>+bPP</td>
<td>8.4</td>
<td>99</td>
<td>0.07</td>
</tr>
<tr>
<td>+pAmp</td>
<td>8.4</td>
<td>99</td>
<td>0.07</td>
</tr>
<tr>
<td>REVs only</td>
<td>7.4</td>
<td>85</td>
<td>0.07</td>
</tr>
<tr>
<td>+melitin</td>
<td>7.4</td>
<td>20</td>
<td>0.07</td>
</tr>
<tr>
<td>+nPP</td>
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<td>30</td>
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<tr>
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<td>7.4</td>
<td>35</td>
<td>0.07</td>
</tr>
<tr>
<td>+pAmp</td>
<td>7.4</td>
<td>80</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Given are the % fractions of Rh diffusing with the vesicles (entrapped in POPG/POPC [30:70] REVs or covalently bound to POPG/POPC [30:70] RLVs) or freely diffusing (non-bound) in the absence, or presence, of the peptides. The rhodamine (Rh) concentration in solution was 200 nM. The vesicles (with a phospholipid concentration of 100 mM for both REV and RLV samples) and the peptides (at a concentration of 10 \( \mu \)M, i.e. PL=0.1, except for melitin, which was at 5 \( \mu \)M, PL=0.05) were incubated for a period of 1 h. The fraction (%a) of Rh diffusing with a certain \( \tau_0 \) was calculated using a two-component fitting of the autocorrelation curves.

\(^1\) The \( \tau_0 \) for this component was fixed during the fitting procedure.

aggregation. In contrast, with POPG/POPC [30:70] RLVs, a small increase in \( \tau_0 \) was observed, and then only at the higher peptide concentrations (P/L > 0.1). Thus, under the conditions of the experiments carried out with POPC and POPC/POPG [30:70] LUVs in this study, vesicle aggregation does not occur. LUV aggregation was also observed with POPG RLVs in the presence of the prion peptides, and aggregation comparable to that induced by pAmp was observed at the higher P/L values (Fig. 5a).

Fig. 5b shows the FCS kinetics of the aggregation of POPG RLVs in the presence of pAmp (P/L = 0.05 and 0.1). With P/L = 0.05 only a partial aggregation is observed. The aggregation process is completed within 5 min. Once aggregation takes place, the aggregates are stable up to 24 h (Fig. 5b inset), contrary to what has previously been reported from light scattering studies, where gradual disaggregation was observed, over a period of 1–2 h [49]. The higher degree of \( \alpha \)-structure induced in pAmp with the POPG LUVs than with the POPG/POPC [30:70] LUVs (Table 2) was highly stable. We did not detect any transient secondary structure conversion back to \( \alpha \)-helix under the present conditions.

Similar extreme properties of POPG LUVs with pAmp is observed with steady-state fluorescence: a significant increase in DPH polarization, and an increase in the calcein leakage (Table 2). The total fraction of free Rh increases to 60% upon addition of pAmp to POPG REVs (FCS data not shown).

Fig. 4. CD Spectra of 4 \( \mu \)M of nPP (---), bPP (---), pAmp (---) and melitin (---) in different media: (a) 50 mM phosphate buffer, and LUVs composed of (b) 100 \( \mu \)M POPC, or (c) 100 \( \mu \)M POPC/POPG [30:70]. The medium for the LUVs was 50 mM phosphate buffer (pH 7.4). The temperature was set at 20°C.
integrity. However, like certain CPPs, particularly the amphipathic chimeras [22,23], the prion peptides produced membrane perturbations in cells, resulting in morphological stress [20], as well as hemolysis of red blood cells and leakage from CHO and neuronal N2A cells (Magzoub et al., unpublished results).

We have now studied the membrane perturbation effects of the prion-derived peptides in vesicle model systems, comparing the results with those of the archetypal CPP pAnp, and the pore-forming melittin. pAnp does not perturb the LUVs to any great extent (Figs. 1 and 2; Tables 2 and 3). Melittin, on the other hand, causes pronounced membrane disturbances. The peptide induces maximal leakage of entrapped calcein, and causes considerable release of Rh from the REVs (Figs. 1 and 3; Tables 2 and 3). Leakage from the LUVs can be caused either by completely destroying the vesicles (lysis) or by making channels into them (pore-formation). As the translational properties of the REVs remain constant in the presence of melittin, under conditions where leakage from the REVs was observed in terms of P/L (Table 3), and an increase in membrane ordering is observed (Fig. 2), we conclude that pore-formation rather than lysis is the explanation for the observed effects. This agrees with numerous literature reports on melittin [38,46,47,50].

The two prion peptides, mPrPp and bPrPp, behave similarly to melittin, giving rise to substantial leakage of the vesicle contents (Figs. 1 and 3; Tables 2 and 3). The overall integrity of the vesicles is not affected, as indicated by the unchanged diffusion times of the REVs (Table 3). This again suggests pore-formation caused by mPrPp and bPrPp. It is worth noting here that there is at present no consensus on the definition of the ‘pore’. While some reports use the term to describe a defined structure, composed of a number of helices in a parallel alignment, others use it to describe a peptide-induced structural defect in the membrane through which ions or molecules can pass [38,51]. In the present context, we use the latter, broader definition. The orientation of melittin in lipid bilayers is dependent on a number of parameters, including P/L and lipid type. At the relatively high concentrations used here it is likely that melittin adopts a transmembrane orientation [52]. In an NMR study of bPrPp associated with neutral DHPC micelles [20], the results indicated that the induced α-helical structure observed under those conditions should have a transmembrane orientation, a positioning favouring pore-formation.

The kinetics of peptide-induced calcein leakage exhibits saturation (Fig. 1b). Such phenomena have been observed in several other studies of peptides interacting with membranes, yielding leakage curves which reach a plateau as a function of time at a constant peptide concentration [53–55]. The observations have been interpreted in terms of transient pore formation, involving a nucleation step during an initial bilayer perturbation period, followed by a transient restabilization of the peptide/lipid bilayer structure, which gives rise to the observed leveling off of the spontaneous leakage [56]. The saturation of the leakage curves for mPrPp and bPrPp as a function of peptide concentration (Fig. 1) may have a similar background. Alternatively, this behavior could be due to increased self-aggregation of the prion peptides taking place.
at the higher peptide concentrations (Magzoub et al., unpublished results) resulting in attenuation of peptide activity.

The two prion peptides differ from melittin in that a higher membrane surface charge density leads to a greater effect on the membrane integrity. At the higher LUV surface charge density the peptides, with higher β-structure content (possibly in an aggregated state), have a more potent effect on the membrane integrity than observed with neutral membranes. This suggests that peptide aggregation and concomitant β-structure induction may be one prerequisite for pore-formation caused by the prion peptides.

When interacting with fully charged LUVs, pAnp adopts a β-structure [48] and causes vesicle aggregation and leakage (Fig. 5; Table 2). The high charge density of the fully charged LUVs promotes extreme behavior in both the vesicles and the peptides. The high surface charge density may lead to peptide aggregation (manifesting itself as β-structure), which in turn leads to vesicle aggregation. This could explain the increase in membrane DPH polarization observed here with fully charged LUVs (Table 2), as well as the previously reported large increase in the DPH polarization with the fully charged SUVs induced by pAnp [40]. The β-structure and vesicle aggregation are not completely interdependent, since we observe some β-structure even when there is no obvious vesicle aggregation (data not shown).

In this study, we have attempted to complement the traditional calcein release assay with a more novel release assay using FCS. Although the parameters measured in both cases are different—fluorescence ‘de-quenching’ as opposed to translational diffusion—while the concentration gradients involved are also rather different, the two methods obviously give similar and complementary results.

Calcein-release is an established method. Self-quenching of calcein has been attributed to a combination of dimerization and energy transfer [57]. However, the high concentrations of the entrapped calcein used (∼55–70 mM) is problematic. For instance, at higher prion peptide concentrations, a decrease in the calcein fluorescence intensity was observed (data not shown). This might be due to self-quenching of the released calcein, and/or binding of the negatively charged calcein to the basic peptides (which also have a high propensity to aggregate) leading to formation of large peptide–calcein complexes, which results in further quenching of the calcein quantum yield. Such phenomena can distort the analysis.

With FCS, and the low concentrations of Rh required (in the nM range), this problem is mostly avoided. However, the affinity of Rh for phospholipids complicates the analysis (Table 3), suggesting the need for an alternative fluorophore. In addition, the presence of two forms of fluorescent particles with very different fluorescence intensities (in this case, Rh free in solution and Rh entrapped in the REVs) distort the quantitative evaluations [45].

The long N-terminal part of the PrP sequence is unstructured in aqueous solution [7]. The interaction of the signal peptide-containing domain with a membrane mediates secondary structure conversion towards β-sheet for the domain. Perhaps, this moeity can serve as a ‘seed’ for a structure conversion involving a larger part of the unprocessed PrP, possibly taking place at a membrane surface. A membrane-induced structure conversion in the N-terminus may also be important for the transformation from PrP⁰ to PrP⁴⁰. The membrane-induced aggregation and structure conversion may be a common process for several amyloid forming proteins and peptides, as a general phenomenon leading to toxic effects in cellular systems (cf. [58,59]). Among the common amyloid proteins [58], only the unprocessed PrP has a hydrophobic signal sequence directly followed by a basic sequence, the combination of which is related to the CPP property of the N-terminal domain. This inherent property may facilitate intercellular prion trafficking, and furthermore contribute to the membrane perturbations, as observed in this study.

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References
N-terminal peptides from unprocessed prion proteins enter cells by macroinocytosis

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Abstract

A peptide derived from the N-terminus of the unprocessed bovine prion protein (bPrPp), incorporating the hydrophobic signal sequence (residues 1–24) and a basic domain (KKRPKP, residues 25–30), internalizes into mammalian cells, even when coupled to a sizerable cargo, and therefore functions as a cell-penetrating peptide (CPP). Confocal microscopy and co-localization studies indicate that the internalization of bPrPp is mainly through macroinocytosis, a fluid-phase endocytosis process, initiated by binding to cell-surface proteoglycans. Electron microscopy studies show internalized bPrPp–DNA–gold complexes residing in endosomal vesicles. bPrPp induces expression of a complexed luciferase-encoding DNA plasmid, demonstrating the peptide’s ability to transport the cargo across the endosomal membrane and into the cytosol and nucleus. The novel CPP activity of the unprocessed N-terminal domain of PrP could be important for the retrotranslocation of partly processed PrP and for PrP trafficking inside or between cells, with implications for the infectivity associated with prion diseases.

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Keywords: Prion protein; N-terminus; Cell-penetrating peptide; Endocytosis; Macropinocytosis; Proteoglycan

Transmissible spongiform encephalopathies (TSE), also known as prion diseases, are associated with the conversion of the benign, cellular form of the prion protein (PrP<sup>C</sup>) into an abnormally folded, aggregated scrapie isoform (PrP<sup>Sc</sup>), which accumulates in the diseased brain [1–3]. Prion diseases may have an infectious, sporadic or familial origin. Although substantial evidence indicates that the conversion process of PrP<sup>C</sup> into PrP<sup>Sc</sup> is a key molecular event in the pathogenesis of prion diseases, PrP<sup>Sc</sup> need not necessarily be the primary toxic species [2].

When undergoing cellular trafficking, PrP travels through the secretory pathway of the ER on its way to the cell-surface. However, PrP has been detected in the cytosol [4,5], as well as in nuclear compartments [6,7]. The presence of PrP in the cytosol occurs either as a result of retrotranslocation from the ER [8,9], or following unsuccessful import to the ER [10]. In the latter case, the signal sequence is not cleaved off. A retained signal peptide of PrP has also been reported for certain forms of membrane associated PrP [11–13]. The presence of an uncleaved signal peptide is almost always associated with manifestations of various forms of prion disease [10,12–14].

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In an earlier study we highlighted various unusual properties of an N-terminal segment of the unprocessed mouse PrP (mPrP) [15]. The mPrP sequence, which is composed of the N-terminal signal sequence (residues 1-22) and an NLS-like sequence (residues 23-28), is similar to that of certain chimeric cell-penetrating peptides (CPPs), or protein transduction domains (PTDs), which are able to internalize into various cells, even with a large conjugated hydrophilic ‘cargo’ [16]. We demonstrated that mPrP internalizes into cultured cells [15]. However, as was common at the time, the internalization assays were carried out with a cell-fixation step in the protocol, which is now understood in many cases to result in artificial uptake and internalization patterns, leading to incorrect conclusions [16].

In the present study we have investigated the CPP-properties, in live (unfixed) mammalian cells, of an analogous peptide with a sequence derived from the N-terminus of the unprocessed bovine prion protein, denoted bPrP (residues 1-30: MVKSKIGSVLWYLFVAMWSDPVGLC2,KKRNP63), where residues 1-24 are the signal sequence and residues 25-30 constitute the highly basic segment of the N-terminus. We show that bPrP is internalized into cells through macropinocytosis—a non-selective form of fluid-phase endocytosis [17]—initiated by binding to cell-surface proteoglycans, and can transport a large anionic cargo, such as DNA plasmid. These results raise the possibility that the rest of the full PrP could also be a cargo for the N-terminal CPP, which may have implications for PrP trafficking, as well as the prion disease-related infectivity.

Materials and methods

The bPrP peptide (without fluorescence label) was produced by NCSysm System, Braunschweig, and Innogenex, Lund. The peptide was amidated at the C-termirsum to remove the negative charge of the carboxyl group. bPrP N-terminally coupled to fluorescein isothiocyanate moiety was synthesized in a stepwise manner on an automated peptide synthesizer (Applied Biosystems, Model 431A) using t-Boc solid-phase strategy. Tetramethylrhodamine-labelled transferrin and rhodamine B-labelled dextran were purchased from Molecular Probes, The Netherlands. Lipofectamine was obtained from Invitrogen, Wurtmannin and cytochalasin D were from Sigma. The luciferase-encoding plasmid pGL3 was a gift from Dr. A. Oldberg (Lund University, Lund, Sweden). The luciferase assay kit was from Promega.

Fluorescence microscopy of bPrP in live cells wildtype and mutant CHO cells. Cells were washed three times with HKR (Hepes Krebs Ringer, 5 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 0.05 mM MgCl2, 1.8 mM CaCl2, and 1 g/L glucose, pH 7.4), after which the fluorescein-labelled bPrP peptide was added at a final concentration of 5 pM in HKR. After 45 min incubation at 37 °C the cells were briefly washed with HKR, mounted on glass slips in HKR to achieve live cell pictures, and examined using a Leica TCS-SP laser scanning confocal microscope (Leica, Germany) with a 488 nm laser line from an argon laser (20 mW). Mutant ppsA/75 cells were seeded out in NUCN chambers to reach 80% confluence on the day of the experiment. Two days post-seeding the cells were washed three times with HKR after which the peptide was added at a final concentration of 2 µM. After 30 min of incubation the cells were washed in HKR three times and pictures were obtained using UltraView ERS confocal live cell imager (Perkin-Elmer Ltd, Upplands Väsby, Sweden) connected to an Axiovert 200 (Zeiss, Götttingen, Germany). Argon laser excitation at 488 nm was used for fluorescein.

Co-localization with endosomal markers and effects of endocytosis inhibitors. CHO cells were seeded out in NUNC chambers to reach 80% confluence on the day of the experiment. Prior to peptide addition, the cells were washed three times with HKR. For the co-localization studies, either tetramethylrhodamine-labelled transferrin (50 µg/ml) or chlorophyll b-labelled dextran (5 mg/ml) was co-incubated with 2 µM of bPrP. For inhibition of endocytosis, the cells were pre-incubated with cytochalasin D for 30 min, after which the peptide was added at a final concentration of 2 µM in cytochalasin D containing HKR. After 30 min of incubation, the cells were washed three times and pictures were obtained using the live cell imager. Laser excitation at 485 nm (argon) for fluorescein, and 560 nm (argon-krypton) for tetramethylrhodamine and rhodamine B was used.

Electron microscopy of bPrP-DNA complexes. CHO cells were grown to sub-confluence in a 24-well plate. 10 nM of crude DNA oligonucleotides (DNAON, SIGMA) with an average size of about 200 bp (120 KDa) was mixed with 22 nM of a biotin-tagged looped single strand DNA construct (B01, Integrated DNA Technologies, USA) with a molecular weight of 30.4 KDa (altogether 32.1 nM, DNA = 2 µg/ml), and 1 nM streptavadin-gold conjugate (20 nm, BBI International, UK) in incubation medium (F12K supplemented with 3 nM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). The sample was pre-incubated for 30 min at room temperature with or without the addition of 2.0 µM (10 µg/ml) bPrP. Cells were then incubated for 6 h with either only fresh incubation medium, or the above-described DNA gold or bPrP-DNA gold complexes. A thorough rinsing procedure to remove extracellular complexes was then applied to the cells: 1x PBS, 3x PBS with 100 µg/ml dextran sulphate (Mw, 500 KDa), 2x PBS, trypsinization in PBS with 0.1% trypsin (w/v), inactivation of trypsin with 10% fetal calf serum in F12K, and finally addition of 2 ml PBS 1% BSA (w/v). Cells were then fixed by 4% formaldehyde for 5 min at 500 ng/ml in a Beckman JS 7.5 rotor. The cell pellet was fixed for 1 h at room temperature and then overnight at 4 °C in 2.5% glutaraldehyde in cacodylate buffer (0.15 M sodium cacodylate, pH 7.4). Samples were washed with cacodylate buffer, postfixed for 1 h at room temperature in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded series of ethanol, and then embedded in Epon 812 using acetone as an intermediate solvent. Specimens were sectioned into 50-nm-thick ultrathin sections with a diamond knife on an LKB ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate. Specimens were observed in a JEOL JEM 1230 electron microscope operated at 80 Kv accelerating voltage, and images were recorded with a Gatan Multiscan 791 CCD camera.

Luciferase reporter gene assay. pGL3-luciferase plasmid (5 µg/ml) was dissolved in F12K with or without bPrP, and incubated with pre-timed, sub-confluent CHO cells in 24-well plates for 4 h, followed by another incubation period of 48 h in growth medium. Cells were then washed with PBS and solubilized in 100 µl of Promega cell lysis reagent (25 mM Tris-HCl, pH 7.8, 2 mM CDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100). Translation by Lipofectamine was performed according to instructions from the manufacturer. Luciferase expression was quantified in 25 µl of the cell lysate using a luciferase assay kit. Light emission was measured by integration over 10 s at 25 °C using BMG Fluostar Optima equipment (Laborvision).

Results

Uptake of bPrP by wild-type and proteoglycan-deficient live CHO Cells

A fluorescent derivative of bPrP was incubated for 45 min with live CHO cells and visualized using a laser confocal microscope. As shown in Fig. 1a-c, most of the peptide fluorescence arises from association with the plasma
membrane, although a significant fraction of the peptide was found to internalize. Even though the peptide contains an NLS-like motif (amino acids 25–30), none of the peptide was observed in the nucleus, but rather in the cytoplasm. As previously observed with mPrPp [15], bPrPp caused morphological perturbations of the cells, suggesting cellular toxicity. Internalization of fluorescently labelled bPrPp into live HeLa cells was also investigated, yielding similar localization (Fig. S1). Again, morphological perturbation of the cells was observed.

It has previously been shown that CPPs, such as the HIV-Tat peptide, enter cells in a proteoglycan (PG)-dependent manner [18,19]. To investigate whether this is also the case for bPrPp, pgsA-745 cells (CHO mutants that are PG-deficient due to loss of xylosyl transferase activity, i.e., the enzyme that catalyzes the first step in PG assembly) were used. These cells showed a much lower internalization with less peptide residing on the cell boundaries (Fig. 1d–f). Flow cytometry studies comparing wild-type CHO cells with mutant pgsA-745 cells confirmed that the association/uptake is dependent on cell-surface proteoglycans (data not shown). Therefore, cell-surface PGs are important for bPrPp binding and uptake, as has also been shown for the full-length PrP [20–22].

Co-localization with endosomal markers and effects of endocytosis inhibitors

Co-localization studies of bPrPp with endosomal markers were carried out in CHO cells using dextran (a marker for fluid-phase endocytosis) and transferrin (a specific marker for clathrin-mediated endocytosis). Fig. 2a–c shows substantial overlap of the fluorescein-labelled peptide and the rhodamine B-labelled dextran, suggesting
strong co-localization of bPrPp and dextran. In contrast, little co-localization was observed for fluorescein-labelled peptide and tetramethylrhodamine-labelled transferrin (Fig. 2d–f). This indicates that while fluid-phase endocytosis is a major route of entry, clathrin-mediated endocytosis is not a prominent pathway for the internalization of the peptide.

The effects of inhibitors of endocytosis on the uptake of bPrPp into CHO cells were then assessed. In the presence of cytochalasin D, a specific inhibitor of F-actin elongation involved in macropinocytosis [23,24], most of the peptide was located on the cellular membrane, with very little punctate staining observed in the cytoplasm (Fig. S2a–c). Wortmannin, which inhibits endocytosis by affecting phosphatidylinositol 3-kinase dependent early endosome fusion [25], was found to inhibit uptake but to a lesser extent (data not shown). These observations support the observations of co-localization with endosomal markers and underline the role of macropinocytosis for the uptake of bPrPp.

Evidence for bPrPp-mediated internalization and nuclear translocation of DNA

Ultra-thin sectioning and transmission electron microscopy (EM) was used to observe the localization of bPrPp-DNA-gold complexes in CHO cells. Although most of the complexes reside on the surface, in the form of aggregates, a fraction is internalized and resides within vesicles (Fig. 3, right). As a control, vesicular localization of DNA–gold in the absence of bPrPp was determined and was found to be approximately 100 times less abundant than in the presence of the peptide (Fig. 3, left).

The cell-surface bound aggregated bPrPp-cargo complexes suggest a similarity to PrPSc; adding a trypsin treatment step to the protocol failed to remove these aggregates, while using very harsh rinsing protocols (100 µg/ml dextran sulfate three times and 1 M NaCl two times) only resulted in partial removal of the aggregates. However, a much lower abundance of aggregated complexes was observed on the surface of mutant pgsA-745 cells (data not shown), indicat-
ing that peptide-cargo complex aggregation is dependent on cell-surface proteoglycans.

To investigate whether bPrPp is able to deliver its cargo to the nucleus, a luciferase-encoding plasmid was employed as cargo. The luciferase assay provides a highly sensitive means of detection of any DNA cargo that has reached the nucleus. Fig. 4 shows that bPrPp mediates uptake and nuclear delivery of a non-covalently complexed DNA cargo in a dose-response dependent manner. In the absence of peptide, no detectable luciferase is expressed. At 7 μM bPrPp (corresponding to peptide/DNA ratio (w/w) of ~5), luciferase expression is observed, and increasing the peptide concentration 4-fold results in a greater than 15-fold increase in yield. The highest level of luciferase expression by bPrPp is orders of magnitude lower than the yield obtained by a commercial transfecting agent, e.g., the cationic lipid Lipofectamine at 10 μg/ml. However, it should be noted that in order for luciferase expression to occur, the plasmid needs to dissociate from the peptide, and since the bPrPp-cargo complexes formed are highly stable (data not shown), it could be envisaged that only limited dissociation takes place, which would account for the poor expression efficiency compared to Lipofectamine. Hence, bPrPp is able to, not only facilitate endocytotic uptake of a sizeable cargo but also, transport the cargo across endosomal membranes into the cytosol, followed by the transport into the nucleus in live cells.

Discussion

The present study is a continuation of our work focusing on the hitherto less investigated N-terminal domains of PrP, comprising the uncleaved signal sequence and the neighboring NLS-like sequence. Since the N-terminus is flexible and unstructured in PrP [26-28], these domains should be freely accessible for molecular interactions in an aqueous solution, for instance with cell-surface proteoglycans or membrane bilayers.

Our studies reveal highly interesting facets of these N-terminal peptides, mPrPp, and bPrPp. The peptides have a high propensity for aggregation, particularly in the presence of a membrane (Figs. 1 and S1), readily undergoing a conformational change from α-helix to β-sheet secondary structures [29]. This suggests a role for these domains to act as the seed for further aggregation and amyloid formation of PrP. In addition, the peptides induce membrane perturbations (Figs. 1 and S1), probably through transient pore-formation [29]. Pore-formation has been proposed as
the underlying mechanism of neurotoxicity associated with prion diseases [30]. This is supported by a recent study showing that the interaction between cytoplasmic PrP and the hydrophobic lipid core of membranes is correlated with neurotoxicity [31].

mPrP and bPrP internalize through macropinocytosis (Figs. 2 and S2). While macropinocytosis has been shown to be the primary route of entry for arginine-rich CPPs [32,33], the current study presents the first evidence implicating this particular form of endocytosis in PrP internalization. Cell-surface proteoglycans appear to play a dual role: they initiate the internalization of the peptides, by facilitating their binding to the cell-surface (Fig. 1); they also enhance aggregation of the peptides, particularly in the presence of a sizeable cargo (Fig. 3).

The novel CPP-like activity of these N-terminal domains reported here could be important for intra- and inter-cellular trafficking of PrP. Since the peptides can carry a sizeable cargo into cells (Figs. 3 and 4), one could speculate that this cargo may also be the rest of PrP during its retrograde translocation. In this respect, the unprocessed PrP may be considered similar to classical proteins with “transduction domains”, such as the Antennapedia homeodomain from Drosophila [34] or the HIV-Tat protein [35]. Furthermore, this raises the intriguing possibility that the N-terminus may facilitate transport of PrP in its scrapie, PrPSc, form, in which case the CPP property may account for the infectivity associated with prion diseases. The presence of these domains exclusively in PrPs, amongst common “amyloid” related proteins [30], may explain the unique infectivity associated with prion diseases.

In conclusion, the N-terminal domains of unprocessed PrP have previously unidentified properties that may be of relevance to both the neuropathology and transmission associated with prion diseases.

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Appendix A. Supplementary data


References


NMR Solution Structure of the Peptide Fragment 1–30, Derived from Unprocessed Mouse Doppel Protein, in DHPC Micelles

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ABSTRACT: The downstream prion-like Doppel (Dpl) protein is a homologue related to the prion protein (PrP). Dpl is expressed in the brains of mice that do not express PrP, and Dpl is known to be toxic to neurons. One mode of toxicity has been suggested to involve direct membrane interactions. PrP under certain conditions of cell trafficking retains an uncleaved signal peptide, which may also hold for the much less studied Dpl. For a peptide with a sequence derived from the N-terminal part (1–30) of mouse Dpl (mDpl(1–30)) CD spectroscopy shows about 40% α-helical structure in DHPC and SDS micelles. In aqueous solution it is mostly a random coil. The three-dimensional solution structure was determined by NMR for mDpl(1–30) associated with DHPC micelles. 2D 1H NMR spectra of the peptide in q = 0.25 DMPC/DHPC bicelles only showed signals from the unstructured termini, indicating that the structured part of the peptide resides within the lipid bilayer. Together with 2H2O exchange data in the DHPC micelle solvent, these results show an α-helix protected from solvent exchange between residues 7 and 19, and suggest that the α-helical segment can adopt a transmembrane localization also in a membrane. Leakage studies with entrapped calcine in large unilamellar phospholipid vesicles showed that the peptide is almost as membrane perturbing as melittin, known to form pores in membranes. The results suggest a possible channel formation mechanism for the unprocessed Dpl protein, which may be related to toxicity through direct cell membrane interaction and damage.

Prion proteins are associated with neurodegenerative diseases called spongiform transmissible encephalopathies (TSE) (1) occurring in a variety of mammals. The diseases are characterized by the accumulation of a pathological form of the host encoded prion protein (PrP) in the infected mammal’s brain (2–4). Prion diseases are associated with the conversion of the nontoxic cellular form of the prion protein (PrPC) into the abnormally folded aggregated scrapie isoform (PrPSc). PrPSc is monomeric and easily digested by proteasome K, while PrPC forms highly insoluble aggregates and shows a high resistance to proteolytic digestion (5).

The Doppel (Dpl) protein is a homologue related to PrP. It is expressed in the brains of mice that lack expression of PrP and causes neuronal cell death. Accumulating evidence suggests that whereas PrP has a neuroprotective role, Dpl is neurotoxic (6–9). The causes of Dpl protein toxicity have been ascribed at least partly to oxidative stress and disturbance of the nitric oxide metabolism (9). However, other mechanisms of toxicity have also been suggested, which take into account the antagonistic effects of PrP and Dpl in causing toxicity. Such models involve competition for a common ligand which in the case of Dpl binding would induce cell death, or unknown antagonistic functions of PrP and Dpl (6) or multimeric pore formation by Dpl giving rise to toxic channels in the ER or cell membrane, which might be made defective by the inclusion of PrP components (10).

NMR studies of the solution structure of the mature form of bovine PrP (residues 23–230) in aqueous solution show a disordered N-terminal segment (residues 23–124), and a globular C-terminal domain extending from residue 125 (11). A basic sequence (residues 23–28) at the N-terminus, resembling a nuclear localization sequence (NLS), appears to have an important role for the internalization of the PrP via endocytosis in neurons. By mutating one of the Lys residues in the N-terminal NLS-like sequence it was shown that the internalization by endocytosis was completely abolished (12). The N-terminal sequence, ranging from residue 23 to around 100, has been shown to be responsible for internalization of the entire prion protein. The cellular trafficking, turnover, and membrane interactions of PrP are believed to be of crucial importance for the infection as well as the structure conversion associated with disease (13–19).

It has been shown that under certain circumstances the signal peptide is not cleaved off from the prion protein, but the association of such forms of the protein with infectivity or toxicity remains unclear (20, 21).

Because of the potential involvement of an intact N terminus in the pathology of PrP, we previously decided to...

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Table 1: Amino Acid Sequences of Dpl- and PrP-Derived Peptides from Different Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOUSE Dpl(1-30)</td>
<td>MKRKLQCVKLPGLASSVLYVARKGK</td>
</tr>
<tr>
<td>BOVINE Dpl(1-30)</td>
<td>MKRKLQCVKLPGLASSVLYVARKGK</td>
</tr>
<tr>
<td>HUMAN Dpl(1-30)</td>
<td>MKRKLQCVKLPGLASSVLYVARKGK</td>
</tr>
<tr>
<td>SHEEP Dpl(1-30)</td>
<td>MKRKLQCVKLPGLASSVLYVARKGK</td>
</tr>
<tr>
<td>MOUSE PrP(1-28)</td>
<td>MA-NLGVWLLFPLFVTMDVQDGLCKRPK</td>
</tr>
<tr>
<td>BOVINE PrP(1-28)</td>
<td>MA-NLGVWLLFPLFVTMDVQDGLCKRPK</td>
</tr>
<tr>
<td>HUMAN PrP(1-28)</td>
<td>MA-NLGVWLLFPLFVTMDVQDGLCKRPK</td>
</tr>
<tr>
<td>MOUSE PrP(23-50)</td>
<td>KKEFKPGQMDGTGSHYPQGGPSGRYP</td>
</tr>
</tbody>
</table>

study an N-terminal segment of PrP and its interaction with biliary membranes. We could observe that the N-terminal part (residues 1–28) of the mouse PrP (mPrP1–28), including the signal sequence, functions as a cell penetrating peptide (CPP), able to facilitate the transport of large hydrophilic cargoes through a cell membrane (22). It was further demonstrated that the peptide could adopt a wide range of secondary structures, ranging from α-helical in neutral vesicles to mostly β-sheet structure in negatively charged vesicles (22). Recently an NMR solution structure of the bovine N-terminal (1–30) fragment of PrP (bPrP1–30) associated with DHPC micelles was determined, and the α-helical form of the peptide was suggested to reside orthogonal to the phospholipid bilayer in bichelles (23).

The structure of the mouse Dpl protein, which has a 23% identity to PrP in the primary structure, but has 179 amino acids instead of 254 for mPrP, has revealed a similar overall topology to that of PrP. The globular C-terminal parts have similar folded structures, whereas the N-terminal parts are largely unstructured in both cases (24). In the case of Dpl, the N-terminal part lacks both the octarepeat region and the hydrophobic region around residue 120 which are present in PrP. Like PrP the mature Dpl is expressed on the cell surface and anchored there with a glycosylphosphatidylinositol moiety (25), and Dpl also contains a basic sequence at the N-terminus (25–30). Much less is known about the cell trafficking of Dpl than of PrP, but in view of their homologous properties it is not unlikely that also Dpl under certain conditions may appear in an unprocessed form containing the signal peptide. Since the intriguing antagonism of the PrP and Dpl proteins may have a basis in their membrane interactions, we decided to study the segment of Dpl that corresponds to the unprocessed N-terminus of PrP, and its properties in a membrane-mimicking environment. We also compare their membrane perturbation potencies in terms of causing leakage from liposomes and conclude that mDpl(1–30) is indeed a highly potent inducer of leakage in model membranes.

In the present study we have investigated the structure and membrane-association properties of the peptide containing 30 amino acid residues derived from the mouse Dpl protein (mDpl(1–30)). Table 1 shows the sequence of this peptide, and the corresponding sequence of mPrP(1–28), as well as some variants from other species. We discuss the structure similarities and differences between the Dpl peptide and the corresponding peptides derived from bovine and mouse PrP in a DHPC micellar solvent, mimicking a membrane environment.

**MATERIALS AND METHODS**

**Sample Preparation.** The peptides melittin, mouse Doppel peptide mDpl(1–30) derived from the mouse Dpl protein, and mouse prion peptides mPrP(1–28) and mPrP(23–50) from the mouse PrP protein were obtained from Neoystem Laboratories (Strasbourg, France; immunograde purity) and used without further purification. The peptide concentration was determined by light absorption on a CARY 4 spectrophotometer with a theoretically calculated molar absorptivity of Trp of 5500 M⁻¹ cm⁻¹ at 280 nm. Nondeterated dihexanol-sn-glycero-3-phosphatidylcholine (DHPC) and undeterated dihexanol-sn-glycero-3-phosphatidylcholine-d₂₅ (DHPC-d₂₅), 1,2-dimyristoyl-d₂₅-sn-glycero-3-phosphocholine (DMPC-d₂₅), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-rac-(1-glycerol) sodium salt (POPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Deterated SDS-d₂₅ was purchased from Cambridge Isootope Laboratories, Inc. Calcein, a fluorescein derivative, was purchased from Molecular Probes, The Netherlands (Proacet N 481). Triton X-100 was obtained from Sigma. Packed PD-10 columns, Sephades G-25 M, were obtained from Amersham Biosciences.

For the 1H NMR experiments, a sample containing 1.5 mM peptide dissolved in 100 mM DHPC-d₂₅ was used. 10% 2H₂O was added for field/frequency lock stabilization. The pH was 3.5. For 3H₂O exchange measurements a sample prepared in H₂O was freeze-dried for 24 h and subsequently dissolved in 2H₂O immediately prior to conducting NMR experiments. Bicelle samples with q = 0.25 were prepared by mixing DMPC-d₂₅ with water and 10% 2H₂O. The inhomogeneous mixture was vortexed repeatedly and dissolved by the addition of 1 M DHPC-d₂₅ stock solution to a final concentration of 25 mM DMPC-d₂₅ and 0 mM DHPC-d₂₅.

CD spectra were recorded for mDpl(1–30) in aqueous solution, or in solvents containing 150 mM SDS or DHPC, or 10 and 40 mM DHPC, and in 2 mM Na phosphate buffer. For comparison, CD measurements were also performed for the mPrP(1–28) peptide derived from the 28 N-terminal amino acid sequence of the mouse PrP in the same solvents. Large unilamellar vesicles (LUVs) were prepared by dissolving the phospholipids—POPC and POPG in a desired ratio—in chloroform. The chloroform was evaporated using an argon flow, thus leaving a lipid film. Calcein (55 mM) in 50 mM potassium phosphate buffer (pH 7.4) was used to disperse the lipid film during 10 min of vortexing. The fluorescence of calcein is well self-quenched at 55 mM. A final concentration of 10 mM lipids was achieved. The dispersion was freeze–thawed five times using liquid nitrogen/hot water and then passed 21 times through two polycarbonate membranes of 100 nm pore size using an Avestin pneumatic actuator. The LUVs were then passed through three Sephades-G25 columns in order to remove the non-entrapped calcein. The vesicles were diluted with 50 mM potassium phosphate buffer (pH 7.4) to a desired concentration (~40) µM and kept on ice when not involved in measurements. They were used directly after preparation.

**Peptide-Induced Calcein Leakage from LUVs Studied by Steady-State Fluorescence Spectroscopy.** The fluorescence measurements were conducted on a Perkin-Elmer LS 50B luminescence spectrometer with FL WinLab software. The
experiments were run at room temperature in 4 × 10 mm quartz cuvettes. Calcein was excited at 490 nm, and its emission was scanned from 510 to 530 nm at a rate of 250 nm/min. The bandwidth for excitation and emission was 4 nm. Each measurement was averaged over five times, baseline-corrected, and compensated for small differences in peptide concentration. Three series of experiments were done for each peptide.

The calcein entrapping LUVs (POPG:POPC, 2:8) were diluted to a phospholipid concentration of ~400 µM, and 750 µL was used for each experiment. The peptides were titrated into the sample with an incubation time of 3 min at 20 °C. The vesicles were lysed at the end of each experimental series with 5 µL of 10% (v/v) Triton X-100 to establish the maximum fluorescence intensity corresponding to each preparation.

**CD Spectroscopy.** The CD measurements were made on a Jasco J-720 CD spectrometer with a 0.05 mm quartz cell or on an Aviv model 202 CD spectrometer with a 2 mm quartz cell. A PTC-343 controller regulated the temperature. CD scans ranging from 5 to 75 °C were performed to check for the temperature dependence of the secondary structure.

**NMR Spectroscopy.** The two-dimensional 1H NMR measurements were performed on a Varian Inova spectrometer equipped with a triple-resonance probe head operating at 800 MHz 1H frequency or on a Bruker Avance spectrometer operating at 500 MHz equipped with a cryoprobe. Two-dimensional TOCSY spectra (26) were recorded with mixing times of 30, 60, and 80 ms, and two-dimensional NOESY spectra (27) were recorded with mixing times of 50, 150, and 300 ms. Water suppression was achieved with the WATERGATE pulse sequence (28). The spectra were processed with the program Felix (version 2000, Accelrys Inc.). For the 1H exchange experiments a series of TOCSY spectra with mixing times of 30 or 80 ms as well as 1D experiments were performed. The exchange was monitored for 24 h in about 5 h intervals. All 2D NMR experiments were performed at 45 °C. A 2D 1H TOCSY spectrum with a mixing time of 80 ms was recorded for 1 mM mDPi(1−30) in a q = 0.25 bicele solution.

Diffusion measurements were performed on a Bruker Avance 400 MHz spectrometer as well as on a Varian Inova 600 MHz spectrometer, using a pulsed field gradient spin–echo experiment (29). To correct for inhomogeneities in the gradient, a distribution function was included for the gradient, a procedure which significantly improves the accuracy of the diffusion coefficients as previously described (30). To avoid flow convection effects, the measurements were carried out at a lower temperature (25 °C) as compared to the 2D 1H NMR experiments. The diffusion coefficient derived from this data was used in combination with the Stokes–Einstein relation to calculate an approximate aggregate radius size. For a simple sphere of radius R the linear translational diffusion is given by

\[
D = \frac{kT}{6\eta_0R}
\]

where \(k\) is the Boltzmann constant and \(\eta\) is the dynamic viscosity of the medium. A hydration shell of 2.8 Å was assumed for the aggregates. To test the effect of the micelles on the peptide, a 100 mM DHPC solution was prepared and divided into two parts. The first sample was used for diffusion measurements of the lipid micelle alone. mDPi(1−30) (1 mM) was added to the other and was used for measuring the diffusion of the peptide and the lipid micelle in the presence of peptide. The same was done with a bicele solution. Measurements were also performed for the mDPi(1−30) alone in aqueous solution and of the monomeric DHPC at 0.5 mM, which is much lower than the critical micelle concentration. 10% H2O was added to all samples for field frequency lock stabilization.

**Structure Calculation.** Distance constraints were generated from quantifying NOE cross-peak intensities as described earlier (31). Cross-peaks were categorized into five different groups, with upper distance limits of 3.0, 3.5, 4.0, 4.5, and 6.0 Å, respectively, based on their intensities. The upper distance limits were normalized against known distances (32). A total of 263 distance constraints (93 intra, 89 sequential, and 81 medium range) were obtained from the assigned NOE spectrum with \(r_{max} = 150\) ms. Structures were calculated with the program DYANA (35) version 1.5, using torsion angle dynamics. Standard annealing algorithms were used, and a total of 60 structures were calculated. Out of these, 20 structures were selected, based on their target function and constraint violations, to represent the solution structure. The quality of the structure was checked with the program PROCHECK_NMR (34). Visual analyses of the solution structures were made with PyMol (version 0.98rc5). The coordinates of the final structure together with the input constraints have been deposited in the PDB under accession code 1ZFS.

The chemical shifts assignments were also deposited in the BMRB database under the deposition code BMRB-6598.

**RESULTS**

**CD Spectroscopy.** The structure induction caused by DHPC in mDPi(1−30) was compared to that in mPrP(1−28), for which earlier studies had revealed striking variations in secondary structure depending on conditions (22). Figure 1 shows the CD spectra of mPrP(1−28) in water and weak (2 mM, pH 7) phosphate buffer (a) and in 10 mM and 40 mM DHPC (c). The corresponding spectra of mDPi(1−30) are shown in Figure 1, b and d. The CD spectra show that in water mPrP(1−28) is close to random coil secondary structure, whereas there is a somewhat higher structure propensity for mDPi(1−30). In 2 mM phosphate buffer both peptides display a mixed partly α-helical structure. When DHPC is added up to 40 mM, the mPrP(1−28) gradually shifts toward a β-sheetlike CD spectrum (Figure 1c), whereas the mDPi(1−30) remains a stable partial α-helix (Figure 1d). We conclude that the mDPi(1−30) peptide lacks the structure variability of mPrP(1−28). It remains α-helical under a variety of conditions, and this probably reflects its weaker tendency for aggregation with concomitant β-structure induction.

Using CD we investigated the secondary structure of mDPi(1−30) under conditions similar to those used in NMR spectroscopy. Figure 2 shows the CD spectra of 1.33 mM peptide in an aqueous solution with 150 mM DHPC, and also includes CD spectra recorded for less concentrated peptide in water and in 150 mM SDS. Also under these conditions there is α-helical structure to around 40% in DHPC, as well as in SDS solvents. The structures are stable in the temperature range from 5 to 75 °C (data not shown).

**NMR Spectroscopy and Structure Calculation.** We chose to study the mDPi(1−30) structure in a DHPC micelle.
environment. The NMR spectra in DHPC micelles had sufficient quality to yield sequence specific assignments using standard procedures. The NMR spectra observed in SDS micellar solvent were less well resolved. For the peptide in DHPC solvent, chemical shifts for the backbone protons were obtained for all residues, except Met1 and the majority of the side-chains were assigned. The secondary chemical shifts for the Hα protons, calculated according to ref 35, indicated an α-helical central part between residues Thr7 and Ser19 (Figure 3), corresponding to 43% helical content. This is in good agreement with the estimate from the CD spectrum obtained under similar conditions (Figure 2).

Figure 1: CD spectra recorded at 20 °C: [a] (○) 40 μM mDP-I(1–28) in water, (●) 40 μM mDP-I(1–28) in buffer; [b] (□) 40 μM mDP-I(1–30) in water, (■) 40 μM mDP-I(1–30) in buffer; [c] (△) 40 μM mDP-I(1–28) in 10 mM DHP in buffer, (●) 40 μM mDP-I(1–28) in 40 mM DHPC in buffer; [d] (□) 40 μM mDP-I(1–30) in 10 mM DHP in buffer, (■) 40 μM mDP-I(1–30) in 40 mM DHPC in buffer. [G] is the mean residual molar ellipticity, with the dimension [mdeg cm² dmol⁻¹]. The buffer was 2 mM Na phosphate buffer, pH 7.0.

Figure 2: CD spectra recorded for mDP-I(1–30) at 25 °C: (○) 150 μM mDP-I(1–30) in H₂O; (■) 1.33 mM mDP-I(1–30) in 150 mM DHPC at 5 °C; (▲) 84 μM mDP-I(1–30) in 150 mM SDS. [G] is the mean residual molar ellipticity, with the dimension [mdeg cm² dmol⁻¹].

Figure 3: Experimental secondary chemical shifts for the Hα protons, calculated according to ref 35, of mDP-I(1–30) in 150 mM DHPC solvent.

Figure 4: Summary of inter-residue constraints used in the structure calculation for mDP-I(1–30) derived from a 2D NOESY spectrum with τmix = 150 ms. The residues for which Hα–Hα cross-peak intensities are unaffected in the D₂O exchange experiments are also displayed.

From the assigned cross-peaks in the NOESY spectrum, 263 distance constraints were generated and used in the structure calculation. Figure 4 shows a summary of the inter-residue connectivities. The structure, as represented by an ensemble of the 20 best structures, is shown in Figure 5, and the structural statistics are collected in Table 2. Based on analyses of backbone torsion angles and hydrogen bonding patterns, it can be concluded that a helical structure is seen for residues Trp8 through Ser19, again in agreement with the secondary chemical shifts and CD results. It can, however, be noted that there are indications that the helix extends further, both at the N-terminal as well as C-terminal, but that the structure is poorly defined in these regions.
Another interesting feature of the NMR ensemble is the localization and position of the tryptophan residues. First of all, these are close to the N-terminal end of the α-helix. This would position them close to the polar group interface. Also, as we could clearly assign a few Trp side-chain NOEs, we can see that they are positioned on the plane of the rings parallel to the α-helix axis. Therefore, since the α-helix is orthogonal to the lipid bilayer (see below), so are the Trp side-chains, which is a favorable positioning at the membrane interface. It is notable that Trp residues are present in all Doppel variants (Table 1) and particularly in human Doppel Trp is present in three consecutive copies.

Amide proton exchange experiments were carried out by monitoring the remaining peak amplitudes in a series of TOCSY spectra recorded with a 3-h interval. Part of a TOCSY spectrum recorded after 3 h is shown in Figure 6. The $^2$H$_2$O exchange experiments show that signals corresponding to $^2$H protons remain for residues Val18 through Ala18 even after 24 h in $^2$H$_2$O at 45 °C. The signals from Trp$^9$ $^2$H$^5$ are still visible after 3 h but disappear after 6 h. For the remaining residues, the signals disappear in seconds. This most likely indicates that amide protons for which signals remain are protected from the solvent by the DHPC environment, being located in the hydrophobic interior of the micelle, and shielded from direct contact with the $^2$H$_2$O solvent.

To compare membrane mimicking solvents, 2D $^1$H NMR was also carried out for the peptide in $^2$H$_2$O solution. However only $^2$H$^5$--$^2$H$^5$ cross-peaks belonging to the unstructured regions could be observed, while those belonging to the central structured stretch were broadened beyond detection. This further supports the conclusion that the central part forms a stable, rigid helical structure protected from solvent also in a bilayer membrane.

**Peptide–Lipid Interactions.** NMR diffusion experiments were carried out to probe the size of the peptide–micelle complex and any possible change in the DHPC micelle aggregation number caused by association with the peptide. The comparative diffusion measurements of mDpl(1–30), DHPC, and DMPC in various samples are displayed in Table 3. In general all diffusion coefficients measured here have errors of less than a few %. The peptide in water has a
diffusion coefficient of $16.6 \pm 0.4 \times 10^{-11}$ ms$^{-2}$ which according to the Stokes–Einstein relation corresponds to a radius of $R_1 = 14.8 \pm 0.4$ Å. Using the relation given by (36) gives $R_0 = 15.7$ Å, which is close to the hydrodynamic radius expected for a peptide of this size.

The diffusion constant of mDpl(1–30) in DHPC micelles was calculated using various peptide resonances, which were distinctively clear and separated from DHPC proton peaks. The constants were averaged and gave a linear diffusion constant of $D = 8.87 \pm 0.47 \times 10^{-11}$ ms$^{-2}$ for the peptide–DHPC complex. Using the Stokes–Einstein relation for the diffusion of a sphere, we calculated a corresponding hydrodynamic radius of the peptide–DHPC aggregate to be $R_0 = 27.2 \pm 1.4$ Å, clearly larger than for the peptide alone. The error estimate is based on the accuracy of the diffusion constant, as determined from multiple experiments, as well as from the value for the viscosity, $\eta = 0.906 \pm 0.005$ cP, of the medium. The viscosity is derived for a solution containing 5% to 10% H$_2$O at 25 °C.

Assuming a hydration shell of 2.8 Å, a lipid density of 1100 kg/m$^3$, and a peptide density of 1300 kg/m$^3$ we get a total micelle volume of 60 nm$^3$ with the peptide. Subtracting the volume of the peptide yields an apparent micelle size of 56 nm$^3$. That corresponds to an average micellar molecular weight of 37.3 ± 8.8 kDa or an aggregation number of 78 ± 19 in the presence of the peptide. This is substantially larger than a normal micelle. For DHPC the critical micellar concentration has been reported as 14 mM, and the micelle aggregate has an average weight of 15–20 kDa with a narrow size distribution (37). This corresponds to 36 DHPC molecules per micelle.

DHPC has a substantially larger diffusion coefficient than the peptide in the micellar solution, which can be explained by the assumption that there is always some free monomeric DHPC, diffusing much faster than the peptide–micelle aggregate. We can conclude that for the most part the diffusion for DHPC is affected by the monomeric DHPC concentration. Yet we can qualitatively see the effect of the peptide in the micelle from observing a decrease in the diffusion coefficient, which indicates an increase in the micelle size.

Diffusion was also measured for mDpl(1–30) in $q = 0.25$ biciples. From inspecting Table 3 we see that the peptide and DMPC have very similar diffusion coefficients, the same within the experimental error, while DHPC diffuses on average faster. This is due to the fact that a substantial fraction of DHPC exists as monomer in solution (38, 39), while all of the DMPC is biciple-bound. This shows that more or less all peptide is bound to the bicalle particle. From the DMPC diffusion coefficient we estimate a particle radius of $32.9 \pm 3.3$ Å. If we take into account the ratio of [DMPC]/[DHPC] = 0.25 and assume a similar density for the two lipids and take into account their molecular weights, we conclude that on average the bicle consists of approximately 27 molecules of DMPC and 109 molecules of DHPC wrapped around the peptide.

Figure 7 shows the structured peptide enclosed in a modeled DHPC micelle. The diameter of this aggregate agrees well with that determined from the diffusion data, as also determined from the specific volume estimation above. We conclude that the peptide is enclosed by a DHPC micelle. The micelle however may be more accurately modeled as a barrel with the peptide in the interior acting as a nucleation center and the DHPC molecules arranged cylindrically around it. The diameter of this DHPC micelle is around 50 Å. Still, the overall picture of a peptide for which the most hydrophobic segment is enclosed by the hydrophobic part of the lipid chains should be a realistic one.

Membrane Leakage Studies. Figure 8 shows the membrane perturbation caused by mDpl(1–30), mPrP(1–28), mPrPp(23–50), and melittin. Melittin, included as a reference, is a potent toxic peptide known to form pores in membranes.
Peptide Fragment 1–30

It is clear that mDpl(1–30) is even more potent than mPrP-(1–28) in causing leakage of calcein from large unilamellar vesicles (LUVs) composed of phospholipids with a fraction of negatively charged headgroups (POPC with 30% POPG). On the other hand mPrP(23–50), the N-terminus of the progressively processed PrP, causes no leakage at even higher concentrations.

**DISCUSSION**

The existence of an unprocessed signal peptide has been shown under certain conditions for PrP (40). The existence of a corresponding form of the Dpl protein has not been reported, but since its cell trafficking pathways are similar to PrP we may expect this form to appear also for Dpl. The present study deals with the properties of these unprocessed segments. They should be considered as extensions of the N-termini of the proteins, which are unstructured in aqueous solution and should be free to interact with membranes in a cell environment.

The NMR structure of mDpl(1–30) peptide associated with DHPC micelles is, under similar conditions, similar to that of the bPrP(1–30) peptide, derived from the N-terminal sequence of the bovine PrP (23). bPrP(1–30) is suggested to act as a cell-penetrating peptide, and may be responsible for the internalization of the entire protein. Interestingly, the peptide derived from the bovine PrP was observed to affect a bilayer membrane in phospholipid bicelles in a way that is characteristic of a transmembrane configuration (23). In that case amide proton exchange experiments showed clearly that a central part of the peptide dissolved in DHPC was protected from solvent. In the present study there is a clear similarity between the amide exchange results for the mDpl-(1–30) and bPrP(1–30) peptides. No resonances could be observed for the helical part of either mDpl(1–30) or bPrP(1–30) in small fast-tumbling bicelles, indicating that both peptides are rigidly attached to the bicelle, with the helical part residing within the bicelle interior. This suggests that also mDpl(1–30) should adopt an orthogonal orientation in a phospholipid bilayer.

These results are in contrast to what has been observed for other helical membrane-interacting peptides, like the typical CPPs penetratin or transportan, which have amphipathic character and reside within the headgroup region in a phospholipid bilayer. For these peptides amide proton exchange occurs within minutes (41, 42). The sequences of both mDpl(1–30) and bPrP(1–30) do not have the characteristics of amphipathic helices, but instead contain quite hydrophobic central regions, with an N-terminal flanking Trp residue (Figure 9). Interestingly, the amphipathic peptides penetratin and transportan gave resolved and assignable spectra in bicelles, and were found to be localized in the headgroup region of the phospholipids (41, 42). Although we cannot at this stage give a detailed explanation for the different NMR characteristics, we believe that they have to do with different mobility and exchange properties of a surface bound peptide compared to a transmembrane peptide.

The diffusion data together with the structure of mDpl-(1–30) provides us with a model for the DHPC–peptide complex (Figure 7). The size of the aggregate indicates that the hydrophobic part of the peptide forms a helix, surrounded by the lipid chains of the DHPC micelle. The indication that the flanking residues at both the N- and C-terminal parts of

![Figure 9: Hydrophobicity plot for mDpl(1–30) (C) and bPrP(1–30) (●), calculated according to ref 44.]

the helix also have partly helical character suggests that the helix may be longer in a real membrane where the lipid chains are longer.

There are notable differences in the membrane interaction between the N-terminal sequences of the PrP and Dpl proteins. The CD results presented here for mDpl(1–30) and mPrP(1–28) (Figure 2) show that the peptide derived from the Dpl protein has a higher propensity for forming stable α-helical structures, as this structure remains under a variety of conditions in terms of peptide/DHPC stoichiometry. The peptide derived from PrP, on the other hand, is seen to have the possibility to form a wider range of structures, including α-helix and β-sheet depending on parameters such as peptide/DHPC stoichiometry. For both peptides, the results suggest that membrane interactions of the N-termini of the whole proteins may be important for structuring of the sequences. Formation of a stable α-helix in the N-terminus can be taken to indicate that the corresponding segment is not prone to uncontrolled peptide aggregation, which may be the case when β-sheet induction is observed. The observed differences in structure propensity between the N-terminal segments of PrP and Dpl may provide part of the background for their different biological activities, particularly when direct membrane interaction effects are concerned. On the other hand, transmembrane pores formed by well-defined oligomeric aggregates of α-helices are typically associated with membrane-toxic peptides such as melittin derived from bee venom (43). In that case the oligomeric, aggregated form of the peptide assembles in the bilayer and forms a channel. A similar situation may occur for the N-termini of unprocessed Dpl proteins interacting with bilayer membranes. This hypothesis is supported by the membrane leakage experiments shown in Figure 8. mDpl(1–30) was found to be almost equally potent as melittin in inducing leakage in LUVs. We conclude that the special combination of fairly hydrophobic signal sequences followed by a highly basic patch is very efficient in causing membrane leakage. These results argue for further cell biology experiments where the trafficking of Dpl is compared with that of PrP and the possible existence of unprocessed Dpl is investigated in experiments where reasons for the observed neurotoxicity are explored.
An animated visualization of mDpl(1–30) in micelles and bicalcis together with the corresponding coordinate files and images of the peptide structure and constraints used. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

Relevance of the N-terminal NLS-like sequence of the prion protein for membrane perturbation effects.

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Abstract: We investigated the nuclear-localization-like sequence KKRPKP, corresponding to the residues 23-28 in the mouse prion protein (mPrP), for its membrane perturbation activity, by comparing effects of two mPrP-derived peptides, corresponding to residues 1-28 (mPrPp(1-28)) and 23-50 (mPrPp(23-50)), respectively. In erythrocytes, mPrPp(1-28) induced ~60% haemoglobin leakage after 30 min, whereas mPrPp(23-50) had negligible effects. In calcein-entrapping, large unilamellar vesicles (LUVs), similar results were obtained. Cytotoxicity estimated by lactate dehydrogenase leakage from HeLa cells, was found to be ~12% for 50 μM mPrPp(1-28), and ~1% for 50 μM mPrPp(23-50). Circular dichroism spectra showed structure induction of mPrPp(1-28) in the presence of POPC:POPG (4:1) and POPC LUVs, while mPrPp(23-50) remained a random coil. Membrane translocation studies on live HeLa cells showed mPrPp(1-28) co-localizing with dextran, suggesting fluid-phase endocytosis, whereas mPrPp(23-50) hardly translocated at all. We conclude that the KKRPKP-sequence is not sufficient to cause membrane perturbation or translocation but needs a hydrophobic counterpart.

Keywords: prion protein; membrane translocation; calcein leakage; endosomal escape; NLS-like sequence; haemoglobin leakage.
Abbreviations: NLS, Nuclear Localization Sequence; PrP, Prion Protein; PrP<sup>c</sup>, cellular isoform of PrP; PrP<sup>Sc</sup>, scrapie isoform of PrP; mPrP<sup>PrP-(1-28)</sup>, residues 1-28 of the mouse PrP sequence; mPrP<sup>PrP-(23-50)</sup>, residues 23-50 of the mouse PrP sequence; bPrP<sup>PrP-(1-30)</sup>, residues 1-30 of the bovine PrP sequence; C, Circular Dichroism; LUVs, Large Unilamellar Vesicles; hRBC, Human Red Blood Cell; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPOP 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt; LDLH, Lactate Dehydrogenase; CPP, Cell-Penetration Peptide; PTD, Protein Transduction Domains; HeLa cells, Henrietta Lacks cells; CHO cells, Chinese Hamster Ovary cells; TSE, Transmissible Spongiform Encephalopathies.

1. Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal neurological disorders of humans and animals that can occur sporadically, by infection, or can be hereditary. The disorders are thought to be caused by a conversion of the cellular form of the neural prion protein (PrP<sup>c</sup>), into an infectious scrapie isoform (PrP<sup>Sc</sup>) [1-3]. The conversion is posttranslational and mutations of the primary amino acid sequence are not needed for the conversion to take place. PrP<sup>c</sup> forms insoluble aggregates that show high resistance to proteinase K digestion, whereas PrP<sup>Sc</sup> is monomeric and is readily digested via proteolytic processes [4].

The prion protein consists of an unstructured N-terminal domain (composed of a signal peptide (1-22 in the mouse protein), a basic region (23-28), a procaseptapeptide region (29-50), an octapeptide region (51-105) prone to bind copper-ions, and a hydrophobic stretch (106-125), a globular C-terminal domain (126-231) comprised of three α-helices and two β-turns, and a C-terminal signal peptide (232-254) [5].

The full length prion protein is rarely examined together with its signal peptide (residues 1-22) since the signal peptide part is normally cleaved off during maturation. It has however been shown that in some cases this part can be retained [6], and that this particular sequence might be associated with functions such as targeting and topogenesis [6-8]. It has also been proposed that the uncleaved signal peptide could cause the prion protein to change its conformation and thus become neurotoxic [6, 9]. Hypothetically the mature PrP<sup>c</sup> might even play a neuroprotective role, reversing neurotoxicity induced by the doppel (Dpl) protein, which is a PrP homologue. Deletion of the charged region PrPA (23-28) left the mutated PrP incapable of rescuing cerebellar neurons in transgenic mice [5].

The signal peptide is by itself hydrophobic and relatively insoluble in water, but taken together with the highly positive, nuclear localization-like (NLS-like) [10] KKKRPKP-sequence (residues 23-28), it becomes water soluble. The corresponding peptide has a sequence that resembles the composition of certain primary amphiphatic cell-penetrating peptides (CPPs). These peptides have a primary structure that contains distinct hydrophobic and hydrophilic regions. Transporter (a chimeric peptide composed of galactamin interlinked with mastoparan by a lysine) and MAP (an amphiphatic model peptide) are two well-known CPPs that are considered to be members of the above mentioned class [11-14]. CPPs, also known as protein transduction domains (PTDs), in general have been used as transmembrane delivery vectors of hydrophilic molecules. Different mechanisms have been debated, but endocytosis followed by endosomal escape seems to dominate. There have been few sequence similarities found between different classes of CPPs, but nevertheless attempts to predict CPPs based on bulk properties of the constituent amino acids have been conducted with fair success [15]. The most common definitions of CPPs include a length restriction around 30 amino acids, a net positive charge and often either primary or secondary amphipathicity.

It has been shown that both the bovine and the mouse prion protein-derived peptides (abbreviated bPrP<sup>PrP-(1-30)</sup>, and mPrP<sup>PrP-(1-28)</sup> respectively) can translocate over cell membranes even when coupled to large hydrophilic cargoes. Concomitantly these peptides cause cell toxicity and are thus not good candidates as useful CPPs [9, 16]. However, the capability of crossing biomembranes suggests a plausible explanation for how oral prion infection might occur in vivo; an unprocessed PrP could in theory cross physiological barriers – like the gut epithelium or the blood brain barrier (BBB) – via a process where the CPP-like part of the protein would drag the rest of the prion protein (or an aggregated form of it) along as cargo. This type of in vivo protein transduction through the BBB has been demonstrated earlier for the Tat-β-galactosidase fusion protein (120 kDa) [17]. The Tat-peptide is a CPP derived form the protein transduction domain of the human immunodeficiency virus 1 (HIV-1) Tat protein [18].

This particular study focuses on the relevance of the NLS-like part of the PrP, i.e. residues 23-28 forming the KKKRPKP-sequence. A new peptide, denoted mPrP<sup>PrP-(23-50)</sup> (see Table 1 for peptide sequences), with the same length as mPrP<sup>PrP-(1-28)</sup> and containing the KKKRPKP-sequence was constructed. Its properties are compared to mPrP<sup>PrP-(1-28)</sup> regarding membrane perturbation and translocation ability, both in membrane mimetic and living cell systems. The KKKRPKP sequence has been suggested to guide the cellular trafficking of PrP and to be responsible for direct internalization of the prion protein via endocytosis [19]. A recent study also shows that the PrP<sup>PrP-(1-28)</sup> sequence can selectively bind to PrP<sup>PrP-(Sc)</sup> and not to PrP<sup>c</sup> [20].

The present results that show great differences between the high and low membrane perturbing activities of mPrP<sup>PrP-(1-28)</sup> and mPrP<sup>PrP-(23-50)</sup>, respectively, indicate that the KKKRPKP segment may be necessary, but is not
sufficient for the membrane perturbing activities of the unstructured part of PrP.

2. Materials and Methods

2.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-
rac-(1-glycerol)] sodium salt (POPG) were purchased from Avanti Polar Lipids, Alabaster, of best quality and used without further purification. Triton X-100 was obtained from Sigma. The pre-packed PD-10 Columns, Sephadex™
G-25 M, were obtained from Amersham Biosciences. Calcium, a fluorescein derivative, was purchased from Molecular Probes, The Netherlands (product no. C-481). 5(6)-carboxyfluorescein and rhodamine-B labelled dextran (70 kDa) were obtained from the same company.

The peptides (without fluorescent labels) were either purchased from NeoSystem Laboratoire (Strasbourg, France) and used without further purification, or synthesized on a peptide synthesizer from Applied Biosystems (model 431A, USA). See table 1 for sequences. The tert-Butyloxy-carbonyl amino acids and the pamo-methylbenzhydrolamine (MBHA) resin were also acquired from NeoSystem Laboratoire. Table 1 shows the sequences, relative hydrophobicities and Gibbs free energies of the studied peptides.

HeLa cells were obtained from the American Type Culture Collection (Manassas, USA). The cells were cultivated in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. All cell culture reagents were purchased from Invitrogen (Stockholm, Sweden).

2.2 Peptide synthesis, purification and analysis

2.2.1 Peptide synthesis and carboxyfluorescein labelling

The amino acids were linked in a stepwise manner in a 0.1 mmol scale on a peptide synthesizer using t-Boc strategy of solid-phase peptide synthesis. tert-Butyloxy-carbonyl amino acids were coupled as hydroxybenzotriazole (HOBr) esters to a p-methylbenzhydrolamine (MBHA) to obtain C-terminally ammated peptides. The peptides were fluorescein labelled by the use of 5 molar equivalents of 5(6)-
carboxyfluorescein, 5 molar equivalents of DIC, 5 molar equivalents of HOBt and 20 molar equivalents of DIEA in DMF (dimethylformamide) overnight. Deprotection of the formyl protecting group on tryptophan was carried out in 20% piperidine in DMF for 60 min. The peptides were finally cleaved from the solid phase with HF (hydrogen fluoride) at 0 °C during 1 h in the presence of p-cresol, or p- cresol and p-thiocresol (1:1) if the peptide sequence contained cysteine or methionine residues.

The cleaved peptides were purified using a reversed-phase HPLC formega C18 column and analyzed using a Perkin Elmer proTOF™ 2000 MALDI O-TOF mass spectrometer.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net Charge</th>
<th>Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>DGDYGIKFLGDGLGLGLGLGLGQQ</td>
<td>+5</td>
<td>0.27</td>
</tr>
<tr>
<td>mPrP(1-28)</td>
<td>MKLMLKMLKTDIYVLGLGFTP</td>
<td>+3</td>
<td>0.30</td>
</tr>
<tr>
<td>Penetratin</td>
<td>DGDYGIKFLGDGLGLGLGLGLGQP</td>
<td>+7</td>
<td>-1.73</td>
</tr>
<tr>
<td>mPrP(23-50)</td>
<td>KRDLGQKDDKMLDDK</td>
<td>+6</td>
<td>-1.86</td>
</tr>
</tbody>
</table>

2.2.2. HPLC purification and analysis of synthesized peptides

2.2.3 Determination of peptide concentration

Peptides were dissolved in distilled water and the concentration of the solutions was established using a quartz cuvette of 1 cm path length in a CARY 4 spectrophotometer at 280 nm. The molar extinction coefficients used were 5690 M⁻¹cm⁻¹ for tryptophan, 1280 M⁻¹cm⁻¹ for tyrosine, and 120 M⁻¹cm⁻¹ for cysteine [21].

2.3 Large Unilamellar Vesicles

2.3.1 Preparation of LUVs

Large Unilamellar Vesicles (LUVs) were prepared by dissolving the phospholipids POPC and POPG in a desired molar ratio in chloroform. The solution was vortexed in order to ensure proper mixing of the components, and the chloroform was evaporated using argon gas. The remaining lipid film was then placed under vacuum for a minimum time of 1 h in order to ensure the complete removal of residual chloroform. The dried lipid film was dispersed in 50 mM potassium phosphate buffer, pH 7.4, by vortexing (10 min) resulting in a final lipid concentration of either 1 or 10 mM (based on weight to volume). The dispersion was freeze-thawed five times using liquid nitrogen/heat water and then passed through two membranes of 100 nm pore size 21 times using a pneumatic Avanti extruder. The vesicles were kept on ice when not involved in measurements and always used within 24 h.

2.3.2 Preparation of calcine-entrapping LUVs

A 55 mM calcine solution in 50 mM potassium phosphate buffer pH 7.4 was prepared. The solution was passed once through two 100 nm pore sized membranes before used to disperse a dried lipid film of desired composition. The resulting 10 mM lipid dispersion was vortexed, freeze-thawed and extruded as described above. The formed calcine-entrapping vesicles were passed through three sequential Sephadex-G25 columns in order to remove the non-entrapped calcine. The dilution factor of each column is estimated to be ~ 1.5 according to the manufacturer.

2.4 Steady-state fluorescence spectroscopy
The calcein leakage measurements were conducted on a Perkin Elmer LS 50B Luminescence Spectrometer using FL WinLab software. The experiments were run at room temperature in a 4x10 mm quartz cuvette. The samples were excited at 490 nm and their emission was scanned from 510 to 600 nm. The scan speed was 250 nm/min and the bandwidth for excitation and emission was set to 4 nm. Each spectrum was baseline corrected and averaged over five accumulated scans. The fluorescence of calcein is well self-quenched at 55 mM.

2.4.1 Peptide-induced calcein leakage from calcein-entrapping LUVs

Calcein-entrapping LUVs POPC:POPG (4:1) were diluted with 50 mM potassium phosphate buffer pH 7.4 to a phospholipid concentration of ~400 mM and 750 µM were used for each experiment. Each peptide (stock solution 100 µM) was titrated into the sample (3 µl at a time) with an incubation time of 3 minutes preceding each measurement. The vesicles were lysed with 5 µl of 10% (v/v) Triton X-100 at the end of each titration series in order to establish the maximum fluorescence intensity corresponding to each sample. The % leakage was determined according to:

\[
\text{% leakage} = 100 \left( \frac{F - F_0}{F_{\text{max}} - F_0} \right)
\]

where \(F_0\) represents the background fluorescence, \(F_{\text{max}}\) the fluorescence after lysis of the vesicles, and \(F\) the fluorescence upon 3 min incubation of the vesicles with the peptide.

2.5 Spectrophotometry

For the measurement of hemoglobin leakage, a CARY 4 Spectrophotometer was used combined with a quartz cuvette with a 1 cm light path. The absorption was measured at the hemoglobin absorption maximum of 540 nm and averaged over three scans for each measurement.

2.5.1 Peptide-induced haemoglobin leakage from hRBCs

Peptides were tested for haemolytic activity against human red blood cells (hRBCs) taken from fresh venous blood. The hRBCs were washed three times with buffer (150 mM NaCl, 0.1 mM EDTA, 20 mM Tris, pH 7.4) by centrifugation (10 min, 3000 rpm, 10 °C) and re-suspension in the same buffer. The rinsed blood cells were then diluted with buffer to a concentration of about 5% v/v hRBCs, (haematocrit) and distributed to 15 ml falcon tubes (1 ml of diluted blood in each). Peptides (10 µM) were added to the falcon tubes and incubated for different time periods during rocking at room temperature. The blood was then centrifuged again (2 min, 3000 rpm, 10 °C) and the supernatant analyzed by spectrophotometry as described above. 0% and 100% haemolytic activity was defined by the absorption of the supernatant of hRBCs suspended in buffer and water respectively. The apparent percentage haemolysis was calculated according to:

\[
\text{% haemolysis} = 100 \left( \frac{A_t - A_0}{A_{\text{max}} - A_0} \right)
\]

where \(A_0\) is the background absorption level, \(A_{\text{max}}\) the level after cell lysis, and \(A_t\) the measured absorption at time \(t\) after addition of the peptide.

2.6 Circular Dichroism spectroscopy (CD)

CD measurements were conducted on a Jasco-750 CD Spectropolarimeter with a 1 mm quartz cuvette. The spectra were acquired from 260 to 190 nm with a data pitch of 1 nm. The scanning speed was 50 nm/min, the response time 1 second and the slit width 2 nm. Each spectrum was background corrected and averaged over 15 accumulated scans. The mean residual molar ellipticities were calculated with respect to peptide concentration, light path length and number of residues. The LUVs (POPC:POPG (4:1) or POPC) where diluted with 50 mM phosphate buffer, pH 7.4, to a total lipid concentration of 100 µM. Peptides (total concentration 20 µM) where incubated at room temperature for 30 min with the LUVs before measurements. Reference spectra where run in water and buffer. All experiments were conducted at 25 °C.

2.7 Peptide translocation and toxicity studies on HeLa cells

2.7.1 Co-localization of mPrPp(1-28) and mPrPp(23-50) with dextran in live HeLa cells

HeLa cells were seeded in NUNC 8-well chambers and one day post seeding, at an 80% confluence, the cells were washed with serum free DMEM, followed by addition of the peptides at a final concentration of 2 µM. After 30 min incubation, the cells were washed three times with HKR (Hepes buffered Krebs-Ringer) buffer (containing 125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgSO₄, 1.33 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, and 10 mM d-glucose, pH 7.4). After washing, fluorescein-labeled peptide and rhodamine-B labelled dextran were added simultaneously at a final concentration of 2 µM and 1 mg/ml, respectively. After 30 min incubation in serum-free DMEM at 37 °C, the cells were washed three times with HKR buffer and examined using UltraView ERS confocal live cell imager (PerkinElmer Ltd, Upplands-Väsby, Sweden) connected to an Axiovert 200 (Zeiss, Göttingen, Germany). Negative controls were made without the presence of dextran.

2.7.2 Cytotoxicity of prion protein-derived peptides measured by LDH-leakage

HeLa cells were seeded out in 48-well plates 2 days before the experiments, and at 80% confluence the membrane integrity was assessed using the Promega CytoTox-ONE® assay. Cytotoxicity was expressed as the percent extracellular lactate dehydrogenase (LDH) activity out of the total LDH activity measured after cell lysis. HeLa cells were incubated with different concentrations of peptide at 37°C during 30 min. The cytotoxic effects of the PrP-derived peptides were compared to the ones of penetratin and melittin. The experiments were repeated three times on independent in duplicates.
3. Results

Previous studies [22] concerning mPrPp(1-28), penetratin and melittin in partially charged large unilamellar vesicles (LUVs) showed significant differences in their abilities to cause leakage of vesicle entrapped calcine. Melittin is the main constituent of the toxin from honeybee, *Apis Mellifera*, and known to be a very potent membrane-perturbing peptide [23]. Penetratin is a relatively non-toxic, but efficient CPP [24]. Melittin was found to cause rapid and complete calcine leakage in the LUVs, followed by mPrPp(1-28) with intermediate activity, and penetratin being relatively inert [22]. In the present study we investigated the effects of mPrPp(23-50) in a similar, partially negatively charged membrane model system comparing the results to mPrPp(1-28) and penetratin. The peptide-induced release of calcine from the calcine-entrapping LUVs is shown in Figure 1 as a function of peptide concentration. In these experiments increasing amounts of peptide were titrated onto the vesicle sample, which was incubated for 3 min before each measurement. As expected, the results show that mPrPp(1-28) is more potent than penetratin in leakage induction potency. In contrast to the two peptides which were able to induce vesicle leakage, mPrPp(23-50) did not give rise to any significant leakage from the LUVs.

![Figure 1. The induced calcine leakage in 400 μM POPC:POPG (4:1) LUVs, pH 7.4. 3 μl of 100 μM peptide was added and incubated for 3 min before each measurement. All experiments were conducted at room temperature.](image)

In another experiment, the haemolytic activities of the three peptides were investigated. Here, the peptide-induced haemoglobin leakage in human erythrocytes was followed at 10 μM peptide concentrations. Figure 2 shows the time course of haemoglobin leakage for mPrPp(1-28), mPrPp(23-50) and penetratin. Under these conditions there is significant haemoglobin leakage caused only by mPrPp(1-28). mPrPp(23-50) is even somewhat less haemolytic than penetratin.

Next we investigated a more complex cell system for the sensitivity towards the peptides. Lactate dehydrogenase (LDH) leakage from HeLa cells was used as a measure of the cytotoxic effects of the two PrP-derived peptides. In this case we chose to compare the effects on both penetratin and melittin, the latter used to give a high degree of leakage close to what is observed after cell lysis. The LDH-activity was measured outside the HeLa cells after an incubation time of 30 min and compared to the total LDH activity measured upon cell lysis, defining 100% cytotoxicity.

![Figure 2. The percental haemoglobin leakage from RBC after incubation with 10 μM peptides during different time periods. Experiments were conducted at room temperature at pH 7.4.](image)

![Figure 3. The percental cytotoxicity of four different peptides based on lactate dehydrogenase (LDH) leakage in HeLa cells. The incubation time was 30 min at 37 °C for all measurements. The enzyme activity after peptide treatment was expressed as the percentage of extracellular LDH activity as compared to the total LDH activity measured upon cell lysis. The standard error of mean was calculated based on three independent duplicates of experiments.](image)

Figure 3 shows that melittin reaches a level of almost 80% cytotoxicity already at a concentration of 1 μM, whereas penetratin and mPrPp(23-50) stay at levels of 1-3% at a concentration of 50 μM. The more active mPrPp(1-28) on the other hand, reaches levels that could be considered cytotoxic (i.e. above 10%) at 50 μM concentration, while at 10 μM the cytotoxicity can still be considered low – around 5%. The behaviour of the four peptides in this experiment approximately follows the calculated hydrophobic index of respective peptides, as indicated by Table 1.

![Figure 4. The CD spectra of the two mPrP-derived peptides, 20 μM, in water, 50 mM sodium phosphate buffer pH 7.4, and in the presence of POPC:POPG (4:1) and POPC LUVs at pH 7.4. No significant change from random coil is induced by neither buffer nor the presence of vesicles in case of mPrPp(23-50) after an incubation time of 30 min. This result suggests overall very weak (and/or slowly occurring) interactions (if any) with the vesicular membranes, even with the charged LUVs, mPrPp(1-28) on the other hand, changes its secondary structure depending on its environment.](image)
water, it predominantly takes on a random coil structure, while buffer seems to stabilize an α-helical conformation. In both zwitterionic and negatively charged LUVs we observe a conversion towards a mixture of β-sheet and α-helix for mPrP(1-28). At 50 μM concentration both peptides have however shown a propensity towards aggregation β-sheet formation and after prolonged room temperature exposure in buffer containing samples (data not shown).

In order to establish whether the two mPrP-derived peptides show different translocation abilities upon contact with biological membranes, confocal fluorescence microscopy was used to study their internalization potency in live HeLa cells. For this experiments, fluoresceinyl-labelled peptides had to be used. Figure 5 shows the confocal microscopy images of live HeLa cells together with mPrP(1-28) in the left column, and mPrP(23-50) in the right column. The cells still look intact after 30 min of incubation with both peptides.

Distinct fluorescent spots are visible for mPrP(1-28), showing membrane translocation into the cells for this peptide, in agreement with previous studies [9, 16]. Under the same conditions for mPrP(23-50), the absence of fluorescent spots inside the cells shows that mPrP(23-50) has a much weaker ability to enter into the cells. Although such images are difficult to interpret quantitatively, we suggest that the experiment shows a significant difference between the two peptides in their cell penetrating ability, with mPrP(1-28) being significantly more potent. Further confocal microscopy experiments with the labelled peptides confirmed the quantitative difference in their uptake into live HeLa cells (Figure 5).

Figure 5. Confocal microscopy images of live HeLa cells showing the internalized fluoresceinyl-labelled mPrP(1-28) and mPrP(23-50). mPrP(1-28) is effectively internalized, whereas mPrP(23-50) is just barely internalized. The incubation time was 30 min at 37°C and the final peptide concentration 2 μM.

These images also showed that mPrP(1-28) colocalizes with dextran, a fluid-phase endocytosis marker, suggesting fluid-phase endocytosis to be a dominant mechanism of internalization for this peptide. In some experiments very small traces of mPrP(23-50) could be observed inside the HeLa cells (data not shown), which may suggest that this peptide is internalized by random incorporation of outside materials during endocytosis.

4. Discussion

In this study we investigate the significance of the basic KKRPKP sequence, residues 23-28 of the mouse prion protein, by constructing a peptide mPrP(23-50), with this basic sequence overlapping with mPrP(1-28). Three types of experiments to investigate membrane perturbation caused by mPrP(23-50) comparing it to mPrP(1-28) were performed. Calcein leakage from partially charged LUVs, leakage of hemoglobin from erythrocytes as well as LDL leakage from HeLa cells showed very similar patterns: compared to the rather potent mPrP(1-28) which had been studied earlier [22], mPrP(23-50) was found to be inactive. The parallel membrane perturbations effects in model systems as well as in cell systems suggest that despite the obvious differences in membrane composition in the experiments, there is a fundamental phospholipid bilayer interaction which may be the major factor to determine the extent of cell leakage and toxicity.

The importance of direct membrane perturbation under an induced cell response, is suggested also from comparing the membrane perturbation results (Figures 1-3) to the secondary structures obtained by CD (Figure 4). mPrP(1-28) interactions with vesicle membranes, uncharged or partially charged, are seen as changes in secondary structure. The peptide interacts with both types of membranes in the same conformation, suggesting that the mode of action is similar as well. mPrP(23-50) does not show any obvious interaction with the vesicles judging from the lack of structure induction observed by CD. In itself an unchanged CD spectrum is not a proof of absence of membrane interaction, since for example the dopamine...
opioid peptides [25, 26] are known to translocate through cell membranes and still retain their random coil formations. We conclude however, that the weak membrane perturbations exhibited by mPrPPr(23-50) in general, are in line with the absence of secondary structure induction by the vesicle membranes.

Figure 5 compares the ability of fluorescein-labelled mPrPPr(1-28) and mPrPPr(23-50) to enter live HeLa cells. Again mPrPPr(23-50) is much less active than mPrPPr(1-28). The mPrPPr(1-28) peptide is highly homologous to its bovine counterpart bPrPPr(1-30), which has previously been shown to enter live mammalian CHO cells [16] using macropinocytosis (fluid phase endocytosis) as the main mechanism of action. The same conclusion is drawn here for mPrPPr(1-28) based on colocalization experiment with dextran shown in Figure S1. The significantly different membrane perturbation activities of the mPrPPr(1-28) and mPrPPr(23-50) correlate well with their respective cell membrane translocation abilities.

The signal sequence of the prion protein, residues 1-22 in mPrP, is normally cleaved off during maturation, but it has been shown that this process might fail at times leaving the signal sequence in place [6]. The retention of the signal sequence has been suggested to prompt the prion protein’s conformational change from the cellular, benign form, to the neurotoxic scrapie form [6, 9]. In the native protein, the signal sequence is followed by a basic region (KKRKP) referred to as the NLS-like sequence. The results presented in this study show that in a peptide or unstructured part of a protein the KKRKP sequence in itself is not sufficient to cause potent membrane perturbations or to mediate cell translocation. We propose that the KKRKP-sequence needs a hydrophobic partner sequence in order to attain membrane perturbation and translocation properties. One can question whether the hydrophobic segment has to be adjacent like the signal peptide (residues 1-22) in the present case, or whether the membrane activity can be efficiently increased by a hydrophobic segment present elsewhere in the protein sequence. Possibly the putative transmembrane region around residue 115 (still a part of the unstructured N-terminus) could fulfill this role in PrP. The hydrophobicity of mPrPPr(1-28) is higher than that of mPrPPr(23-50) (Table 1) which is in line with the observation that mPrPPr(23-50) has more difficulty to dissolve itself into the bilayer. Although the net positive charge is higher for mPrPPr(23-50) (which would facilitate initial contact with the negatively charged membranes via electrostatic interactions) this is obviously not the major determining factor for the membrane activities investigated in the present study.

The mPrPPr(1-28) has the theoretical ability to form an amphipathic helix based on helical wheel projections and may therefore, in parallel to the endocytotic mechanism, internalize by transient pore formation or disruption of the bilayer according to the carpeting model. These latter mechanisms are related to what has been described earlier for anti-microbial peptides among others [27, 28]. Since the CD-data clearly shows a mixture of induced α-helix and β-sheet for mPrPPr(1-28) when interacting with the LUVs, we cannot rule out these as complementary mechanisms. The presence of an unprocessed signal peptide in the PrP may cause not only toxicity through transient pore formation, but also mediate aggregation that might lead to the structure conversion of PrP0 to PrPSc.

In addition, the CPP-like activity of the N-terminus of the unprocessed protein, could be important in the transmission process of TSE diseases. Binding to negatively charged cell surface heparan sulphates, would provide the main route of entry to the cell by endocytosis [16]. How PrP conversion takes place is still unknown, but several studies suggest that lipid rafts might play a key role in the process [29] and cholesterol depletion has been shown to decrease conversion of PrP0 to PrPSc [30]. An infection propagation by direct cell membrane contacts has also been proposed [31]. It is worth noting that among the common “amyloid” related proteins, only PrP has a hydrophobic signal sequence directly followed by a basic sequence [32] - the combination of which should be responsible for the CPP-like property of this domain.

5. Acknowledgements

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6. References


Membrane Binding of pH-sensitive Influenza Fusion Peptides. Positioning, Configuration and Induced Leakage in Lipid Vesicle Models

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ABSTRACT: pH sensitive HA2 fusion peptides from influenza virus hemagglutinin have potential as endosomal escape-inducing components in peptide-based drug delivery. Polarized light spectroscopy and tryptophan fluorescence were used to assess conformation, orientation, effect on lipid order and binding kinetics of a wild-type peptide HA2(1-23) and a glutamic acid-enriched analog (INF7) in large unilamellar POPC or POPC/POPG (4:1) lipid vesicles (LUVs). pH sensitive membrane leakage was established for INF7 but not HA2(1-23) using an entrapped-dye assay. A correlation is indicated between leakage and low degree of lipid chain order (assessed by linear dichroism, LD, of membrane orientation probe retinoic acid). Both peptides display poor alignment in zwitterionic POPC LUVs compared to POPC/POPG (4:1) LUVs and it was found that peptide-lipid interactions display slow kinetics (hours), resulting in reduced lipid order and increased tryptophan shielding. At pH 7.4, INF7 displays tryptophan emission and LD features indicative of a surface-orientated peptide, suggesting that its N-terminal glutamic acid residues prevent deep penetration into the hydrocarbon core. At pH 5.0, INF7 displays weaker LD signals indicating poor orientation, possibly due to aggregation. By contrast, the orientation of HA2(1-23) peptide backbone supports previously reported oblique insertion (~60-65° relative to membrane normal) and aromatic side-chain orientations are consistent with an interfacial (pH independent) location of the C-terminus. We propose that conformational change upon reduction of pH is limited to minor rearrangements of the peptide “hinge region” around Trp14, and repositioning of this residue.

Peptide-based drug delivery vectors, named cell-penetrating peptides (CPPs)1 or protein transduction domains (PTDs), have emerged as promising efficient and non-toxic tools in enhancing intracellular delivery of cargo molecules ranging from small organic compounds and small oligonucleotides to larger proteins, plasmid-DNA, liposomes and nanoparticles (/). While these vectors were originally thought to deliver cargo into cells via direct membrane penetration mechanisms, it has become increasingly clear that uptake of peptide-cargo constructs is in fact mainly occurring via endocytotic pathways (2-4). However, since cargo molecules evidently can induce the desired biological effect inside cells (5), the peptide-cargo constructs must have a certain capability to escape from endosomes before lysosomal degradation.

Since CPPs such as penetratin (pAnp) and Tat-peptides are already functional for internalizing cargo via endocytosis a natural step forward in the development of more efficient peptide-based vectors would be to improve endosomal escape characteristics of peptide-cargo constructs. There are several examples in the literature of strategies to enhance escape from endosomes, all based on utilizing the acidification process that brings down pH inside the endosome from the physiological 7.4 to around 5 to selectively

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destabilize endosomal membranes (see (6) for a recent review).

The influenza virus is an archetypical example of a virus that uses endosome acidification to enter the cytoplasm. This process has been extensively explored and the escape mechanism is by membrane fusion, initiated at low pH due to conformational changes in the viral hemagglutinin membrane protein HA2, resulting in exposure of its 20-25 amino acid long N-terminal pH-sensitive and membrane disruptive segment. This segment is thought to constitute an individually folded domain and has been named simply the fusion peptide due to its ability to mediate fusion of lipid vesicles on its own, even in the absence of the rest of the protein. Comparison of fusion peptide sequences in various subtypes of influenza virus A strains reveals that the sequence is highly conserved (7). It is believed that pH-sensitivity of this peptide is a result of inability to form a stable α-helix upon membrane binding at neutral pH due to electrostatic repulsion between the negatively charged residues Glu11, Glu15 and Asp19. Several attempts have been made to improve pH-sensitivity by introducing additional glutamic acid residues in the N-terminal part of HA2 (8, 9).

The structure of a fusion peptide corresponding to residues 1-20, HA2(1-20) bound to DPC micelles has been determined by NMR both at physiological (pH 7.4) and endosomal (pH 5) conditions (10). The peptide was concluded to bind to the micelles in a crescent-shaped fashion, with the N-terminal part forming an α-helix that penetrates into the micelle core in a tilted (oblique) conformation. Oblique insertion angles of HA2 peptides have also been suggested from polarized infrared spectroscopy studies on supported lipid bilayers as well as from EPR spectroscopy studies with spin-labeled peptides associated to lipid vesicles membranes (10, 11). In addition to the oblique insertion, HA2 fusion peptides are thought to form a hinge at Gly11 and Asn12 which is stabilized by hydrogen bonds and this structure has been concluded essential for biological activity (12). In the above-mentioned NMR model the C-terminal part of HA2(1-20) is seemingly unstructured at pH 7.4 but adopts a short 3_10 helix at pH 5.

The “hinge region” creates a hydrophobic pocket in which Trp14 is positioned and which is thought to aid in membrane destabilization by imposing negative curvature on the membrane. A similar structure has been observed for a glutamic acid-enriched peptide (E5), also in DPC micelles (13).

Whereas membrane fusion is a viable endosomal escape strategy for viruses as well as for drug delivery using liposomes or even lipoplexes, membrane fusion may not be applicable in increasing endosomal escape of CPP-cargo constructs. However, in addition to promoting membrane fusion, HA2-peptides do act destabilizing on liposomal membranes as well as erythrocytes which results in leakage of entrapped content (9, 14, 15). Therefore, their utility in promoting endosomal escape of non-lipid-based delivery systems has been tested (9). Particularly for peptide-based drug delivery the concept of using a pH sensitive peptide segment that can be fused to a cell-penetrating peptide using automated peptide synthesis is an attractive approach for enhancing delivery of cargo to the cytosol. Proof-of-principle for this strategy has already been demonstrated using a Tat-HA2 peptide for delivery of a Tat-Cre fusion protein (5).

We have studied the binding geometry, degree of orientation, membrane association and membrane disruptive properties of a wild type HA2 fusion peptide (residues 1-23 in the HA2-protein of influenza virus strain X-31 (H3N2)) and a suggestively more pH-sensitive mutant (denoted INF7) enriched in glutamic acid residues in the N-terminal part (see Table 1 for sequences) (9) using polarized light spectroscopy (flow linear dichroism and circular dichroism) and fluorescence spectroscopy techniques. Large unilamellar vesicles (LUVs) with a diameter of 100 nm composed of synthetic phospholipids were used as membrane models. As mentioned, the fusion activity of HA-peptides has already been extensively explored in several contexts.

Moreover, the enhancement of endosomal escape in peptide-based drug delivery will not involve fusion events, but rather be a consequence of the general membrane destabilizing properties of the peptide construct. Therefore, this study was undertaken to explore structure-activity relationships for these peptides in the context
of membrane disruption and all experiments have been performed under conditions where we observe significant leakage of entrapped dyes from LUVs.

From binding geometries and degree of alignment as well as effects of lipid chain order and positioning of aromatic tryptophan residues we have been able to pinpoint differences between wild-type HA2(1-23) and the glutamic acid enriched analog INF7, important for the understanding of their membrane disruptive characteristics. In addition, the binding geometry of HA2(1-23) backbone and aromatic residues will, obtained here from linear dichroism experiments, be compared to existing NMR models for slightly shorter HA2(1-20) and the positioning of the C-terminal part of HA2 peptides will be discussed.

**Materials and Methods**

**Materials.** Peptides (≥90% purity) were purchased from NeoMPS (Strasbourg, France) and dissolved at high concentration (up to 1 mM) in 0.1M bicarbonate/acetonitrile/H2O (1/1/2). See table 1 for sequences.

**Table 1.** Amino acid sequences of HA2(1-23) and INF7. Aromatic residues are marked in bold and acidic residues in italic. The peptides were synthesized with acetylated N-terminus and amidated C-terminus.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>HA2(1-23)</td>
<td>Ac-GLF-GAIA-AGFL-IE-EN-GE-GE-MD-GW-YY-NH;</td>
</tr>
<tr>
<td>INF7</td>
<td>Ac-GLF-EEIAE-GEFL-IE-N-EN-GE-GE-MD-GW-YY-NH;</td>
</tr>
</tbody>
</table>

Peptide stock solutions were kept frozen or on ice. Lipids (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine (POPC) and 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidyl-rac-glycerol (POPG)) were purchased from Larodan (Malmö, Sweden). Buffer chemicals (sodium-phosphate salts and citrate) were from Fluka. Sucrose, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were from Sigma. The buffers were 10 mM sodium phosphate for pH 7.4 conditions and 10 mM sodium citrate/sodium phosphate for pH 5.0. Deionized water from a MilliPore system was used. In all linear dichroism experiments 50% (w/w) sucrose was dissolved into the buffer to reduce light scattering from the LUVs (vide infra).

**Large unilamellar vesicle preparation.** Large unilamellar vesicles (LUVs) were prepared by mixing appropriate amounts of lipids, dissolved in chloroform in a round-bottom flask, and thereafter removing the solvent by rotary evaporation. Remaining trace amounts of solvent were removed by placing the lipid film in vacuum for at least 2 hours. Thereafter the lipid film was dissolved in the appropriate buffer by vortexing. The lipid suspension was subjected to five freeze-thaw cycles and thereafter extruded 21 times through polycarbonate filters with a pore size of 100 nm using a Liposofast pneumatic extruder or a hand-held syringe extruder (Avestin, Canada). The lipid vesicles used were composed of either 100% POPC or POPC/POPG at a molar ratio of 4:1 (20 percent negative charge).

**Circular dichroism.** Circular dichroism (CD) was used to examine the secondary structure of HA2(1-23) and INF7 in buffer and bound to LUVs (POPC or POPC/POPG (4:1)) at pH 7.4 and pH 5.0. Spectra were recorded between 190 and 260 nm in 1 nm increments on a JASCO J-810 spectropolarimeter at 25°C using a scan speed of 50 nm/min and a bandpass of 2 nm. 20 scans were recorded and averaged by the computer. Spectra were corrected for background contributions by subtracting appropriate blanks. The path length of the quartz cell was 1 mm. The peptide-to-lipid molar ratio was 1:50 and the peptide concentration was 10 μM. The method of Chen et. al. (16) was used to estimate the fraction of peptide that adopts α-helical conformation from the mean molar residue ellipticity, [θ]MR, at 222 nm according to:

\[
X_{\text{helix}} = \frac{[\theta]_{222\text{nm}}}{39500 \times (1 - 2.57/n)}
\]

with n the number of residues in the peptide.

**Linear dichroism.** Linear dichroism (LD) is the differential absorption of linearly polarized light parallel and perpendicular to an orientation axis (Eq. 2) and requires a macroscopically aligned sample.

\[
LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda)
\]

Liposomes can be deformed by shear flow in a rotating Couette cell device resulting in slightly ellipsoidal vesicles which align in the
flow (17). Peptides that bind to the lipid surface in a non-random fashion will hence also be aligned and their transition moments will exhibit LD in the 190-300 nm spectral region. Information on binding geometry can be obtained by normalizing the LD spectrum with respect to the isotropic absorption (Aiso). The normalized LD is thus a pathlength- and concentration-independent quantity and is called reduced LD (LD'). For a non-overlapping transition moment, the relation between LD' and its angle relative to the membrane normal (the only unique axis in a membrane system) is given by:

$$LD' = \frac{LD}{A_{iso}} = \frac{3}{4} S(1 - 3 \cos^2 \alpha) \quad (3)$$

with $S$ the macroscopic orientation parameter describing the ordering of the sample. $S$ can vary between 0 and 1, with the latter denoting a perfectly aligned sample.

Linear dichroism was measured using a JASCO J-720 spectropolarimeter equipped with an Oxley prism to obtain linearly polarized light (18). The samples were oriented in an outer-rotating Couette cell with a total light path of 1 mm under a shear rate of 3100 s⁻¹. Spectra were recorded from 190 to 500 nm using a scan speed of 100 nm/min and a bandpass of 2 nm. Three successive scans were recorded and averaged by the computer. Spectra were corrected for background contributions by subtracting a spectrum that was collected without rotation of the Couette cell (isotropic sample). In order to reduce light scattering from the lipid vesicles and to improve the macroscopic orientation 50% (w/w) sucrose buffer was used in all LD experiments. The sucrose buffer matches the refractive index of the lipid vesicles and increases the viscous drag in the Couette flow cell (19).

We have previously shown for a number of cell-penetrating peptides that the use of sucrose buffers does not alter the secondary structure of membrane-bound peptides (20). The lipid concentration in all samples was 5 mM and peptide was added at a peptide-to-lipid molar ratio of approximately 1:100 (corresponding to 50 μM peptide). Absorption spectra on all samples were recorded on a Cary 4000 UV-Vis spectrophotometer (Varian Inc.) in a quartz cell with 1 mm path length. Prior to measurements on the peptides, the LUV suspensions were incubated with approximately 12.5 μM of retinoic acid (corresponding to 1 chromophore per 400 lipids) for at least 1 hour. Retinoic acid inserts parallel to the lipid chains in a well-oriented manner and is thus an excellent spectroscopic probe of membrane lipid order, suitable for estimation of the orientation parameter $S$ (21). It absorbs light in one single gauss-shaped absorption band centered on 352 nm and its transition moment is oriented parallel to the molecular long axis. LD and absorption spectra of retinoic acid were recorded before addition of peptide. LD spectra were then recorded immediately after addition of peptide (the time taken to record one LD spectrum with the setting used in this study is approximately 15 minutes) and thereafter every thirty minutes for up to two hours.

The absorption of retinoic acid overlaps to some extent the transitions from the aromatic side-chains (tryptophan and tyrosine) and to more clearly show the true LD of these transitions the contribution to LD from retinoic acid was subtracted in all LD spectra except for those shown in Fig. 5. Since the LD of retinoic acid changed upon addition of peptide due to changes in microscopic ordering in the lipid vesicles (in general a decrease was observed) the retinoic acid spectrum had to be scaled at 352 nm to subtract the correct amount. Since the magnitude of LD is concentration-dependent the obtained peptide LD spectra were thereafter corrected for small (few μM) differences in peptide concentration between samples, so that each spectrum corresponds to exactly 50.0 μM.

To determine the orientation parameter in each sample the LD from retinoic acid at 352 nm (the absorption maximum) was normalized with respect to the isotropic absorption to obtain the LD' for the long axis transition in retinoic acid. Using $\alpha = 0°$ (insertion parallel to the membrane normal) for this transition $S$ was estimated using Eq. 2.

**Peptide transition moments in UV-Vis linear dichroism.** LD in the UV-Vis region of the spectrum can be used to qualitatively assess the orientation of the peptide backbone through the low energy π-π* transition which is in the plane of the peptide bond (190-210 nm) and the n-π* transition which is
perpendicular to the plane of the peptide bond (220-230 nm). In an α-helix the π-π* transitions of neighboring peptide bonds will interact through exciton coupling and, due to the α-helix symmetry, split into two transitions, one at longer wavelengths (200-210 nm) oriented parallel to the helix axis and one at shorter wavelengths (<200 nm) oriented perpendicular to the helix axis (22-24). The latter transition cannot be resolved using our experimental setup due to the high absorptivity of the samples at such short wavelengths. The n-π* transitions will be perpendicular to the helix axis. In addition, it is possible to use the LD from the aromatic side chains in the 220-300 nm region to obtain detailed information on their orientation relative to the membrane normal.

**Figure 1.** Schematic view of electronic transition moments of an α-helical oligopeptide. Peptide bond π-π* low energy exciton transitions are polarized parallel to the α-helix and weaker n-π* transitions are polarized perpendicular to the α-helix. The tryptophan side chain has three transitions localized on the indole chromophore: B0 which is directed slightly off the pseudo-symmetry axis at 225 nm, Lα with a broad absorption band in the 250-300 nm region and Lβ which has a vibrational structure with two peaks close to 290 nm. Tyrosine has two transitions in its phenol side chain: a long-axis polarized Lβ at 276 nm and a (mainly) short-axis polarized Lα at 276 nm.

The orientation of transition moments relative to the molecular framework of a model peptide is shown in Fig. 1. Tryptophan has two overlapping electronic transitions that absorb light between 250-300 nm. The Lα transition is broad and largely unstructured and is the only absorbing transition above 295 nm whereas the Lβ transition has a characteristic structure with two distinct peaks near 290 nm. LD measurements on the tryptophan side-chain 3-methyl indole in stretched films have revealed that these transitions are oriented 85° apart in an essentially symmetric fashion around the indole pseudo-symmetry long axis. Lα (ε~3800 M⁻¹cm⁻¹ at 280 nm) is oriented in the direction of the nitrogen in the indole ring (−44°) and Lβ (ε~1900 M⁻¹cm⁻¹ at 280 nm) is oriented in the direction of the methyl substituent (+42°) which links indole to the peptide backbone (25). Tryptophan also has a strongly absorbing B0 transition at ~225 nm (ε~36000 M⁻¹cm⁻¹) which is oriented +11° off the pseudo-symmetry long axis (in direction toward the methyl substituent of the indole ring). Tyrosine has two perpendicular transition moments, the Lβ at 276 nm and the Lα at 230 nm. The Lβ transition is oriented parallel to the long axis of the methyl phenol side chain (26).

**Assignment of orientation angles and LD‘ values of peptide transition moments from LD spectra.** As a first step in the interpretation of LD spectra the sign of each absorption band gives a rough picture of the insertion of the peptide. For example, in Fig. 5 all spectra display positive peaks at approximately 208-210 nm indicating that the average orientation of the α-helix is more parallel to the membrane surface than to the membrane normal. The average insertion angle of the peptide backbone was estimated from the LD‘ value of this peak, but due to the competing high absorptivity at wavelengths below 210 nm from species other than the peptide existing in the sample (including lipids and buffer components) it was difficult to obtain reliable absorption spectra showing the exact absorption of the peptide bond π-π* transitions. We therefore used an approximation for the extinction coefficient of the peptide bond π-π* transition (~2000 M⁻¹cm⁻¹ at 210 nm), measured for a polyllysine peptide in aqueous solution (27), as the theoretical absorbance for each of the 22 peptide bonds in HA2(1-23) and INF7 at the wavelength where the low energy exciton coupling display its maximum LD. With the peptide concentration used in our LD experiments (50 μM peptide, 1 nm light path) the total absorbance in this absorption band should be approximately 0.2. It should be noted that the LD of this transition is readily measured as it is the only one giving rise to dichroic absorption at 200-210 nm.

To access more detailed information on the orientation of other peptide transition moments from LD it is necessary to resolve
overlapping transitions. Eq. 2 allows for straight-forward calculation of the angle relative to the membrane normal of an absorbing transition if there is no spectral overlap, but to use this equation for transitions that overlap their relative contribution to LD must first be deduced. In this study we determined the orientation parameter S in Eq. 2 using retinoic acid (vide supra) and for the assignment of LD values it is convenient to divide the LD spectra with this constant to obtain LD/S.

Using this quantity rather than LD the determined reduced linear dichroism values (now LD'/S) will be scaled to those of a perfectly aligned sample and hence the maximum negative LD'/S is -1.5 (α=90°) and the maximum positive LD'/S is +0.75 (α=0°). Since the three transitions that absorb in the “aromatic” region have different spectral profiles, it is possible to relatively accurately determine the contribution of each transition to the LD/S spectrum by least-squares analysis, using concentration-scaled absorption spectra for La(Trp), La(Trp), and La(Tyr) (here corresponding to 100 μM tryptophan and 50 μM tyrosine) as reference spectra. The fraction of each reference spectrum needed to reconstruct the measured LD/S spectrum corresponds directly to LD'/S.

The resolved LD'/S values for the transition moments in tryptophan and tyrosine were used to qualitatively assign sign and magnitude of the Bα(Trp), Lα(Tyr) and the peptide backbone n-pi* transitions. These three transition moments absorb light in the 220-230 nm region and have unstructured gauss-shaped absorption bands which overlap, thus complicating accurate analysis of the LD in this spectral region.

The procedure to define the orientation of Bα(Trp) was as follows: The orientation of the plane of the indole side-chain was defined by assigning directors of unit length for Lα(Trp) and Lα(Trp) in the X-Z plane of a Cartesian coordinate system using the information on their angles relative to the membrane normal (the Z-axis). The Lα(Trp) director was allowed to rotate around the Z-axis (in the X-Y plane) until the geometrically defined angle between Lα(Trp) and Lα(Trp) was 85°. The vector cross product of these two directors defines the normal vector to the indole plane. To obtain the orientation of Bα(Trp), the director for Lα(Trp) was rotated 30° around the indole plane normal and thereafter the angle between the Bα(Trp) director and the Z-axis could be determined. It should be emphasized that in all cases except when either Lα(Trp) or Lα(Trp) is close to any of the extreme values (0° or 90°) two unique orientations of Bα(Trp) are possible since all transition moments are uniaxially oriented around the membrane normal and LD cannot distinguish between positive and negative angles. In addition, both HA2(1-23) and INF7 contain two tryptophan residues and since these are indistinguishable in the LD technique the recorded LD and all parameters derived thereof are ensemble averages of the orientations of these two residues.

**Tryptophan fluorescence experiments.** All fluorescence experiments were performed on a wavelength corrected SPEX Fluorolog +3 (Jobin Yvon Horiba, France) thermostated at 25°C. The settings for each type of experiment are detailed below.

**Acrylamide quenching.** Quenching of tryptophan fluorescence by water-soluble acrylamide was used to monitor the accessibility of tryptophan to the aqueous media. A peptide or peptide-LUV solution (peptide-to-lipid molar ratio was 1:100) containing 1 μM peptide was titrated with small aliquots of acrylamide and the concomitant quenching of tryptophan fluorescence was measured. The samples were excited at 295 nm to avoid inner filter effects due to the absorbance of acrylamide at lower wavelengths and emission was monitored during 30 s at 350 nm for peptide in solution and at 337 nm for peptide associated with lipid membranes. The excitation bandpass was 1 nm and the emission bandpass was 4 nm. The ratio of the emission intensity of the unquenched sample (F0) and the emission intensity of the quenched samples (F) was plotted against the molar concentration of quencher and analysed according to the Stern-Volmer equation:

\[ \frac{F_0}{F} = K_{sv} [Q] + 1 \]  

with \( K_{sv} \) the Stern-Volmer quenching constant representing the slope of a straight line fitted to the data points.
Binding kinetics monitored by tryptophan fluorescence. The time-course of peptide association to lipid vesicles at pH 7.4 and pH 5.0 was monitored by following the change in fluorescence intensity with time after addition of 100 μM lipid vesicles to a 1 μM peptide solution. The time required for mixing and starting the experiment was 15-20 seconds. The excitation and emission wavelengths were set to respectively 280 nm and 335 nm and the excitation bandpass was set to 1 nm to avoid photobleaching and light scattering. The emission bandpass was 8 nm.

 Leakage of LUV-entrapped dyes. Peptide-induced leakage of entrapped vesicle content was measured using the dye/quencher pair ANTS/DPX (28). LUVs were prepared essentially as described above, but 12.5 mM ANTS and 45 mM DPX were added to the buffer prior to dissolving the lipid film. The LUVs were separated from non-encapsulated material by gel filtration on a Sephadex PD-10 column (Amersham Biosciences). The eluting buffer was supplemented with 100 mM sucrose to match the osmolarity of the encapsulated ANTS/DPX buffer solution. The osmolarities of the encapsulated and exterior buffers were determined using a freeze-point osmometer (Advanced™ 3300 Micro Osmometer, Advanced Instruments). The amount of vesicle content leakage was monitored by measuring the dequenching of ANTS that occur upon leakage when ANTS and the quencher DPX become diluted (28).

The excitation and emission wavelengths were set to 353 nm and 526 nm respectively. The bandpass of the excitation and emission monochromator was 1 nm and 4 nm respectively. The ANTS emission was monitored with time in 1 s time increments. At least 50 data points were recorded to establish the background emission level in each experiment, prior to addition of peptide. The maximum level of leakage was obtained by adding 10 μl Triton X-100 (from a stock solution, 10 % (w/v)) to the sample, which had a total volume of 1 ml. The total lipid concentration was 100 μM in all experiments. The data were corrected for background contributions by subtracting appropriate blanks. The percentage of leakage was calculated according to:

\[
\text{Leakage} = \frac{I(t) - I_{\text{I0}}}{I_{\text{I0}} - I_{\text{I0}}\text{max}} \times 100
\]

with \(I(t)\) the emission intensity at time \(t\), \(I_{\text{I0}}\) the initial intensity and \(I_{\text{I0}}\text{max}\) the final intensity recorded after addition of Triton X-100.

Results

Figure 2. Leakage of entrapped ANTS/DPX from (A) POPC and (B) POPC/POPG (4:1) large unilamellar vesicles as a function of time (logarithmic scale) after addition of peptide. The peptide-to-lipid ratio was 1:100 and the lipid concentration was 100 μM. Each trace represents the average of three independent experiments, (the spread between experiments is represented by the noise).

Vesicle content leakage. The amount of peptide-induced leakage of the encapsulated quencher pair ANTS/DPX was assessed as a measure of the membrane-disruptive capacity of HA2(1-23) and INF7 in POPC and POPC/POPG (4:1) LUVs. At a peptide-to-lipid ratio of 1:100, which is representative for all experiments in this study, these peptides are highly leakage-inducing at both physiological (pH 7.4) and endosomal (pH 5.0) conditions, as is shown in Fig. 2. More than 85% leakage is observed in POPC LUVs within two minutes (Fig. 2A) and HA2(1-23) caused near 100% leakage within as little as 10 seconds. Leakage induced by HA2(1-23) in POPC vesicles is insensitive to pH which is in agreement with similar studies on leakage of encapsulated calcine from the same type of vesicles (9). Noticeably, in POPC/POPG (4:1)
vesicles HA2(1-23) induced leakage is more prominent at pH 7.4 than at pH 5.0 (Fig. 2B).

INF7 is more potent at pH 5.0 than at pH 7.4 which confirms the hypothesis that introduction of glutamic acid residues in the N-terminal part of HA2 peptides has an effect on the pH sensitivity (9). The leakage half-life is approximately one order of magnitude longer for INF7 at pH 7.4 than at pH 5.0 in both vesicle types. To further explore the difference between these peptides in terms of induced leakage activity in LUVs the peptide-to-lipid ratio was reduced until a significant difference in maximum leakage level was observed. Fig. 3 shows time traces for leakage of ANTS/DPX from POPC and POPC/POPG (4:1) LUVs at a peptide-to-lipid molar ratio of 1:1600. As expected, leakage occurs with a much slower rate at this ratio, but still reaches relatively high levels. This clearly points out the strong membrane-destabilizing properties of HA2 fusion peptides. Also here leakage from POPC/POPG (4:1) LUVs in presence of HA2(1-23) is faster and more efficient at pH 7.4 than at pH 5.0, which is indeed the opposite to what would be expected for this type of peptide (see Fig. 3B).

![Figure 3](image-url) **Figure 3.** Leakage of entrapped ANTS/DPX from (A) POPC and (B) POPC/POPG (4:1) LUVs as a function of time (logarithmic scale). The peptide-to-lipid ratio was 1:1600 and the lipid concentration was 100 µM.

**Circular dichroism.** Fig. 4 shows circular dichroism (CD) spectra of HA2(1-23) and INF7 at pH 7.4 and pH 5.0 in buffer, POPC LUVs and POPC/POPG (4:1) LUVs. The degrees of peptide α-helicity (in percent), estimated as described in Materials and Methods, are indicated in each panel. HA2(1-23) adopts α-helical conformation (approximately 40%) under all tested experimental conditions, but there is none or little effect of lowering pH since both the spectral shapes and the degrees of α-helicity, as determined from the mean molar ellipticities at 222 nm, are practically unchanged.

![Figure 4](image-url) **Figure 4.** CD spectra of HA2(1-23) (A,B) and INF7 (C,D) in buffer (solid line) and in lipid vesicles composed of POPC (dashed line) or POPC/POPG (molar ratio 4:1) (dotted line) at pH 7.4 (A,C) and at pH 5.0 (B,D). The peptide-to-lipid ratio was 1:50 and the peptide concentration was 10 µM. Degree of α-helicity, calculated from the mean molar residue ellipticity at 222 nm according to Chen (16) is indicated in percent.

The secondary structure content is in agreement with what has previously been reported for HA2(1-20) (29) as well as for HA2(1-20) analogs and wild-type HA2(1-23) (14), but while we observe HA2(1-23) to adopt secondary structure already in buffer, Lear et al. report their peptide to be random coiled in absence of lipid. Our CD observations could indicate that the peptide self-assembles in solution under the conditions used in this study. However, we never experienced any problem with insolubility or precipitation, not even at acidic pH and saw no signs of β-sheet formation which is commonly encountered when HA2 fusion peptides aggregate.

The CD spectrum of INF7 in buffer at pH 7.4 (solid line in Fig. 4C) indicates that INF7
is largely unstructured in absence of lipid membranes. Also, INF7 shows only a modest degree of α-helicity in POPC/POPG (4:1) LUVs at pH 7.4. By contrast, at pH 5.0 INF7 is α-helical in buffer and there is a marked increase in peptide α-helicity upon changing the pH from 7.4 to 5.0 in both types of LUVs. The CD spectrum of INF7 in buffer at pH 7.4 shows little α-helical character and neither is it a spectrum of a typical random coil. In this case it could be misleading to try to estimate α-helicity using the method of Chen et al. (16) and, therefore, the degree of α-helicity given for this particular measurement should be treated with caution. For the purpose of this work we did not find it meaningful to further analyze the secondary structure content of INF7 in buffer at physiological pH.

**Linear dichroism.** The binding geometry of HA2(1-23) and INF7 in POPC and POPC/POPG (4:1) LUVs at a peptide-to-lipid ratio of 1:100 was assessed using linear dichroism (LD). To judge the effect of peptide binding on lipid order and to estimate the orientation factor S in Eq. 2, LUVs were pre-incubated with retinoic acid (one chromophore per 400 lipids) as described in Materials and Methods. Since retinoic acid orients its long-axis parallel to the membrane normal, and hence perpendicular to the flow orientation axis direction (21), it exhibits negative LD (according to Eq. 1). A typical LD spectrum of retinoic acid in POPC/POPG (4:1) LUVs at pH 7.4 is shown in Fig. 5 (dashed line). The solid lines are LD spectra of HA2(1-23) recorded at 0, 30, 60 and 90 minutes after addition of peptide to the LUV-retinoic acid sample.

These spectra show peptide-specific LD features indicative of non-random peptide association to the lipid membrane. Dichroic absorption can be observed from the pi-pi* peptide bond transitions at ~210 nm as well as from the aromatic tryptophan and tyrosine side chains at ~225 nm and in the 250-310 nm region. Furthermore, a marked decrease in LD signal from retinoic acid at 352 nm can be observed immediately after addition of peptide and this signal decreases further with time, indicating that the peptide has an effect on the membrane orientation, an observation that we shall return to.

Figure 5. LD of HA2(1-23) in POPC/POPG (4:1) at pH 7.4 in lipid vesicles preincubated with the membrane orientation probe retinoic acid (solid line spectra). Spectra were recorded at 0, 30, 60 and 90 minutes after addition of peptide (black to light gray). Dashed line is LD of retinoic acid prior to addition of peptide. Arrows indicate trends of spectral change with time. The peptide-to-lipid ratio was 1:100 and the lipid vesicles contained approximately 1 retinoic acid chromophore per 400 lipids. Total lipid concentration was 5 mM.

**General LD features of HA2(1-23) and INF7.** Fig. 6 shows LD spectra of HA2(1-23) and INF7 in POPC and POPC/POPG (4:1) LUVs at pH 7.4 and pH 5.0. All spectra show a positive peak at 208-210 nm emanating from the low-energy exciton pi-pi* transition, whose moment is oriented parallel to the long axis of a peptide α-helix. Thus, at least the α-helical parts of the peptides are oriented more parallel to the surface than to the membrane normal which immediately excludes stable transmembrane peptide configurations. LD will be zero for a transition that is at an angle of 54.7° (magic angle) to the membrane normal limiting the possible average backbone orientation to the interval between 54.7° and 90° relative to the membrane normal. The magnitude of the LD from both HA2(1-23) and INF7 is, in this spectral region, small compared to surface-oriented penetratin peptides, which under similar conditions display approximately 5-10 times stronger LD in the pi-pi* absorption band (20, 30). This suggest either tilted (oblique) insertion of the peptide α-helix (which would be in accord with the NMR data of Han et al. (10)) or general poor alignment of these peptides.
Figure 6. LD spectra of HA2(1-23) (A, B) and INF7 (C, D) bound to POPC lipid vesicles (gray lines) and POPC/POPG (4:1) lipid vesicles (black lines) at pH 7.4 (A, C) and pH 5.0 (B, D), recorded within 15 minutes after addition of 50 µM peptide to a lipid vesicle suspension with a total lipid concentration of 5 mM. The spectra have been magnified 10 times in the 250-310 nm region to facilitate visualization of spectral features from the aromatic amino acids in the peptides. All spectra were recorded in presence of membrane orientation probe retinoic acid added prior to peptide addition at a chromophore-to-lipid ratio of 1:400. For clarity, the spectral contribution from this chromophore (see Figure 5) has been subtracted.

LD magnitudes are markedly weaker in all absorption bands for peptides in zwitterionic POPC LUVs (gray lines) compared to acidic (negatively charged) POPC/POPG (4:1) LUVs (black lines), indicating that poor alignment in POPC membranes. With INF7 at pH 7.4 being an exception, LD can only be observed from the peptide backbone in POPC LUVs suggesting that the aromatic side-chains are completely random. We have tried to vary the peptide-to-lipid ratio in POPC LUVs to find conditions where an ordered orientation could be obtained, but without success. The descriptions of the binding geometries of HA2(1-23) and INF7 will thus below be limited to what was observed in POPC/POPG (4:1) LUVs.

Most LD spectra in Fig. 6 display two conspicuous positive LD peaks at ~290 nm emanating from the L_0 transition moment in tryptophan. We have previously shown that tryptophan side-chains (indole chromophores) associated to LUV membranes will intrinsically adopt such orientation as a consequence of their preferred positioning at the membrane interface (31).

**LD of HA2(1-23) in POPC/POPG (4:1) LUVs.** The LD spectra of HA2(1-23) at pH 7.4 and pH 5.0 in POPC/POPG (4:1) LUVs have been analyzed in detail, since these two samples display discernable LD features in the aromatic region of the spectrum and since we have here a possibility to compare our results with NMR structures for slightly shorter HA2(1-20) docked in bilayers as constructed by the group of Lukas Tamm (10).

The orientation factors of these two samples were estimated to 0.026 and 0.029 at pH 7.4 and pH 5.0, from the LD of retinoic acid at 352 nm. Oblique insertion angles of the peptide backbones were estimated to 60-65° relative to the membrane normal from LD of the π-π* transitions at 210 nm, using a theoretical value for the absorption at this wavelength (see Materials and Methods). This agrees well with the oblique angle of 65° degrees obtained using EPR on HA2(1-20) bound to lipid vesicles equivalent to ours at pH 7.4 (10). However, our study provides no indication of a change in oblique insertion upon lowering pH whereas, Han et al. (10) report a as much as 15° steeper insertion for HA2(1-20) at pH 5.

Figure 7. Resolved LD spectra in the aromatic region of HA2(1-23) in POPC/POPG (4:1) lipid vesicles at pH 7.4 (A) and pH 5.0 (B). Measured (solid gray lines) and reconstructed (solid black lines) LD spectra and also LD spectral components of L_0(Trp) L_0(Tyr) and L_0(Tyr) (dashed, dotted and dash-dot respectively) are shown.

The average orientation of Trp14 and Trp21 and the orientation of Tyr22 was
assessed by estimating the contributions to the 
LD spectrum from \( L_a \) and \( L_b \) in the two 
tryptophan residues and \( L_b \) in tyrosine, 
(thereafter denoted \( L_b(Trp) \), \( L_a(Trp) \) and \( L_b(Tyr) \)) using a least-squares approach 
described in Materials and Methods. Fig. 7 
shows experimental LD/S curves, resolved 
contributions to LD from \( L_a(Trp) \), \( L_b(Trp) \) and \( L_b(Tyr) \) and reconstructed LD/S curves. The 
good agreement between experimental and 
reconstructed LD/S curves indicate that the 
shape of the LD spectrum in the aromatic 
region is well accounted for by the above-
mentioned transitions, Table 2 displays LD/S 
values and corresponding estimated angles to 
the membrane normal. Tyrosine is at the C-
terminus of HA2(1-23) (residue 22) and given 
that the α-helicities in these peptides are far 
from 100% (see Fig. 4) we reasoned that 
possibly this end of the peptide is unstructured 
and flexible, resulting in tyrosine not being 
oriented at all. We therefore also tried to 
resolve the LD spectra using absorption 
reference spectra for tryptophan only. It was, 
however, not possible to accurately describe 
the LD in the 280-300 nm region without 
including a contribution of \( L_a(Tyr) \). Trying 
to do so inevitably resulted in poor fits with 
strong overrepresentation of \( L_a(Trp) \) at 
wavelengths above 280 nm. Therefore we 
conclude that all three aromatic residues in 
HA2(1-23) must contribute to LD and thus 
have a defined orientation within the 
membrane. This suggests that the C-terminal 
part of HA2(1-23) adopts an at least partly 
ordered orientation in POPC/POPG (4:1) 
LUVs at both pH 7.4 and pH 5.0.

From the LD/S values and insertion angles 
for \( L_a(Trp) \) and \( L_b(Trp) \) theoretically possible 
limiting orientations for \( B_0(Trp) \) were 
calculated (see Materials and Methods). At pH 
7.4 the \( B_0(Trp) \) transition moment is 
estimated to have an average orientation that 
is either very close to the magic angle or close 
to 90°, that is its LD should either be zero 
or close to the maximum possible. The 
magnitude of the LD at ~225 nm is not large 
enough to allow the orientation of \( B_0(Trp) \) to 
be 90°, therefore we accept the near magic 
angle alternative as the true one (see Table 2). 
At pH 5.0, \( B_0(Trp) \) was estimated to be 60-65° 
from the membrane normal.

Table 2. LD values and concluded angles (α) of 
peptide-specific transition moments angles relative to 
the membrane normal for HA2(1-23) in POPC/POPG 
(4:1) large unilamellar vesicles at pH 7.4 and pH 5.0. 
LD′ for each sample, normalized with respect to the 
orientation factor determined by the retinoic acid probe. 
The maximum possible LD/S = ~0.75 (α=90°) and 
the maximum negative LD/S = -1.5 (α=0°).

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Transition</th>
<th>pH 7.4</th>
<th>pH 5.0</th>
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<tr>
<td></td>
<td>LD/S</td>
<td>α</td>
<td>α</td>
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<tr>
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<tr>
<td></td>
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<td>( L_b )</td>
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<tr>
<td>Peptide bond</td>
<td>p-i-p_i*</td>
<td>0.27^c</td>
<td>62</td>
</tr>
</tbody>
</table>

^a Parameters in bold were resolved by least-squares 
projection of LD spectra, normalized with respect to 
order parameter S, onto reference absorbance spectra 
in the aromatic region (255-310 nm) as shown in Figure 7.

^b Parameters in italic were estimated by applying 
geometric constraints for planar chromophores of 
tryptophan and tyrosine side-chains.

^c The LD/S values defining the oblique insertion 
angles of the peptide backbone were estimated from 
experimental LD and extinction coefficient for the 
p-i-p_i* transition at 210 nm as described in text (Results).

The \( L_a(Tyr) \) transition moment is oriented 
35° from the membrane normal at pH 7.4 as 
well as at pH 5.0, indicating that Tyr22 does 
not significantly change its binding geometry 
in the lipid membrane when pH is lowered. 
The \( L_b(Tyr) \) and \( L_a(Tyr) \) transition moments 
are at right angles to each other in the plane of 
the tyrosine side-chain and this geometric 
constraint prohibits that both simultaneously 
display negative LD. Therefore \( L_a(Tyr) \) must 
also contribute to the positive LD signal in the 
220-230 nm region. We explain the LD in the 
220-230 nm as follows: The n-p_i* 
transitions in the peptide backbone gives rise to a weak 
negative LD contribution both at pH 7.4 and 
P6.0. According to our estimate of the 
insertion angles of the peptide backbone from the 
p-i-p_i* transition the peptide backbone does 
not change its orientation to any significant 
extent upon lowering pH and thus, also the 
p_i* transition should display LD of invariant 
magnitude. The \( L_a(Tyr) \) transition contributes 
to a positive LD at both pH 7.4 and pH 5.0 
and due to that Tyr22 does not change its 
conformation this contribution is of constant
magnitude. We therefore assign the observed increase in LD (see black lines in Fig. 6A-B) at -225 nm upon changing pH from 7.4 to 5.0 to a change in orientation for B(Trp) from approximately magic angle insertion (~55°) at pH 7.4 to 60-65° at pH 5.0. This change corresponds to an increase in LD/S of +0.25 which when recalculated into LD units corresponds almost exactly to the observed increase in LD signal at 225 nm.

**LD of INF7 in POPC/POPG (4:1) LUVs.** At pH 7.4 INF7 (Fig. 6C, black line) displays significantly larger LD in the pπ-pπ* absorption band at 210 nm compared to HA2(1-23). The average insertion angle of the peptide backbone relative to the membrane normal was estimated to ~70° indicating that INF7 inserts more parallel to the membrane surface, possibly related to a difficulty for this peptide to insert the negatively charged Glu4 and Glu7 residues into the hydrocarbon core. In the aromatic region there is a clear positive contribution to LD from L(Trp) whereas the LD is essentially zero between 260 and 280 nm indicating that neither L(Trp) nor L(Tyr) give rise to net LD in this region. The negative contribution to LD at 225 nm is in part due to the relatively weak nπ-pπ* transitions, orientated perpendicular to the peptide α-helix, but the magnitude of this peak suggest that also the aromatic side-chains contribute to this region, presumably this peak is enlarged due to a negative LD from B(Trp).

At pH 5.0 INF7 displays no discernable LD in the 250-310 nm region and the magnitude of the pπ-pπ* transition at 210 nm is reduced approximately 4 times compared to at pH 7.4 (Fig 6C, black line). It is thus clear that the interaction between INF7 and the POPC/POPG (4:1) LUV membrane is dramatically different from the interactions observed for HA2(1-23). The lack of orientation for aromatic side-chains indicate multiple binding modes and the weak LD at 210 nm indicate that at least some fraction of the peptides penetrate deeper into the membrane, resulting in that the average insertion of the peptide backbone is close to the magic angle (54.7° from the membrane normal).

**Time-dependent LD of lipid order probe retinoic acid.** The evolution of LD from retinoic acid with time was followed by recording LD spectra every 30 minutes after peptide addition and Fig. 8 shows the retinoic acid LD at the wavelength of maximum absorption normalized with respect to the initial signal obtained before addition of peptide. The retinoic acid LD decreases markedly immediately after addition of peptide to POPC LUVs (Fig. 8A). The POPC membranes are least perturbed by HA2(1-23) at pH 5.0. In POPC/POPG (4:1) LUVs the retinoic acid LD is significantly less reduced than in POPC LUVs, which is in agreement with the over all better peptide orientations observed in Fig. 6. The change in retinoic acid LD in POPC/POPG (4:1) LUVs is pH dependent and at pH 5.0, addition of HA2(1-23) even results in a minor increase in retinoic acid LD with time.

![Figure 8](image.png)

**Figure 8.** Relative LD of membrane probe retinoic acid, LD(t)/LD(0) showing the change in membrane orientation of shear-deformed large unilamellar vesicles (LUVs) with time upon addition of peptide (added at time 0). Each trace starts at LD(t)/LD(0)=1 which represents the initial retinoic acid LD recorded on the LUV sample prior to addition of peptide. Data is shown for HA2(1-23) (squares) and INF7 (circles) at pH 7.4 (open symbols) and at pH 5.0 (filled symbols). The LUV lipid composition was POPC (panel A) or POPC/POPG (4:1) (panel B).

A change in retinoic acid LD is a consequence of changed overall orientation in the sample and may be caused by either a change in lipid chain order, in vesicle deformability, or in vesicle alignment. The trivial cause that the decreased LD is due to a decreased number of vesicles in the light path due to for example vesicle lysis or precipitation must first be excluded. From experiments using the detergent Triton-X we know that complete vesicle lysis results in a total loss of LD (results not shown). Absorption measurements indicate that no precipitation occurred since the peptide concentrations in the samples (measured after
the LD experiments were completed) were always the expected.

As a control, dynamic light scattering was used to establish that the vesicle size distribution remained constant. Addition of peptide to the LUVs at the peptide-to-lipid ratios and total concentrations identical to those used in the linear dichroism as well as all fluorescence spectroscopy experiments had no detectable effect on count rate (scattering intensity) or shape of the autocorrelation functions. Estimated hydrodynamic radii and polydispersity indexes calculated using second order cumulant analysis remained constant even after more than 2 h of incubation (data not shown). In addition, while performing fluorescence experiments, static light scattering intensity from LUV samples was routinely monitored with time after addition of peptide using a SPEX Fluorolog τ-3 fluorimeter with excitation and emission monochromators set to 600 nm. The scattered intensity remained constant indicating that vesicle aggregation did not occur (32). Taken together the above controls indicate that the LUVs maintained integrity during experiments and we therefore exclude both complete lysis and vesicle aggregation as causes of the observed loss in retinoic acid LD.

**Peptide-lipid interaction kinetics probed by tryptophan fluorescence.** Binding of peptide to LUVs was assessed by monitoring the tryptophan emission intensity with time as well as by recording emission spectra. Transfer of peptide from buffer to the LUV membrane resulted in blue-shifted tryptophan emission and a concomitant intensity increase. Fig. 9 shows time traces of the tryptophan fluorescence from peptide samples recorded after addition of POPC or POPC/POPG (4:1) LUVs. The intensity is expressed in relative units with respect to the emission intensity of the peptide in buffer. None of these traces can be fitted with a monoeponential function which suggests that the binding of HA2(1-23) and INF7 to both POPC and POPC/POPG (4:1) lipid membranes is a multi step process.

![Figure 9](image.png)

**Figure 9.** Kinetics of peptide binding to lipid vesicles monitored as the relative change in intensity of peptide tryptophan fluorescence, I(t)/I₀, I₀(t) is the emission intensity at time t after addition of lipid vesicles (POPC circles) and POPC/POPG (4:1) (squares) to samples containing HA2(1-23) (A, B) or INF7 (C, D) at pH 7.4 (A, C) or pH 5.0 (B, D). I₀ is the emission intensity of the same sample prior to addition of peptide. The peptide-to-lipid ratio was 1:100 and the lipid concentration 100 µM.

The shapes of the curves indicate a fast first step, in which the peptides presumably associate to the lipid bilayer. It should be noted that the leakage of entrapped content reported in Fig. 2 is completed during this phase. Thereafter follows one or several slower steps which may be related to peptide rearrangement in the membrane. The emission intensity increases on average ~3 times which must be regarded as extreme compared to measures obtained for membrane-bound penetratin peptides (~1.5 times increase) (20). This effect is more pronounced in POPC LUVs than in POPC/POPG (4:1) LUVs. Binding of INF7 to POPC/POPG (4:1) LUVs at pH 7.4 (Fig. 9C) is associated with a noticeably smaller increase in intensity than all other peptide-lipid vesicle combinations but it is, as indicated above, readily comparable to what we have previously observed for the surface-bound CPP penetratin (20). At pH 5.0 (Fig. 9D), INF7 first displays a fast initial binding step, resulting in an expected increase in emission intensity, but thereafter a slow but steady decrease follows,
implying that the tryptophan residues slowly become more accessible to water again.

The emission maximum wavelength of tryptophan can give a rough estimate of how deep this residue penetrates into the membrane (33). For a peptide that associates in the membrane head-group region the emission maximum is centered at 337-340 nm (20). We observe emission maximum wavelengths typically below 333 nm for membrane-bound HA2(1-23) and INF7 in agreement with previous reports for HA2(1-20) (29, 34). This indicates some penetration into the hydrocarbon core for the tryptophan residues, but does not correspond to a completely apolar environment where the emission maximum can approach 320 nm (26). INF7 at pH 7.4 in POPC/POPG (4:1) LUVs is an exception and exhibits tryptophan emission maximum at 341 nm, again indicating that its tryptophan residues are in an environment similar to that of tryptophan residues of surface-bound peptides.

Tryptophan emission quenching by acrylamide. Acrylamide quenching experiments were performed to further assess the shielding of tryptophan residues from water. Fig. 10A shows Stern-Volmer plots for the quenching of tryptophans in HA2(1-23) at pH 7.4 in buffer, POPC vesicles and POPC/POPG (4:1) vesicles. The quenching of free tryptophan in buffer is shown as comparison. Similar linear Stern-Volmer plots were obtained in all experiments, indicative of a relatively homogeneous longitudinal distribution of the tryptophan residues (26), therefore suggesting that Trp14 and Trp21 are equally susceptible to quenching and thus reside in similarly protected environments. Fig. 10B shows Stern-Volmer quenching constants, $K_{SV}$, for HA2(1-23) or INF7 at pH 7.4 or pH 5.0 in buffer, POPC LUV's or POPC/POPG (4:1) LUV's as well as for tryptophan in buffer (this $K_{SV}$ value was independent of pH).

Fig. 10B reveals that tryptophan residues in both HA2(1-23) and INF7 are significantly shielded from water already in buffer which in turn strongly indicates that these peptides assemble in solution.

This is further supported by our observations on the maximum wavelengths of tryptophan emission for the peptides in buffer were significantly less than the ~352 nm measured for tryptophan alone (below 340 for HA2(1-23) and below 345 for INF7 at pH 5.0). INF7 in buffer at pH 7.4 is an exception: Displaying maximum emission at 348 nm this peptide may probably not be significantly aggregated in water which is also manifested in the larger $K_{SV}$. From helical wheels constructed for the peptide sequence it is evident that the two tryptophan residues in both HA2(1-23) and INF7 should be positioned at the edge of the nonpolar face if the entire peptide was α-helical and it is thus envisaged that the tryptophan residues will be significantly shielded from water if the peptides associate to avoid displaying their hydrophobic side to the aqueous surrounding. Lowering pH from 7.4 to 5.0 lowers the $K_{SV}$ for the free peptides even further, which is to be expected since the water solubility of the peptides should be significantly reduced upon protonation of the glutamic acid side chains. When bound to liposomes HA2(1-23) displays $K_{SV}$~2 and there is none or little effect of varying LUV type or pH. The values reported here are somewhat lower compared to earlier observations for HA2(1-25) in DMPC vesicles.
(34). The $K_{av}$ values for INF7 bound to LUVs are similar to that of HA2(1-23) at pH 5.0, except for INF7 in POPC/POPG (4:1) LUVs at pH 7.4 which is significantly higher.

**Discussion**

This work comprises a linear dichroism study of HA2 fusion peptides in zwitterionic and acidic lipid membranes of large unilamellar vesicles (100 nm), where we assess the orientation of the peptide backbone and also gain detailed information on binding geometry from the positioning of the aromatic tryptophan and tyrosine side chains. In addition, we explore the effects of peptides on the vesicle lipid bilayer, properties that we will relate to leakage-induction capacity. As mentioned in the Introduction, HA2 fusion peptides have been extensively studied in the past, foremost with the aim of understanding the mechanisms by which the influenza virus infects cells. Despite this we are here first to report on the orientation of an unlabelled HA2 peptide when bound to a lipid vesicle model membrane and to point out a marked difference in the mode of interaction of HA2 peptides with zwitterionic (POPC) and slightly acidic (POPC/POPG (4:1)) lipid membranes. Finally, we find that HA2 peptides interact with lipid membranes in a time-dependent fashion, the origin of which will be discussed.

**Leakage-inducing capacity.** We set out to explore the relation between structure and membrane-perturbing properties of HA2 fusion peptides with perspective on future implementation of such peptides in existing strategies for peptide-mediated drug delivery in order to enhance endosomal escape. Within this scope, selective membrane destabilization at endosomal pH is a qualifying property of the delivery system. We show in Fig. 2-3 that both HA2(1-23) and INF7 are indeed highly potent in causing leakage of small organic molecules at moderate down to extremely low peptide-to-lipid ratios. However, HA2(1-23) is not significantly discriminatory to pH in terms of induced leakage. This has been observed before (9) and is in fact not so surprising since it is likely that the HA2 fusion peptide itself is buried within the hemagglutinin protein at pH 7.4 and only exposed at the membrane surface at pH 5.0 (35). In addition, even though Glu11 and Asp19 are highly conserved in influenza A virus strains, studies on point-mutated HA proteins expressed on the surface of CV-1 cells imply that neutralization of these residues may not even be necessary for targeting lipid bilayers and initiating fusion (36). Thus, in nature, built-in pH sensitivity in the fusion peptide sequence may not be essential.

By contrast, INF7 is pH-sensitive in terms of induced leakage, reaching similar levels as the wild-type HA2(1-23) at pH 5.0 but significantly reduced levels at pH 7.4. At a peptide-to-lipid ratio of 1:100 this primarily manifests in slower establishment of maximum leakage levels, but since the turnover time for lipids in the plasma membrane is relatively short in mammalian cells with normal endocytic activity (for example: 50 percent of the surface area of fibroblasts is interiorized in one hour (37)), this type of kinetic discrimination may be sufficient to minimize leakage at the surface of the plasma membrane relative to leakage obtained in the endosome. Despite that both HA2(1-23) and INF7 induce near 100% leakage we see no signs that LUVs may burst in presence of peptide which may indeed be a limitation when it comes to release of larger endosome-entrapped cargo such as DNA or full-length proteins.

**Secondary structure and pH sensitivity.** A relationship between secondary ($\alpha$-helical) structure and fusion ability of HA2 peptides was early proposed: The basis of this mechanism may be steric clash or electrostatic repulsion between glutamic acid residue side-chains in the folded state preventing stable $\alpha$-helix formation at physiological pH (9, 14). From CD we confirm that the Glu4 and Glu7 introduced in the mutated INF7 indeed prevent efficient $\alpha$-helix formation in buffer at pH 7.4, but since addition of lipid immediately induces $\alpha$-helicity already at physiological pH it seems less likely that electrostatic repulsion between the glutamic acid residues could be the only origin of pH sensitivity of the INF7 peptide, at least not in zwitterionic membranes where the negatively charged side-chains could interact with positively charged choline head-groups and thus screen their charge. In POPC/POPG (4:1), where pH sensitivity is more pronounced the situation is somewhat different. The $\alpha$-helical content increases from 18% to 52% when pH is lowered from 7.4 to
5.0. Under similar conditions HA2(1-23) is 39% and 44% α-helical. Thus Glu4 and Glu7 are
carried to repel each other and prevent helix formation of the N-terminal.

**Binding geometry of HA2(1-23) in POPC/POPG (4:1) LUVs.** HA2(1-23) in
POPC/POPG (4:1) exhibits a remarkably high degree of orientation which allowed us to
resolve orientation details of its aromatic residues (see Table 2). The fact that the
insertion angle of the Lα(Trp) transition at pH 5.0 conditions could be estimated to 84°,
despite that it represents an average of the two tryptophans in HA2(1-23), suggests that both
these two residues have a strong propensity to orient themselves with this transition close to
parallel to the membrane surface. Further, this suggests that at least these residues, and very
likely the entire peptide, is well-aligned in the lipid membrane since any heterogeneity
among the peptides would result in canceling effects and decreased average LD. The
observed oblique insertion angles (Table 2) of the peptide backbone are in agreement with
previous reports and support that HA2(1-23) could be inserted in a crescent-shaped
“boomerang” fashion (10). HA2(1-23) undergoes some conformational change upon
reduction of pH (see Fig. 6 and Table 2), but as concluded in Results this has in our hands
no visible effect on the oblique insertion angle of the peptide backbone. In addition, our LD
results indicate that the orientation of Tyr22 at the C-terminal end of HA2(1-23) is virtually
unaffected by pH. Thus it is reasonable to propose that also the neighboring Trp21
maintains its orientation when pH is changed.

Tryptophans and tyrosines have a strong bias to reside in the membrane interfacial
region (38) and the LD of the tryptophan side-chains have the same features as those
observed for tryptophan model compounds with interfacial positions such as indole and 3-
methyl indole when bound to LUVs (positive Lα and Bα negative <, (33)). Therefore we
suggest that interfacial ph-independent interactions of Trp21 and Tyr22 drive the
positioning of the HA2(1-23) C-terminal, serving to anchor this part of the peptide at the
membrane interface. This indicates that the flexibility of HA2 peptide C-termini observed
in NMR for both wild-type and mutated versions of HA2(1-20) (10, 13) is reduced if
the peptide is elongated so that these two
aromatic side-chains are included. This result
may have implications for the understanding
of how the HA2 fusion peptide domain functions when part of the hemagglutinin
protein.

Taken together, LD data suggest that the conformational change in the membrane-bound
HA2(1-23), observed upon lowering pH, does not involve any major repositioning
of the peptide backbone, nor is the C-terminus significantly affected. One may therefore
envisage that a local conformational change occurs in the “hinge region” (10) which
comprises Trp14 and is flanked by pH sensitive Glu11 and Glu15. According to LD
data, this conformational change involves repositioning of Trp14, resulting in the indole
chromophore long axis being rotated towards an insertion more parallel to the membrane
surface at pH 5.0. This rotation could be consistent with that Trp14 “swings out” from
the hydrophobic pocket in the “hinge region” and adopts an orientation similar to that in the
NMR model of HA2 analog E5 proposed by Hsu et al. (13).

**Binding of INF7 to POPC/POPG (4:1) LUVs.** We confirm that INF7 displays some
promising pH-sensitivity regarding capacity to induce leakage, especially in POPC/POPG
(4:1) LUVs (Fig. 2-3). This manifests foremost in reduced leakage activity at pH 7.4
compared to HA2(1-23). However, under these experimental conditions INF7 also
displays the smallest increase in tryptophan fluorescence intensity upon membrane
binding, the lowest Stern-Volmer quenching constant (KSV) and the highest emission
maximum wavelength observed for any of the peptide-lipid-pH combinations tested in this
study. In addition, INF7 is only moderately α-helical (18%). These observations imply pH
sensitivity of INF7 could primarily be due to poor membrane association at pH 7.4, a
property not ideal from a drug-delivery perspective since it could potentially reduce uptake
efficiency of the vector.

Few attempts have been made to characterize the binding affinity of HA2
peptides, presumably since their highly hydrophobic character strongly favor
complete membrane association, but also because their hydrophobicity complicates the
analysis of binding data as noted by Vacekar

16
et al. (39). We did try to construct binding isotherms from tryptophan fluorescence spectra recorded during titration of peptide to a LUV solution. However, this procedure did not yield any informative results due to peptide aggregation in solution and likely also in the membrane (vide infra) obstructing the necessary definition of “free” and “bound” states (40). According to estimates by Lear et al. (29) HA2(1-20) associates completely to small unilamellar vesicles composed of POPC at peptide-to-lipid ratios above 1:75. INF7 may be expected to display lower affinity since Glu4 and Glu7 increase water solubility and decrease hydrophobicity. Using the Wimley and White intrinsic whole-residue hydrophobicity scale (41), the theoretical free energy of partitioning the INF7 peptide from water to the interface of a POPC membrane was calculated to -4.3 kcal/mol taking into account the acetylated N-terminus and amidated C-terminus as well as the observed peptide α-helical content (42). This corresponds to, at room temperature, an equilibrium partition constant of ~10⁴, indicating that the equilibrium is strongly shifted towards membrane association.

Naturally, the membrane binding affinity could be expected to be somewhat lower in presence of POPC/POPG (4:1) LUVs due to that 20 percent of the lipids in these vesicles carry a net negative charge. However, returning to our own results we wish to reemphasize that the tryptophan fluorescence properties of INF7 indeed resemble those of the CPP penetratin which interacts strongly with lipid membranes by interactions in the head-group region (20). In addition, INF7 displays the, for this study, largest LD in the pi-pi* absorption band, which in fact disputes poor membrane association. The LD at 210 nm could be exploited to derive a lower limit of the bound fraction of INF7. Under the assumption that all bound INF7 peptides bind with their entire backbone perfectly parallel to the lipid plane the LD observed in Fig. 6C would correspond to that at least 55% of the peptides are membrane bound. Perfect orientation is not likely achieved because of the dynamic properties of the membrane and due to that far from 100% of the peptide is α-helical. Therefore we can safely conclude that significantly more than 55% has to be bound. Thus, even though direct experimental evidence in terms of binding constants cannot be provided, the above argumentation taken together suggests that the major fraction of added INF7 peptide associate with the POPC/POPG (4:1) LUV membrane at a peptide-to-lipid ratio of 1:100. Therefore the reduced leakage capacity at pH 7.4 cannot be explained solely by poor membrane association even though we cannot exclude that this could have some minor effects.

Possible structural origins of INF7 pH sensitivity. Leaving the discussion whether poor membrane binding may to some extent influence the pH sensitivity of INF7, we shall now focus attention on structural and physico-chemical features differentiating the membrane interactions of INF7 at pH 7.4 and pH 5.0. CD indicates that upon binding to POPC/POPG (4:1) LUV's, INF7 cannot adopt much α-helical structure (18%), whereas HA2(1-23) under similar conditions is 39% α-helical. With the only difference between these two peptides being the introduction of glutamic acid residues in INF7 at positions 4 and 7, it is reasonable to suggest that the N-terminal part of INF7 is hindered from adopting α-helical structure in the membrane milieu, likely due to that these negatively charged residues repel each other. As mentioned above, several tryptophan fluorescence parameters speak in favor of that Trp14 and Trp21 in INF7 reside in the head-group region. In addition, the LD magnitude in the pi-pi* absorption band corresponds to an oblique angle of ~70°, despite that only a small portion of the peptide takes part in the α-helical structure where pi-pi* transitions are expected to be stronger due to exciton coupling. This points to a more surface-oriented binding of INF7 compared to HA2(1-23).

The free energy cost of partitioning one charged glutamic acid residue from water into octanol amounts to ~3.6 kcal/mol (43), indicating that it should be virtually impossible for the N-terminal segment of INF7 to be inserted into the hydrocarbon core. By contrast, the free energy cost for partitioning of a protonated glutamic acid residue is only ~0.1 kcal/mol, indicating that at low pH insertion of this segment may occur. Recent estimates of pKₐ values of each individual negatively charged amino acid in HA2(1-25) and the glutamic acid enriched analogs E5(3,7) and E5(4,8), determined in
negatively charged SDS micelles, indicate that all glutamic acid residues and Asp19 have pKα values at least one log unit above the standard value for glutamic acid residues (pKα = 4.3) (5). By contrast, in zwitterionic DPC micelles the pKα of Glu4 in an 20 residue E5 peptide was estimated to ~4.2 whereas Glu8, Glu11 and Glu15 had pKα = 5.4 (44). These observations suggest that at pH 5.0 70-80% of the negative charge on the glutamic acid residues can be expected to be neutralized. Glutamic acids in the very N-terminal (Glu4) may be an exception but it is possible that negative lipids in the head-group region promotes protonation of such residues and thus insertion into the hydrocarbon core. The driving force could be to minimize electrostatic repulsions between lipid head-groups and peptide.

Two different NMR models have been proposed for the glutamic acid enriched HA2 peptide E5 (GLFEAIAEFIEGGWEGLIEG), both acquired in DPC micelles. Hsu et al. (13) propose that the pH-sensitive mechanism of E5 is essentially the same as the one proposed by Han et al. (10) and points out the importance of the hinge-region around Trp 14. Their model assumes that Glu4 and Glu8 have negligible effect on the insertion of the N-terminus, probably because both these residues are on the hydrophilic phase of the helix and can reside in the head-group region even if the peptide backbone is inserted. By contrast Dubovskii et al. (44) suggest no pronounced hinge motif but suggest that the E5 peptide act as an amphipathic helix and that protonation of foremost Glu11 promotes relocation of the peptide into the hydrocarbon core at low pH.

This is also concomitant with stabilization of α-helical structure. Even though the INF7 primary structure is not identical to E5 they share sufficient analogy to be compared: Both CD and LD data for INF7 indicate that this peptide behave differently from HA2(1-23). The marked increase in α-helix content, the surface oriented structure at pH 7.4 indicated in LD and the transfer of tryptophan residues from the head-group region into a more hydrophobic environment upon lowering pH (indicated from tryptophan fluorescence measurements) speak in favor of that the pH-dependent membrane interactions of INF7 are more resembling Dubovskii’s model than Hsu’s. Unfortunately, it is obvious from LD data (Fig. 6D) that INF7 is no longer well aligned at pH 5, suggesting that in a lipid bilayer as opposed to a micellar environment several orientations become accessible resulting in averaging that inevitably reduces LD. This cannot be explained by overall poor alignment of the sample but must result from different backbone conformations (see Fig. 8B). A possible cause of multiple peptide orientations is self-assembly within the membrane, which has been reported to occur (8, 45, 46). Peptide self-assembly could be driven by interactions between Glu4 or Glu7 on neighboring peptides.

**Long-term changes in peptide-membrane interactions.** The membrane destabilizing effect of both HA2(1-23) and INF7 manifests in that encapsulated small dyes can equilibrate across LUV membranes within minutes after peptide addition. Nevertheless, even after leakage has reached 100 percent we can observe a slow monotonous increase in peptide tryptophan emission intensity (Fig. 9) and a decrease in LD of membrane orientation probe retinoic acid (Fig. 8), suggesting that the peptide-lipid interactions do not reach an equilibrium state with the completion of leakage. The decrease in retinoic acid LD indicates that the upper part of the hydrocarbon core slowly becomes more and more disordered due to interactions with HA2 peptides, a process that is seemingly accompanied by more efficient shielding of peptide tryptophans from water. In POPC LUVs this behavior is coincident with poor peptide alignment, manifested in weak peptide bond LD and indiscernible orientation of peptide aromatic side chains (Fig. 6). Why such poor orientation is obtained in POPC LUVs whereas incorporation of a rather small portion of negatively charge lipid head-groups in the POPC/POPG (4:1) LUVs gives much better order is not easily understood. It seems as if some sort of collapse occurs in the headgroup region of POPC membranes possibly due to interactions between lipid cholines and peptide glutamic acids. Note that Han et al. choose to construct their EPR model of HA2(1-20) in DOPC/DOPG (4:1) LUVs even though their NMR model was obtained zwitterionic DPC micelles (10).

The observations of poor peptide orientations that are gradually worsened with time could indicate peptide self-assembly.
within the membrane, which has been reported to occur (8, 45, 46). The lack of observable aromatic residue LD suggests that such assemblies are of disordered nature. Since the positioning of the peptide backbone is more restricted by the membrane than the positioning of individual side chains, there is still an observable bias towards a preferentially more parallel than perpendicular insertion. Peptide assembly could potentially help shielding negatively charged residues thus allowing the peptide backbone to penetrate deeper into the membrane; the result being smaller LD magnitudes as well as decreased lipid chain order. A correlation between reduced lipid chain order and oblique insertion was found from molecular dynamics simulations of HA2 peptides in POPC membranes (39). As mentioned in Results, we performed control experiments to verify that the LUVs were indeed intact, despite their extensive leakiness and the observed reductions in retinoic acid LD. To our surprise we found no indications of that the LUVs would actually burst or that other types of peptide-lipid aggregates would form.

**Correlation between leakage levels and orientation.** A general conclusion from this work is that leakage is more prominent in zwitterionic POPC LUVs than in slightly acidic POPC/POPG (4:1) LUVs. Interestingly, as probed by retinoic acid, we can also see some correlation between leakage efficiency and the lipid membrane disorder observed immediately after addition of peptide to LUVs.

This correlation may in fact also explain the somewhat surprising observation that HA2(1-23) is even more potent as leakage-inducing peptide at pH 7.4 than at pH 5.0 in POPC/POPG (4:1) LUVs since in Fig. 8 we show that HA2(1-23) does not decrease lipid chain order at all at pH 5.0, whereas a significant effect is observed at pH 5.0. Furthermore, we observe a connection between faster establishment of the maximum leakage level (Fig. 2) and higher relative increase in tryptophan emission intensity (Fig. 9), suggesting that peptide penetration into the hydrocarbon core is a prerequisite for efficient leakage. On the other hand we do not find any correlation between ordered or disordered peptide binding and leakage efficiency; both alternatives result in high leakage levels. To be able to judge on the mode of action by which these peptides cause leakage, i.e. by “carpet” or “detergent” mechanisms, a dedicated leakage study would be required.

**Conclusions**

The influenza virus fusion peptide HA2(1-23) as well as its mutated glutamic acid-enriched version INF7 induce high degrees of leakage in both zwitterionic and slightly acidic LUVs. This study shows that INF7 displays pH sensitive characteristics promising for potential future use in peptide-based drug delivery systems, whereas HA2(1-23) is, expectedly, too active at pH 7.4. The origin of pH sensitivity seems related to that INF7, with five negative charges at pH 7.4, is hindered from deep penetration into the membrane hydrocarbon core under physiological conditions. In addition we a possible correlation between induced membrane lipid disorder, measured using the membrane probe retinoic acid, and leakage capacity. From LD we conclude that HA2 peptides display poor alignment in zwitterionic LUV membranes, compared to slightly acidic LUVs. We also present evidence for slow changes (hours) in peptide-lipid interactions resulting in reduced lipid order and in most cases more efficient shielding of tryptophans, not related to the fast initial peptide-induced membrane leakage of small dyes.

Using linear dichroism spectroscopy we have been able to show that HA2(1-23) in slightly acidic lipid membranes (POPC/POPG (4:1)) exhibits a high degree of orientation and that details regarding the binding geometry can concluded from the orientations of transition moments of the peptide backbone and of the aromatic side-chains. HA2(1-23) displays few pH sensitive characteristics under the experimental conditions of our study. This can be understood from the limited conformational change we propose for this peptide upon reduction of pH from 7.4 to 5.0. We suggest, based on the LD features of Tyr22 that the positioning of the peptide C-terminus is driven by interfacial interactions of Tyr22 and Trp23; this positioning is insensitive to pH. Further, no change in oblique insertion of the peptide backbone is observed suggesting that the pH-induced conformational change is primarily due to minor rearrangement of the peptide backbone.
in the “hinge region” around Trp14. LD of INF7 at pH 7.4 is different from that of HA2(1-23) and suggests a more surface-oriented binding mode, perhaps providing an explanation of its pH sensitivity. Due to that INF7 display poor membrane orientation at pH 5.0, informative information on the relation between structure and leakage inducing capacity could not be obtained, but it can safely be concluded that INF7, due to the glutamic acid residues at positions 4 and 7, interacts with lipid membranes rather differently than HA2(1-23).

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References


Linear dichroism studies of geometry and positioning of two α-helical peptides in large unilamellar phospholipid vesicles

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ABSTRACT: Two peptides, transportan and mDplp(1-30), complexed with partly negatively charged shear oriented large unilamellar phospholipid vesicles, were studied by linear dichroism (LD) spectroscopy. The results are compared to earlier NMR studies in simpler membrane mimetic model systems that had suggested diametrically different orientations relative to the membrane normal for the two α-helical peptides in phospholipid bilayers. Transportan is a chimeric cell-penetrating peptide (CPP) that was proposed to bind in the head-group region of zwitterionic or negatively charged lipid membranes as a partial amphipathic helix, while mDplp(1-30), derived from the 30 N-terminal residues of the mouse Doppel protein, was implicated to associate to lipid membranes as a transmembrane helix. The present LD results agree with these orientations and for mDplp(1-30) give further detailed orientation information regarding two tryptophan residues which are located at the membrane/solvent interface of the vesicles with their side chain long-axes oriented close to parallel to the bilayer surface.

KEYWORDS Cell-penetrating peptide • transportan • doppel protein derived peptide • linear dichroism • peptide-membrane interactions

Introduction

Transportan is composed of the 12 N-terminal residues of the neupeptide galamine, linked to the mastoparan sequence (originating from wasp venom) by a lysine residue. This cell penetrating peptide (CPP) has been shown to internalize into live cells on its own and when linked to cargoes of variable sizes (1-4). When the solution structure of transportan in micelles or zwitterionic bicelles was studied by NMR, two α-helical structures separated by a disordered hinge region were revealed (5). The C-terminal helix was better defined than the N-terminal one, but both helices were suggested to reside in the headgroup region of the bilayer, close to parallel to the surface.

The Doppel protein (Dpl) was the first prion protein (PrP) homologue found in humans (6). The Dpl and PrP proteins have been proposed to have antagonistic effects, i.e. PrP has been suggested to be neuroprotective, while Dpl seems neurotoxic (7-9). The neurotoxicity of Dpl can possibly be related to its N-terminal part, since the peptide mDplp(1-30) has been shown to exhibit similarly potent membrane perturbing effects on phospholipid bilayers as does melittin (a bee venom) (10-12). mDplp(1-30) is composed of the signal peptide sequence (residues 1-24), normally cleaved off during cellular processing of Dpl, and the first five residues (25-30) of the mature Dpl. Circular dichroism (CD) measurements have shown that mDplp(1-30) possesses a high propensity towards α-helix formation and NMR studies in bicelles and micelles suggest a transmembrane orientation for this helix (13). Table 1 shows the amino acid sequences of the two peptides. Transportan has like mDplp(1-30) been shown to cause membrane perturbations of model membranes, but to a much lesser extent (13, 14).
Table 1. The sequences of transportan and the Doppel protein derived peptide, mDplp(1-30). Aromatic residues are written in bold and charged residues in italic.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net charge</th>
<th># residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transportan</td>
<td>GWIINLSAGYLLGKINLKALLAALKKIL</td>
<td>+4</td>
<td>27</td>
</tr>
<tr>
<td>mDplp(1-30)</td>
<td>MKNRLGTWWVAILCMLLASHULSTVKARGIK</td>
<td>+6</td>
<td>30</td>
</tr>
</tbody>
</table>

The present study was undertaken with the aim of investigating if the observed membrane perturbations are correlated to different membrane-induced structures and orientations in bilayers of transportan and mDplp(1-30). We have used polarized linear dichroism spectroscopy (LD) to study the peptides complexed with shear-deformed large unilamellar vesicles (LUVs). Earlier LD studies have established that the orientation of the peptide bond transition moments in α-helical segments as well as transition moments in tryptophan and tyrosine side chains can be resolved by LD using a membrane model system comprised of LUVs (6, 15, 16) hence overall peptide orientations derived form LD studies are compared to suggested orientations from previous NMR studies in simpler membrane mimetic systems (5, 13).

Materials and methods

Chemicals 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] sodium salt (POPG), both of 99% purity, were purchased from Larodan, Malmö, Sweden. The lipids were dissolved in chloroform upon arrival and stored at -20°C. Retinoic acid, used as membrane orientation probe and the buffer chemicals were obtained from Sigma. Transportan and mDplp(1-30), both of 95% purity, were purchased from Neosystem Laboratoire (Strasbourg, France) and used without further purification (see Table 1 for peptide sequences). Deionized water from a MilliQ system was used in all experiments.

Preparation of Large Unilamellar Vesicles (LUVs) The POPC:POPG (4:1) LUVs were prepared by mixing desired amounts of chloroform dissolved POPC and POPG in a round bottom flask.

The chloroform was evaporated using a rotary evaporator and the remaining lipid film placed under vacuum for at least 2 hours in order to get rid of any residual solvent. The film was then hydrated in 10 mM sodium phosphate buffer (50% w/w sucrose), pH 7.4, during 8 min of vortexing, resulting in a final lipid concentration of 7.5 mM. The hydrated lipid film was freeze-thawed five times using liquid nitrogen and hot water. The suspension was passed through a polycarbonate membrane (pore size of 100 nm) 21 times using a Liposofast pneumatic extruder. This procedure results in the formation of a population of unilamellar vesicles with a small size distribution around the membrane pore size (100 nm). Sodium phosphate buffer is chosen for transparency in the UV region and sucrose is added to match the refractive index of the otherwise highly scattering LUVs. In addition, the sucrose-supplemented buffer increases the viscous drag and hence the degree of sample orientation in the Couette cell used in LD (see below) (17). Is has been established in a study on penetratin peptides that sucrose does not alter their secondary structure when membrane-bound (18).

Absorbance The concentration of the peptide stock solutions in MilliQ water was determined from the absorbance at 280 nm using extinction coefficients of 5690 M⁻¹ cm⁻¹ for tryptophan and 1280 M⁻¹ cm⁻¹ for tyrosine (19). Absorption spectra between 190 and 500 nm were recorded for all LD samples on a Cary 4000 UV-Vis spectrophotometer (Varian Inc.) in a quartz cell of 1 mm path length.

Linear Dichroism (LD) LD is defined as the differential absorption of linearly polarized light parallel and perpendicular to an orientation axis.
\[ LD(\lambda) = A_0(\lambda) - A_1(\lambda) \]  

Cotredtation was achieved by placing the LUV-peptide sample (containing 4 mM lipid) in the narrow gap between the two quartz cylinders of an outer-rotating Couette cell with a total light path of 1 mm. During rotation, shear forces act on the vesicles and make them slightly elongated in the laminar flow (shear rate 3100 s\(^{-1}\)), hence creating a net orientation of the sample in the flow direction. Spectra were recorded at room temperature in 1 nm increments between 190 and 500 nm on a JASCO J-720 Spectropolarimeter equipped with an Oxley prism to obtain linearly polarized light (20). The scan speed was 100 nm/min and the bandpass 2 nm. Three consecutive scans were recorded and averaged by the computer for each measurement. All spectra were corrected for background contributions by subtracting corresponding spectra recorded without rotation of the Couette cell (isotropic sample).

The binding geometry of a vesicle-bound (α-helical) peptide can be obtained from its LD' i.e. the reduced LD normalized with respect to the corresponding isotropic absorption (A_{iso}). The relation between LD' and the angle \(\alpha\) of a transition moment relative to the membrane normal (the only unique axis in a membrane system) is:

\[ LD' = \frac{LD}{A_{iso}} = \frac{3}{4} S(1 - 3 \cos^2 \alpha) \]  

where \(S\) is the macroscopic orientation parameter describing the ordering of the sample.

\(S\) varies between 0 (no orientation) and 1 (perfect orientation of the sample). The \(\cos^2 \alpha\) should be considered as an average value.

A total concentration of 10 µM of the membrane probe retinoic acid (from a 5 mM stock solution in ethanol, kept on ice and wrapped in foil to protect from light) was added to the LUV sample as an inner standard to determine the orientation parameter \(S\) as described earlier (21). When the probe reached an equilibrated intercalated position in the membrane (achieved after 1 hour of incubation), the LD was measured to determine the initial sample orientation. Thereafter, peptide was added to the sample (to a total concentration of 40 µM, yielding a peptide-to-lipid molar ratio of 1:100). The blue edge of the retinoic acid absorption band (centred on 350 nm) tails into the 280-300 nm region of the peptide spectrum. Hence all spectra acquired in the presence of membrane-bound retinoic acid were corrected for the contribution of the probe as described previously (16).

LD spectra were recorded with time in 30 minutes intervals to monitor if the peptide needed time to find an equilibrated position. Transportan adopted its position essentially immediately upon addition, whereas mDplp(1-30) needed approximately 30 minutes to equilibrate. All experiments were repeated on three separate occasions and representative spectra are shown in Figure 2.

Information on the positioning of the aromatic residues (particularly Trp8 and Trp9 in mDplp(1-30)) could be obtained from the sign and magnitude of the absorbing transition moments in the 260-300 nm region (\(^1L_a\) and \(^1L_b\) in tryptophan) as well as from the absorption band at ~225 nm (\(^1B_\theta\) in tryptophan) (22). The transitions in the peptide bond absorb at wavelengths < 210 nm (\(\pi\rightarrow\pi^*\)) and at 225-230 nm (\(n\rightarrow\pi^*\)). The first transition corresponds to the lower energy component of the exciton coupled \(\pi\rightarrow\pi^*\) transitions in an α-helix, directed in parallel to the helix axis, while \(n\rightarrow\pi^*\) is oriented perpendicular to the α-helix axis. Figure 1 shows the directions of the transition moments in Trp, Tyr and the peptide bond of in α-helix. The LD signal of especially \(n\rightarrow\pi^*\) is strongly overlapped by the signal of the relatively strong \(^1L_b\) transition in tryptophan as well as \(^1L_a\) in tyrosine.
The LD' values of $^1\text{L}_\text{a}(\text{Trp})$ and $^1\text{L}_\text{d}(\text{Trp})$ in mDplp(1-30) were derived after extracting the orientation parameter $S$ from measurements on retinoic acid incorporated in LUV membranes as described earlier (16, 21, 22). In short, the relative contributions to LD from $^1\text{L}_\text{a}(\text{Trp})$ and $^1\text{L}_\text{d}(\text{Trp})$ were estimated by least-squares projection of the LD signal in the 270–310 nm region onto reference spectra of the corresponding $^1\text{L}(\text{Trp})$ absorption bands. The spectral profile of these absorption bands was obtained from fluorescence anisotropy and excitation spectra of tryptophan in a propylene glycol glass according to the calculations described by Lakowicz (23). An angle of 85° between the $^1\text{L}_\text{a}(\text{Trp})$ and $^1\text{L}_\text{d}(\text{Trp})$ transition moments was assumed based on measurements of the tryptophan side chain analogue 3-methyl indole in stretched polyethylene film (24). The possible orientations for the $^1\text{B}_\text{e}$ transition moments were thereafter derived using a 3D model whose mobility was restricted by the angular constraints determined from the LD' data derived for the two $^1\text{L}(\text{Trp})$ transitions. In addition, to examine the total LD' in the 225 nm absorption band (where $^1\text{B}_\text{e}$ and the $n\rightarrow\pi^*$ peptide bond transitions absorb) a modified version of the method previously described by Thulstrup et. al. (25) was employed. Linear combinations of the isotropic absorption spectrum and the LD spectrum for a series of $k$ values were performed according to:

$$kA_{iso} = LD.$$  

The value of $k$ for which the spectral feature of the $^1\text{B}_\text{e}$ and the $n\rightarrow\pi^*$ transitions was exactly eliminated (i.e., when the shoulder in the absorption spectrum disappears) corresponds to the total LD' in this absorption band.

**Results and discussion**

The LD spectra of mDplp(1-30) and transportan in POPC:POPG (4:1) LUVs, pH 7.4 are shown in Figure 2. CD spectra were also recorded for the two peptides in the same vesicle system, verifying the induction of $\alpha$-helices in both cases (data not shown). Both peptides display strong LD signals around 220–225 nm, which adopt opposite sign. These bands are primarily due to the strong $^1\text{B}_\text{e}$ transitions originating from tryptophans overlapping with the relatively weak $n\rightarrow\pi^*$ transitions of the peptide bond. For transportan, contributions from the $^1\text{L}_\text{a}(\text{Tyr})$ transition moment with an absorption intensity about 20 percent of the $^1\text{B}_\text{e}$ in tryptophan, should also contribute.

![Figure 2](image)

The LD spectrum of mDplp(1-30) shows well-ordered aromatic residues with a positive $^1\text{L}_\text{d}(\text{Trp})$ absorption band (a positive double peak just below 290 nm), a negative $^1\text{L}_\text{a}(\text{Trp})$
absorption band (negative amplitude around 270 nm and above 295 nm) besides the positive $^1B_b$ band (strong positive LD at 225 nm). The LD spectrum of transport is less well defined in the 260-300 nm region, but a positive LD contribution from $^1L_a$(Trp) is nevertheless evident.

The value of the orientation parameter $S$ for the mDplp(1-30) sample was estimated to 0.038 from the LD' of retinoic acid (data not shown), a value that is normal for a LUV membrane of this composition (27) and thus gives an indication that mDplp(1-30) has little effect on the overall macroscopic orientation of the LUV sample. For the evaluation of orientation angles, the LD spectrum was normalized with respect to $S$ so that LD' values would only depend on the insertion angle of the peptide and not on the degree of macroscopic sample orientation. All LD' values discussed from now onwards are thus formally LD'/S.

For mDplp(1-30), the LD' values of the $^1L_a$(Trp) and $^1L_a$(Trp) transition moments were estimated to -0.84 and +0.54, respectively, which correspond to angles of 32° and 72° relative to the membrane normal. The least-squares analysis used to estimate these values (see Materials and Methods) was restricted to the 270-310 nm region since the red wing of the absorption from the very strong positive $^1B_b$ transition contributes to LD at lower wavelengths. This results in that the experimental spectrum is less negative below 270 nm than it should be if the LD was just a linear combination of the $^1L_a$(Trp) and $^1L_a$(Trp) spectral profiles. The reconstructed LD spectrum $LD'(1L_a)*Abs(1L_a) + LD'(1L_a)*Abs(1L_a)$. Abs(1L_a)) is shown in Figure 3.

Apart from the explainable discrepancy below 270 nm, the fit is very good and reproduces the vibrational structure of the $^1L_a$-band at ~289 nm well. Further, based on the orientations of the $^1L_a$(Trp) and $^1L_a$(Trp) transition moments, two possible orientations of the $^1B_b$ transition were derived; one close to the magic angle (54.7°, LD = 0 according to Equation 2) and the other one close to perpendicular to the membrane normal (>75°).

![Figure 3](image)

**Figure 3.** Experimental (solid line) and reconstructed (dotted line) LD spectra for mDplp(1-30) in the aromatic region.

Judging from the magnitude of the positive LD signal centred at 225 nm, $^1B_b$ must contribute significantly to this peak, hence the magic angle alternative was excluded. We conclude that the tryptophans in mDplp(1-30) thus must orient with their pseudo-symmetry axes close to parallel to the membrane surface and with the indole ring plane at the oblique angle defined by the orientations of the $^1L$(Trp) transitions.

The LD signal from the peptide bond transitions in mDplp(1-30) are not as clearly deduced as those for the tryptophan side chains. At 210 nm LD is close to zero but approaches negative values at even lower wavelength. Judging from the shape of the exceptionally strong LD signal at 225 nm it is possible that the absorption from the $^1B_b$ transitions tail out even below 210 nm and hence cancel a negative contribution of the $\pi \rightarrow \pi^*$ peptide bond transitions below 210 nm. There is however no evidence of a positive peak or shoulder on the blue-wing of the 225 nm peak at this wavelength. A positive LD at 210 nm is a distinct signature of a surface-oriented peptide (15, 16, 18). In addition, the strong positive peak at 225 nm has in fact an LD' value above the formal maximum of 0.75 (see below). This strongly suggests that the $n \rightarrow \pi^*$ transitions, even though being of weak intensity, must contribute to the positive LD signal in this region. The absence of positive LD at 210 nm in combination with the large LD' value at 225 nm
thus supports previous NMR data (13) suggesting that mDplp(1-30) should orient in a transmembrane fashion rather than on the surface.

The LD' in the 225 nm absorption band was estimated to be ~0.9, which is in fact larger than the formally allowed maximum that corresponds to 0.75 given by an angle α=90° according to Equation 2. This observation implies that the local orientation of the sample in the immediate surrounding of the tryptophans in mDplp(1-30) is higher than the orientation obtained for the membrane probe retinoic acid. Retinoic acid has up to now exhibited the highest degree of orientation among molecules measured by us in flow-oriented lipid vesicles (21). One possible reason for the high LD' signal for mDplp(1-30) may be an unusually high local order of the phospholipids in the vicinity of the peptide and particularly its tryptophans.

The reported (13) NMR 3H2O exchange experiments performed on mDplp(1-30) in DHPC micelles showed that the NMR signal from Trp8 quickly disappeared, whereas the signal from Trp9 could still be observed after 3 h, but became undetectable after 6 h. This implies that Trp8 is exposed to solvent, while Trp9 is not fully exposed, but is nevertheless accessible. Further, from the NMR study of mDplp(1-30) complexed with a DHPC micelle, the orientations of the ring planes in the relatively disordered tryptophans were suggested to be rather parallel to the helix axis (13). In contrast, the present results for the peptide in a vesicle environment indicate that the relatively well-ordered tryptophan side chains are oriented perpendicular to the helix axis, adopting orientations similar to what have been observed for tryptophan model compounds (indoles) in LUVs (22). It should however be noted that the DHPC micelles used for NMR exhibit a higher surface curvature, thus possibly constraining the orientation angles of the tryptophans at the solvent interface. The combined NMR and LD results indicate that Trp8 and Trp9 in mDplp(1-30), residing at a membrane interface, may be considered as flanking residues of a α-helical, membrane-spanning segment, with their side chains positioned in parallel to the bilayer surface.

The LD spectrum of transportan resembles, especially in the UV region, in certain respects the spectra of penetratin and some penetratin analogues (18). It displays a positive LD peak centred at ~210 nm emanating from the low energy couplet of the exciton couplings between π→π* transitions in the peptide bonds indicating that transportan orient more parallel than perpendicular to the surface. A possible explanation for the low magnitude of the peak at 210 nm is that transportan may not adopt a well-ordered orientation in the lipid bilayer. An additional explanation is that transportan possesses a disordered, flexible hinge region linking its two α-helical segments (5) (PDB structure 1sm2). These two segments can tilt slightly with respect to the bilayer surface. Even a small tilt of one of the segments can result in a relatively large reduction in LD signal, since the LD is very sensitive to the transition moment orientation in the regimes close to parallel or close to perpendicular to the orientation axis as a consequence of its cosα dependence on the orientation angle (see Equation 2).

The spectral features of the aromatic region are not as well resolved for transportan as they are for mDplp(1-30) (Figure 2). The two peaks arising from the 1Lα(Trp) transition are positive, indicating that this transition moment is more in parallel to the surface than to the membrane normal also in transportan. In addition, the LD is close to zero above 295 nm where only 1Lα(Trp) absorbs, indicating that this transition’s contribution to the total LD is relatively small. The negative LD at 270 nm is assigned to the 1Lβ transition moment in Tyr9. However, the LD spectrum in the 260-310 nm region could not be well reconstructed from absorption profiles of the tryptophan 1Lα-transitions and 1Lβ(Tyr) using the least-squares projection approach and therefore no detailed angular information is given.
The negative LD in the 225 nm region for transportan (Figure 2) is too strong to emanate solely form the n→π* transitions which have an extinction coefficient of approximately 100-200 per bond (26) (2500-5000 M⁻¹ cm⁻¹ for the entire peptide). We therefore conclude that either 1B₈(Trp) or 1L₄(Tyr) (or both) must contribute to the negative LD. The orientation of the tryptophan residue in transportan (Trp2) seems less well-defined than of the tryptophans in mDplp(1-30). The NMR structure of transportan shows that the N-terminus is relatively flexible, although having helical structure (5). Our LD data is thus in agreement since Trp2 seems to possess very little motional restriction. The fact that the spectral contributions in the aromatic region could not be readily resolved indicates that this residue can adopt a range of orientations and possibly also reside in slightly different environments. The outcome of such flexibility is foremost an averaging effect of the 1L₄(Trp) and 1B₈ signals that consequently approach zero. The 1L₄(Trp) transition is however less affected since its polarization is almost in line with the bond connecting the indole side chain with the backbone. We suggest that rotational motion around the indole-backbone bond contributes most to the somewhat imprecise LD. For the same reasons it is difficult to give any estimate on the orientation of Tyr9.

In conclusion, the LD studies give detailed information about the structures and orientation of two membrane-interacting peptides bound to LUVs. Both peptides are known to give rise to membrane perturbations and leakage, despite their different membrane- associated geometries. The most potent membrane perturbing of the two, mDplp(1-33), has a transmembrane orientation and seems to be anchored at the bilayer surface by its well-ordered tryptophan residues, which may be thought of as tilted paddles reaching out of the transmembrane helix. mDplp(1-30) can be considered as a general model for a membrane-spanning helical peptide. The somewhat less membrane perturbing peptide, transportan, is localized to the head-group layer of the membrane, where its two aromatic residues are less well ordered. The cell penetrating and less toxic properties of transportan may be due to this preferred orientation in a phospholipid bilayer.

References


