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Protein Structure and Interaction in Health and Disease

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Cover: The structure of the N-terminal thioredoxin domain of human glutaredoxin Grx3 solved by NMR spectroscopy.

During the course of the research underlying this thesis, Cecilia Andresen was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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To Emma

Abstract

This thesis focuses on protein structure, dynamics and interaction and their relation to human disease. In particular, the biophysical and structural properties of both well-ordered and partially disordered proteins are studied using a range of biophysical techniques such as circular dichroism spectroscopy, fluorescence spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy. *Pseudomonas aeruginosa* is a human pathogen due to its multidrug resistance (MDR) caused by overexpression of efflux pump systems. This thesis describes how MDR mutations within the MexR repressor of the MexAB-OprM system reduce the DNA affinity by altering its stability with maintained structure. The oncogenic protein c-Myc is involved in many essential biological functions such as cell proliferation, differentiation and apoptosis and is also highly associated with several forms of human cancers, and where the N-terminal domain is regulated by a plethora of protein interactions. In this thesis the intrinsically disordered N-terminal part of c-Myc and its interactions with the proteins Bin1 and TBP are described. Myc binds Bin1 with maintained disorder in a multivalent manner, which may explain why the onco-protein can interact with such a wide range of binding partners. A similarly dynamic interaction is observed for Myc with the TATA-binding protein (TBP). The essential human multidomain glutaredoxin Grx3 is associated with several biological functions such as redox signaling, proliferation and signal transduction. We have solved the structure and analyzed the dynamic properties in the ps-ns and ms time scale for the two N-terminal domains, providing a platform for further analysis of the Grx3 protein and its interactions. Taken together, this thesis emphasizes the importance of joint structural, biophysical and dynamic studies to better understand protein function in health and disease.

Populärvetenskaplig sammanfattning

Proteiner, naturens kanske viktigaste molekyler, består av endast ett fåtal grundämnen men styr trots det många livsnödvändiga funktioner i alla levande organismer. Proteiner är t.ex. en huvuddel av vårt immunförsvar, muskler och hormonsystem. De olika proteinerna har en specifik struktur som ofta är kopplad till dess funktion. Även rörelser är avgörande för proteinets funktion. Inom proteinet sker rörelser i form av snabba vibrationer i bindningar mellan atomer men även långsammare rörelser som uppkommer när proteinet samverkar med andra proteiner eller DNA. Då ett fel i proteinstrukturen uppkommer kan det leda till sjukdomar som cancer, Alzheimers eller autoimmuna sjukdomar. För att se hur proteinets normala egenskaper har förändrats, så är det av högsta relevans att studera hur funktionen och strukturen hos proteinet förändras vid sjukdom.

I det här arbetet har proteiners struktur, dynamik (rörelse) och bindning till andra proteiner eller DNA studerats. Att se kopplingen mellan proteiners struktur, dynamik och bindningsförmåga är avgörande för att få bättre kunskap om dess funktion. Tre sjukdomsrelaterade proteiner, MexR, c-Myc samt Grx3 har studerats biofysikaliskt och strukturbiologiskt med metoder som kärnmagnetisk resonans (NMR), cirkulär dikroism (CD) samt fluorescensspektroskopi.

Vissa proteiner reglerar produktionen av andra proteiner genom att binda till DNA. Proteinet MexR t.ex., reglerar ett specifikt pumpsystem (MexAB-OprM) i *Pseudomonas aeruginosa*, en bakterie som ger svåra lunginflammationer främst hos patienter med nedsatt immunförsvar, orsakat av sjukdomar som cancer, cystisk fibros och AIDS. Pumpsystemet kan effektivt pumpa ut antibiotika och liknande molekyler från bakterien innan den tagit skada vilket gör bakterien multiresistent. Man har funnit ett flertal mutationer i MexR som leder till ökad produktion av pumpsystemet och därmed ökad resistens mot antibiotika. Mer kunskap om det regulatoriska proteinet MexR och dess mekanismer är därför av yttersta intresse for att kunna utveckla framtida varianter av läkemedel. Vi har genom att biofysikaliskt analysera MexR fått en bättre kunskap om orsakerna till den försämrade förmågan att binda till DNA. Proteinets struktur samt dynamiska egenskaper är avgörande för bindningsförmågan.

Det humana cancerrelaterade proteinet c-Myc, är involverat i en mängd biologiska funktioner, celltillväxt, celldelning och programmerad celldöd för att nämna några. Proteinet är strikt reglerat i normala celler men överproduceras då en förändring i proteinet sker, vilket leder till massiv tumörbildning. Vi har studerat de strukturella och dynamiska egenskaperna hos c-Myc samt dess förmåga att samverka med andra proteiner. Eftersom c-Myc styr ett stort antal funktioner i cellen är analyserna av stort intresse. Vi såg att c-Myc binder andra proteiner då det delvis är ostrukturerat. Det är i sig intressant eftersom det troligen är en del av dess mekanism och kan möjliggöra bindning till ett större antal bindningspartners än om det vore välstrukturerat.

Humant Grx3 är ett multidomänprotein, dvs. består av isolerade delar som kan ha olika funktioner och olika struktur. Proteinet produceras i onormal mängd då kroppens celler upplever stress, vilket indirekt reglerar uttrycket av andra proteiner. Detta kan ske i t.ex. hjärtmuskulatur eller i cancerceller. Vi har bestämt strukturen för två av tre domäner i proteinet för att senare kunna analysera om proteinets struktur är avgörande för funktionen. Vi har även analyserat rörelser i proteinet vilket kan ge information om vilka delar som ingår i bindning till andra proteiner.

För att i framtiden kunna utveckla nya läkemedel eller andra terapeutiska metoder för att bekämpa sjukdomar är det viktigt att kunna se hur ett proteins strukturella och dynamiska egenskaper hör samman och hur förändringar hos dessa egenskaper påverkar funktionen. Denna avhandling bidrar till att samla och utveckla denna kunskap.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-V).

I Cecilia Andresen, Shah Jalal, Daniel Aili, Yi Wang, Sohidul Islam, Anngelica Jarl, Bo Liedberg, Bengt Wretlind, Lars-Göran Mårtensson and Maria Sunnerhagen. "Critical biophysical properties in the *Pseudomonas aeruginosa* efflux gene regulator MexR are targeted by mutations conferring multidrug resistance"
Protein Science, **2010,** 19(4): 680–692

II Cecilia Andresen, Sara Helander, Alexander Lemak, Christophe Farès, Veronika Csizmok, Jonas Carlsson, Linda Z. Penn, Julie D. Forman-Kay, Cheryl H. Arrowsmith, Patrik Lundström and Maria Sunnerhagen. "Transient structure and intrinsic disorder in the c-Myc transactivation domain and its effects on ligand binding"
Submitted

III Cecilia Andresen, Madhanagopal Anandapadmanaban, Sara Helander, Malin Fladvad, Karl Andersson, Daniel Kanmert, Pär Säfsten, Martin Moche and Maria Sunnerhagen."Molecular characterization of the interaction between the disordered c-Myc transactivation domain and the TATA-binding protein (TBP)"
Manuscript

IV Cecilia Andresen, Maria Sunnerhagen, Patrik Lundström. "Structural and dynamic analysis of human glutaredoxin 3"
Manuscript

V Aristi Potamitou Fernandes, Malin Fladvad, Carsten Berndt, Cecilia Andresen, Christopher Horst Lillig, Peter Neubauer, Maria Sunnerhagen, Arne Holmgren and Alexios Vlamis-Gardikas. "A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as a substrate for thioredoxin reductase"
The Journal of Biological Chemistry, **2005**, 280(26): 24544-24552

Papers not included in the thesis

VI Martin Lundqvist, Cecilia Andresen, Sara Christensson, Sara Johansson, Martin Karlsson, Klas Broo and Bengt-Harald Jonsson. "Proteolytic cleavage reveals rnteraction patterns between silica nanoparticles and two variants of human carbonic anhydrase". *Langmuir*, **2005**, 21(25): 11903–1190

VII Janosch Hennig, Lars Ottosson, Cecilia Andresen, Linn Horvath, Vijay K. Kuchroo, Klas Broo, Marie Wahren-Herlenius and Maria Sunnerhagen. "Structural organization and Zn^{2+}-dependent subdomain interactions involving autoantigenic epitopes in the RING-B-box-coiled-coil (RBCC) region of Ro52"
The Journal of Biological Chemistry, **2005**, 280(39): 33250-33261

Abbreviations

aa	Amino acids
CD	Circular dichroism
CSA	Chemical shift anisotropy
DNA	Deoxyribonucleic acid
GRX	Glutaredoxin
IDP	Intrinsically disordered protein
MALDI	Matrix assisted laser desorption/ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
RDC	Residual dipolar coupling
RNA	Ribonucleic acid
TBP	TATA box binding protein
TOF	Time of flight
TRX	Thioredoxin

Contents

Preface

This thesis focuses on protein structure, dynamics and interactions. The proteins studied in this work are all related to diseases, and regulate transcription directly or indirectly. To increase the knowledge of their functionality and biophysical properties, several biophysical and analytical methods have been used. The introduction of the thesis is intended to provide the reader with a short background on the proteins studied and the methods used.

Paper I describes the biophysical properties of the regulatory protein MexR found in the bacteria *Pseudomonas aeruginosa*. Several mutations are found in MexR causing disturbed DNA binding ability. This increases the antibiotic resistance of *Pseudomonas aeruginosa*. In Paper II and Paper III, the dynamical, structural and interactional properties of the transactivation domain of the oncoprotein c-Myc are evaluated. In particular, the interaction between c-Myc and the transcription factor TATA-box binding protein (TBP) as well as the tumor suppressor Bin1 are described. Paper IV and V present the structure and dynamics of two out of three domains in the multidomain human monothiol glutaredoxin 3 (Grx3), which is associated to oxidative stress in the cell.

The work has been performed mainly at the Department of Physics, Chemistry and Biology, Linköping University, but in close collaboration with both national and international scientists. I have had the fortune of working in an inspiring environment where protein science has always been at focus.

1. Introduction

1.1 Protein interaction, dynamics and structural biology

Proteins are nature's most important molecules and are present in all living beings. The human body consists mostly of proteins of different structures, dynamics and functions. Oxygen is transported to the human cells by hemoglobin, a multidomain protein in complex with iron which coordinates the oxygen molecule. The skin, the immune system, and muscle tissues are other examples of protein arrangements essential to human life. The building blocks of proteins are amino acids, small molecules mainly composed of carbon, nitrogen, oxygen and hydrogen. There are 20 different amino acids with specific properties that can be part of the protein structures. The sequence of the amino acids is unique for each protein and determines its fold and the function.

1.1.1 Structural and dynamical elements of proteins

The protein interior is usually composed of hydrophobic amino acids, which thus avoid contact with the hydrophilic environment in the cell. At the protein surface, polar and hydrophilic residues dominate, as they are attracted to the polarity of the solvent. Charged amino acid side chains like those of Asp, Glu, Lys and Arg are hydrophilic, whereas large non-charged residues such as the aliphatic Leu, Ile and Val as well as the aromatic Phe, Tyr and Trp are hydrophobic. The backbone of proteins, which includes the alpha carbons and the peptide bond, is hydrophilic and is thus not favored in the hydrophobic core. Nature has solved this problem by forming secondary structure elements from the backbone, thus preventing their hydrophilic NH and CO groups from

exposure towards the hydrophobic surroundings. The two main secondary structure elements, called α-helix and β sheet, are stabilized by hydrogen bonding between residues that are non-adjacent in sequence (Figure 1).

Figure 1. Structural elements of proteins. Left: α-helical formation, middle: β-sheet and right: tertiary structure of carbonic anhydrase II (pdb-id; 3NJ9).

The orientation in space of the secondary structure elements builds the tertiary structure of the protein. The secondary and tertiary structure uniquely characterizes each protein and is important for its function. For example, many proteins that interact with DNA consist of a dimer where each monomer contains a helix-loop-helix motif. This fold allows the protein to fit the DNA groove, and the affinity is modulated such that the protein can slide along the DNA until the right position is found. Membrane proteins often have a part consisting of helices to be able to penetrate the membrane. Other proteins and/or protein domains can be unstructured in their native form, but become structured on interacting with their interaction partner. The world of protein structure is as diverse as life itself.

Furthermore, proteins require motions at different time scales for their functions. Some examples of functions requiring molecular dynamics are protein folding/unfolding, catalysis, membrane transportation, transcription, and protein-protein complex formation (Mittag & Kay *& et al.*, 2010). Intrinsically disordered proteins are involved in a large range of biological important functions and are at one extreme of the dynamic timescale with a large degree of motion. At the other end, we find localized dynamics within the active sites, which is often critical for reactivity in catalyzed reactions, and in linker regions, which may be essential for bringing different parts/domains of proteins together to form biologically active arrangements.

1.1.2 Intrinsically disordered proteins

Characteristic for intrinsically disordered proteins (IDPs) are their ability to perform critical biological functions in the absence of well ordered (tertiary) structure under native conditions. The IDPs can be related to a large number of critical biological functions such as transcription regulation, cellular signaling and transactivation (Dyson & Wright, 2005). Because of the larger abundance of charges and/or polar residues and the low amount of hydrophobic residues, the IDP does not form a well-defined core. Indeed, IDPs lack enzymatic activity, since a folded scaffold is required to position residues appropriately in an active site (Tompa, 2002). Instead, the molecular function of IDPs, or longer segments of disorder, is often related to essential cell functions such as protein DNA interactions for regulation of processes including transcription, transposition, repair and replication, cell signaling and endocytosis (Dyson, 2011; Dyson & Wright, 2005; Fink, 2005).

Many IDPs use disorder and/or transition between ordered and disordered states to perform their functions (Dunker *et al.*, 2001; Dyson, 2011). IDPs promote molecular recognition through different features, including an unique combination of high specificity and low affinity towards the interaction partner and the ability to bind a high range of binding partners due to a large interaction surface (Liu *et al.*, 2006). Intrinsically disordered proteins often undergo folding on binding when interacting with substrate proteins (Dyson & Wright, 2005; Hazy & Tompa, 2009), examples of such proteins include p21 and p53 (Vise *et al.*, 2005; Wright & Dyson, 2009). Molecular recognition without the transition from disordered to ordered states are also shown for many IDPs where the unstructured conformation is a part of the protein function (Hazy & Tompa, 2009; Tompa, 2002). Thus, the disordered protein structure itself can even be functionally essential, which is contrary to a more old-fashioned view that structure yields function. Flexible linkers between domains are also often disordered thus allowing for interlinked domains to move relative to each other; linkers can also serve as interdomain spacers selective to specific domain orientations (Dunker *et al.*, 2001). Furthermore, many proteins regulated by phosphorylation are found to be disordered or contain intrinsic disordered sequences. One example of such protein is the intrinsically disordered protein Sic1 which contains several potential phosphorylation sites (Mittag & Marsh &

et al., 2010). The Sic protein interacts with Cdc4 in phosphorylated form for regulation of cell cycle progression.

To increase the understanding of the functions of IDPs and the advantages of unfolded states, it is important to analyze the thermodynamic, dynamic and structural properties of these proteins. Although IDPs do not have higher-order structure, several methods can be used to increase knowledge about their function and transiently fluctuating structures. NMR spectroscopy, circular dichroism (CD) spectroscopy, time resolved proteolytic digestion and fluorescence spectroscopy are some example of techniques that can give information of residual secondary structure, dynamics and interactions. Backbone relaxation experiment measured by NMR gives useful information of the state of order on a per-residue basis (Dunker *et al.*, 2001). As an example, the ^{15}N-{^{1}H}-NOE experiment gives positive values (>0.6) for ordered residues and negative (or values <0.6) for more disordered segments in the protein. By CD spectroscopy, ordered, molten globular and random coil states can be distinguished by far UV CD spectroscopy (Greenfield, 2006c), and the presence of non-cooperative denaturation, as found in the absence of a folded core, can be assayed by following ellipticity as a function of temperature (Greenfield, 2006b).

2. Regulation of multidrug resistance

2.1 Bacterial antibiotic resistance

Today, antibiotic and multidrug resistance (MDR) is a serious and worldwide threat to human health, and it is therefore important to increase the understanding of the mechanism of the resistance for development of the new generation of drugs and therapeutic strategies. Treatment of lethal infections with antibiotics has been used since the 1920s, thus dramatically decreasing the mortality caused by common and wide spread diseases like cholera and tuberculosis. However, the use of antibiotics has led to the development of resistant pathogens which are spreading rapidly, especially in the hospital environment (Kumar & Schweizer, 2005).

Antimicrobial agents act on bacteria according to several different mechanisms: inhibition of protein synthesis, improper cell wall synthesis, interference with DNA or RNA synthesis and modification of the cell metabolism (Neu, 1992; Tenover, 2006). Examples of antibacterial drugs that disturb the cell wall synthesis include β-lactames (including penicillin and carbapenems) and glycopeptides (vancomycin, teicoplanin and others). Antibiotics such as tetracyclines, chloramphenicol, and aminoglycosides can inhibit the protein synthesis of the bacteria. The DNA synthesis can be disturbed by fluoroquinoles and sulfonamides.

In order to survive the antimicrobial treatment, bacteria developed a range of mechanisms to survive and to be able to spread. Bacterial antibiotic resistance can be achieved by intrinsic, i.e. naturally occurring, resistance or acquired mechanisms, which includes mutations in gene targets (Alekshun & Levy, 2007). Bacteria can develop genes expressing proteins that destroy the antibiotic molecule before it reaches the desired effect. One example of such a protein is β-lactamase, which inactivates β-lactames before interfering with enzymes required for the synthesis of the cell wall of the organism. Drug resistant bacteria can also possess efflux systems that extrude the foreign drugs out of the cell, or produce an altered cell wall without binding sites for the antibacterial drug. Finally, resistance caused by spontaneous mutations can alter protein binding to the antimicrobial drug, upregulate drug-inactivating enzymes, downregulation of membrane pumps related to entry of the drug into the cell, and upregulate the production of efflux pumps. Increased antibiotic resistance leads to prolonged therapy as well as increased mortality rate both in patients suffering from diseases directly caused by pathogenic bacteria, and in patients with reduced general resistance to bacterial infections due to suppressed immune response by treatment or as a result of other diseases (Tenover, 2006).

2.1.1 *Pseudomonas aeruginosa* and drug resistance

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen, which means that the bacterium exploits some weakness in the host defense to initiate infection. *P. aeruginosa* causes infections in the respiratory system, bone and joints (Deredjia *et al.*, 2011), especially in patients with repressed immune system as a consequence of severe diseases including cancer, AIDS and cystic fibrosis, or patients suffering from burns. The bacterium is gram-negative (two cell membranes), aerobic and belongs to the family *Pseudomanadaceae.* The bacterium is commonly found in soil and water and occurs regularly on the surface of plants and animals. *P. Aeruginosa* has very simple nutritional requirement.

P. aeruginosa is intrinsically multiple resistant to antibiotics, due to the presence of a low-permeability outer membrane as well as the expression of Multidrug-EffluX (MEX) systems with broad specificity. MexAB-OprM (Poole *et al.*, 1993) and MexXY-OprM (Mine *et al.*, 1999; Westbrock-Wadman *et al.*, 1999), are two out of several efflux pumps, responsible for the intrinsic resistance. The

efflux systems recognize antibiotics, biocides, organic solvents, metals including zinc, cadmium and mercury as well as small molecules (Deredjia *et al.*, 2011; Schweizer, 2003).

2.2 Multidrug transporter systems

Multidrug transporters, also named efflux pumps, are regulated by repressors or activators at a transcriptional level, and are often an important factor in multidrug resistance (MDR). If the level of antibiotics increases in the cell, the expression of the efflux pumps increases, which provides multidrug or even complete resistance (Piddock, 2006). The drugs related to resistance are often targets of the transcription factors (Schumacher & Brennan, 2002) and are in some cases identical to the substrate recognized by the transporters in the cell-membrane. It has been shown that antibacterial compounds are often able to penetrate the cell wall but are expelled by the transporters before reaching the intracellular target. The transporters efflux a wide range of substrates such as antibiotics, organic solvents, metabolic products and signaling molecules, requiring an equally wide and structurally diverse range of efflux pump protein families (Grkovic *et al.*, 2001; Piddock, 2006; Schumacher & Brennan, 2002).

The efflux systems in *P. aeruginosa* belong to the resistance/nodulation/division (RND) family and consist of three components, the transporter, the linker and the pore in the outer membrane which prevents the substrate from entering the cytosol (Poole, 2001). The intrinsic resistance of *P. aeruginosa* can be related to expression of the MexAB-OprM pump, regulating the level of fluoroquinolones, other antibiotics and pathogenic organisms. In wild-type cells the MexAB-OprM is expressed at low level, still leading to antibiotic resistance, but when the efflux pump is overexpressed, the bacteria becomes multidrug resistant. The three components of the efflux pump are MexA, MexB and OprM. MexB, the inner membrane protein, recognizes the antibiotic compound and constitutes the proton energized pump. The compound is then transported via MexA, a linker protein, through OprM, which forms the exit channel through the outer membrane to the cell exterior. (Nakajima *et al.*, 2000). Crystal structures of MexA (Akama & Matsuura *& et al.*, 2004), MexB (Sennhauser *et al.*, 2009) and OprM (Akama & Kanemaki *& et al.*, 2004) are available, and ongoing work is detailing the molecular arrangement of these proteins in the membrane (Trepout *et al.*, 2010). In addition to the MexAB-OprM, several of other efflux systems

with a wide range of substrate drugs are found in P. aeruginosa (Schweizer, 2003).

2.2.1 Transcriptional regulation and the MarR family

The expression of transporters is regulated by transcriptional regulator proteins. Known activators are members of the MerR, AraC, and LysC families whereas repressors belongs to TetR, Lacl or MarR families (Grkovic *et al.*, 2001). The transcriptional regulators bind and respond to antibiotic compound and thereby acting as sensors, inhibiting or activating the expression of MDR transporters.

Overexpression of the efflux pumps leading to increased multi drug resistance can be a consequence of mutations in the transcriptional regulator. This has been shown in many proteins, for example MarR, MexR and EmrR. Antibiotics like benzalkonium and chloramphenicol often require higher level of efflux pump expression than wild type bacteria to prevent mortality of the organism.

The members of the multiple antibiotic resistance regulators, MarR, family are winged-helix-winged DNA binding transcriptional regulators from a large variety of species. The dimeric MarR shows an α/β fold, a common feature of MarR family members (Figure 2), consisting of a dimerization domain and a central domain which includes the characteristic winged-helix DNA binding motif. The most common secondary structure arrangement for the DNA binding motif is $\alpha1{-}\beta1{-}\alpha2{-}\alpha3{-}\beta2{-}W1{-}\beta3{-}W2$ where α stands for α–helix, β for β-sheet and W for loop (Wilkinson & Grove, 2006). $\alpha3$ is the so called DNA recognition helix, binding to the major grove of the DNA and contributes to the most contact and determines thereby the sequence specificity. It is also shown that within some family members, the wings are also crucial for DNA recognition by specific contacts to bases and to the DNA phosphate backbone (Wilkinson & Grove, 2006).

Common for the MarR family of proteins is their structure, the transcriptional regulation and the ability to increase the survival of the host organism in antibiotic environments (Perera & Grove, 2010). The active state of the MarR family members is dimeric both in the DNA-bound and free state. Most of the studied MarR family regulators are repressors (MarR, MexR and HucR) but in some case they act as activators (BadR, ExpG) or both (SlyA).

The toggling between binding and releasing DNA is effected by small molecule binding to the dimer region, or to the interface between the dimer and DNA domains (Wilkinson & Grove, 2006). For example, in *Escherichia coli*, MarR interacts with the DNA at two sites, (I and II) within the *marO* operator. It is shown that when MarR binds salicylic acid and other chemical compounds (Alekshun & Levy, 1999; Saridakis *et al.*, 2008) the interaction with *marO* is inhibited due to displacement of the DNA binding domains so that optimal fit with the major grooves can no longer be obtained. Similar observations have been made both for MexR (Lim *et al.*, 2002) and for the HucR protein (Wilkinson & Grove, 2006). It is thus clear that the structure and multidrug binding properties are closely linked to each other (Perera & Grove, 2010; Wilkinson & Grove, 2004).

The crystal structure of HucR reveals that the regulator also comprises the classical MarR family fold with an additional 35 residue long extension in the N-terminal not found for other MarR members. Moreover, for HucR, differences in the regulatory function can be seen compared to other MarR regulators with known structure, where HucR recognizes a single DNA sequence, not two like other HucR homologues (Wilkinson & Grove, 2006).

The SlyA regulator found in *Salmonella enterica* also shows characteristic features of a MarR family fold with a helix-turn-helix motif connected by a β-wing, forming the DNA recognition domain, and α-helical dimerization domain Unlike other MarR family regulators, the protein does not seem to undergo a static conformational change when binding the substrate drug salicylate (Dolan *et al.*, 2011), thus, dynamic mechanisms may be involved.

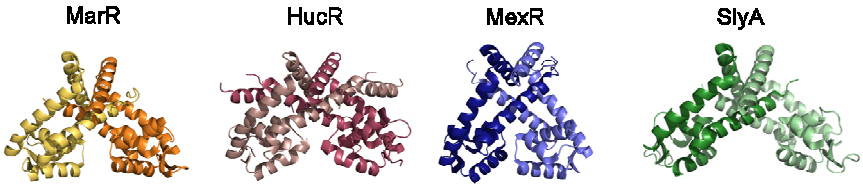

Figure 2. MarR family members MarR (chain A yellow, chain B orange pdb-id; 3BPX), HucR (chain A light purple, chain B purple pdb-id; 2FBK), MexR (chain A darker blue, chain B lighter blue, pdb-id; 1LNW) and SlyA (chain A light green, chain B dark green, pdb-id; 3Q5F). All three dimensional structures are drawn using PyMol (DeLano, 2002).

2.3 MexR

The repressor protein MexR is found in *P. aeruginosa* where it represses the MexAB-OprM efflux system. As mentioned above, the efflux system recognizes a range of antibiotics including β-lactame, tetracycline and chloramphenicol (Grkovic *et al.*, 2001). The compounds may be targets of MexR or to an unknown expression inducer. Over-expression of the efflux pumps result in multidrug resistance (MDR), which can be related to overexposure to antibiotics, including β-lactame, quinolones, tetracycline, chloramphenicol, sulfonamides and novobiocin, as well as oxidative stress signaling, macrolides and biocides (Chen *et al.*, 2010; Lim *et al.*, 2002).

MexR represses the expression of *MexAB-OprM* operon by binding as a dimer to the intergenic region of *MexA-MexR* at two DNA sites (one for each monomer) with inverted repeat of GTTGA sequence, also named PI and PII (Evans *et al.*, 2001). The overlapping binding sites of *MexR* and *MexA* regulate the expression of both MexR and MexAB-OprM.

The crystal structure (Lim *et al.*, 2002) (Figure 3A) reveals that MexR consists of a homo-dimer. Each monomer is composed of six helixes and two shorter β-sheets, and resembles the structure of homologue MarR protein found in *E. coli*. The DNA binding domain shows a characteristic winged helix-turn-helix motif common of DNA binding proteins. The winged helix domain (residue 36-97) consists of α2–β1–α3–α4–β2–W1–β3 where α4 is the recognition helix, interacting with the major groove of the DNA.

The DNA binding domain is connected to the dimerization domain by two helixes, α1 and α5. The N-terminus (residue 5-17) and C-terminus (residue 120-139) of the protein together form the dimerization interface.

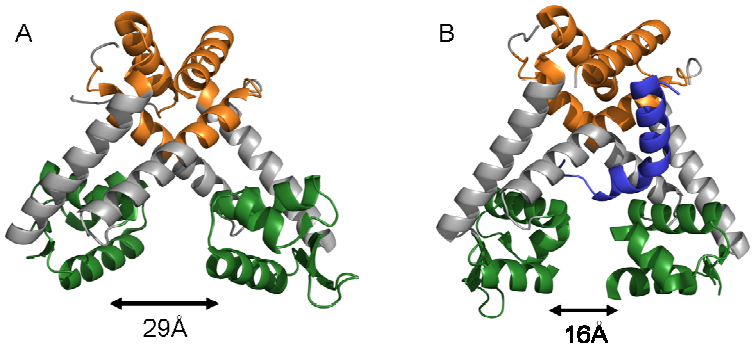

Figure 3. Crystal structure of MexR wt (pdb id: 1LNW). **A)** Chain C and D marked with light grey. The dimerization interface is labeled with orange for both chains C and D. The helix-turn-helix DNA binding motif is colored green. **B)** MexR chain CD (grey) in complex with ArmR peptide (blue). The MexR protein is rotated 180° around the y-axis in this representation compared to figure A. (PyMol software (DeLano, 2002)).

Crystallography data (Lim *et al.*, 2002) shows eight different representations of the MexR monomers (chain A-H) in the asymmetric unit. Not only the monomers show differences in the unit, but also the conformation of the two chains in each dimer is different, revealing some flexibility within the protein. Altered spacing between the DNA binding helices of the dimer might be a mechanism of regulation (Lim *et al.*, 2002) as in the tetracycline repressor and FadR (Orth *et al.*, 2000; van Aalten *et al.*, 2001). Interestingly, the crystal structure of oxidized MexR (Chen *et al.*, 2010) shows no conformational differences between the four dimers in the asymmetric unit.

Three cysteines are found in each monomer, Cys30, Cys62 and Cys138. Cys30 located in chain A can form a disulfide bond with Cys62 in chain B. It has been shown that MexR responds to oxidative stress signals by forming the disulfide bridge and thereby losing the DNA binding ability (Chen *et al.*, 2010) although the spacing between the two DNA binding domains remains. The decrease in

DNA binding is more likely to be a consequence of the repositioning of the recognition helix only, which shows most contact with DNA in the reduced form. This mechanism can explain the ability of repressor proteins like MexR to recognize such a wide range of antibiotics, leading to MDR. The proteins recognize the signal of oxidative stress in the cell caused by the antibiotics (Chen *et al.*, 2010).

MexR is regulated by ArmR. When ArmR is interacting with MexR, the spacing between the DNA binding regions decreases and the repressor protein goes to the more closed conformation and cannot interact with DNA (Chen *et al.*, 2010). Thus, ArmR indirectly regulates expression of the MexAB efflux pump. Upon binding with ArmR, the distance between the two recognition helixes in the DNA binding motifs decreases from 29Å to 16Å (Figure 3A, B). The conclusion of this, together with the fact that MexR respond to oxidative stress signals by altering the conformation, is that the flexibility of the protein monomers can be one of the mechanisms regulating the drug resistance within *P. aeruginosa.*

Several mutations found in MexR lead to increased multidrug resistance including L13M, R21W, C39R, G58E, T69I, L69F, R70W, N79S, R83H, R91C, R91H, L95F, Q106R and R114C (Adewoye *et al.*, 2002; Dupont *et al.*, 2005; Jalal & Wretlind, 1998; Jalal *et al.*, 1999; Saito *et al.*, 2003; Suman *et al.*, 2006). The mutations leading to drug resistance are found both in the dimerization and the DNA binding domains of MexR. Several mechanisms for the increased resistance due to the mutations have been suggested. If the mutation occurs in the dimer interface, the protein stability can be affected, or the orientation of the chains in the dimer can be altered leading to decreased DNA affinity (Suman *et al.*, 2006). It has also been suggested that dimerization can be disturbed (Adewoye *et al.*, 2002). If mutations in the DNA binding motif remove or add charge(s) to the interacting surface, DNA binding will be affected directly (Alekshun & Levy, 1999). The DNA binding of the repressor can both be enhanced and decreased by mutations in this area.

2.4 Unanswered questions regarding MexR function and interaction

Although the MexR protein is well characterized both in reduced and oxidized forms, biophysical studies of the MexR-DNA complex is essential to increase the understanding of the conformational and dynamic changes upon DNA interaction. It is also thought that MexR binds several of the target molecules of the efflux pump MexAB-OprM. Where and how this binding occurs within the repressor protein is highly interesting, since many of these molecules resemble drug molecules which can then be optimized for non-binding. It is purposed that the dimerization domain comprises a hydrophobic pocket that binds the drugs and this pocket might be important for the regulatory mechanism of MexR. Finally, to better grasp how MexR is regulated and how regulation can be distorted, structural and dynamic understanding of mutated MexR proteins that confer antibiotics resistance will provide crucial leads.

3. The c-Myc oncoprotein

3.1 The role of c-Myc in human cancers

A high degree of human malignancy shows amplification or overexpression of *c-myc* at different levels (Liao & Dickson, 2000) and it is today well known that the deregulated expression of c-Myc plays an important role in development of human cancers. To mention a few examples; overexpression of c-Myc is found in approximately 80% of breast cancers, 90% of gynecological cancers and 70% of colon cancers (Gardner *et al.*).

Activated *c-myc* gene is a part of cancer development, which can occur in several ways: gene amplification, increased *c-myc* gene transcription, transcriptional co-activators and post-translational modification mechanisms (Gardner *et al.*, 2002; Lutz *et al.*, 2002). The *c-myc* gene is involved in many biological activities such as cell cycle progression, cell growth, metabolism, apoptosis and genomic instability (Meyer & Penn, 2008; Oster *et al.*, 2002). Since many tumor cells are directly dependent on the c-Myc expression level, c-Myc activity, and its interactions with protein and/or DNA, c-Myc is a potent therapeutic target (Oster *et al.*, 2002).

3.2 The Myc family and the function of the c-Myc oncoprotein

More than thirty years ago, the proto-oncogene *c-myc* was discovered in human chromosome 8, and overexpression of the gene was found in many human cancer tumors (Dang, 1999). c-Myc, n-Myc, l-Myc and b-Myc are all members

of the Myc family of proteins, all comprising two highly conserved regions in the N-terminus named Myc box I and II (Meyer & Penn, 2008; Stone *et al.*, 1987). *c-myc* as well as *MYCN* and *MYCL1* show oncogenic activity and are members of *myc* family of genes. As mentioned above, the c-Myc protein regulates a wide range of functions in the cell such as cell-cycle progression, proliferation and apoptosis (Grandori *et al.*, 2000; Oster *et al.*, 2002). A variety of transcription factors are involved in the regulation of c-Myc expression. NF-*κ*B is an example of transcriptional regulator of the *c-myc* promoter both in human and murine B-cells (Boxer & Dang, 2001). In many cancer types, one of the most well-known being Burkitt´s lymphoma, the *c-myc* gene is translocated to a high-level expression promoter and transcription thus becomes higher than in normal B-cells.

3.2.1 Structure of c-Myc

The C-terminal part of c-Myc (residue 263-439) contains a basic Helix-Loop-Helix-Leucine-Zipper (bHLHLZ) motif and dimerizes with another bHLHLZ containing protein, Max (Figure 4 and 5) (Blackwood & Eisenman, 1992; Nair & Burley, 2003). Max lacks the transactivation part that is found in the c-Myc N-terminus. The heterodimeric Myc-Max protein (Figure 5) binds to specific consensus sequences 5'-CACGTG-3', also named Enhancer boxes (E-boxes) upon transactivation (Blackwood & Eisenman, 1991). The bHLHLZ part of c-Myc has been shown to interact with various cellular factors such as Miz-1 (Peukert *et al.*, 1997), BRCA1 (Wang *et al.*, 1998) and AP-2 (Gaubatz *et al.*, 1995).

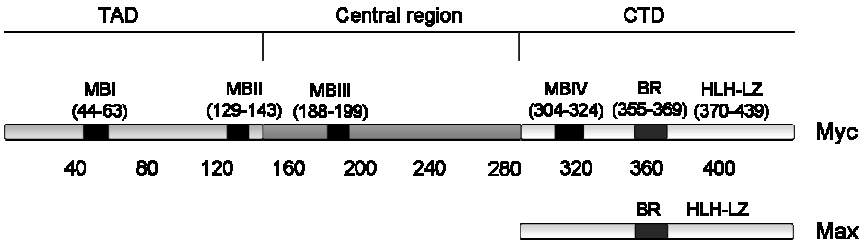

Figure 4. The regions of c-Myc. Residues 1-143 comprise the transactivation domain (TAD), residues 144-262 the central region and residues 263-439 the C-terminal domain (CTD). In the c-Myc sequence, four well conserved regions are named myc boxes (MBI-IV). In the C-terminal domain, the basic region (BR) and Helix-Loop-Helix-Leucine-Zipper (HLHLZ) motifs are essential for interaction with Max.

The Max protein can homodimerize and recognize E-boxes, and repression of the Myc-Max function might be the role of the homodimer (Nair & Burley, 2003). It is also found that Max is not regulated in the cell cycle and is most likely present as homodimer in resting cells (Blackwood & Eisenman, 1992). When the level of c-Myc increases during cell cycle entry, the Max protein starts to dimerize with c-Myc and thereby decreases the level of Max-Max complexes. The level of the Myc-Max complex is likely to be dependent on the rate of c-Myc expression.

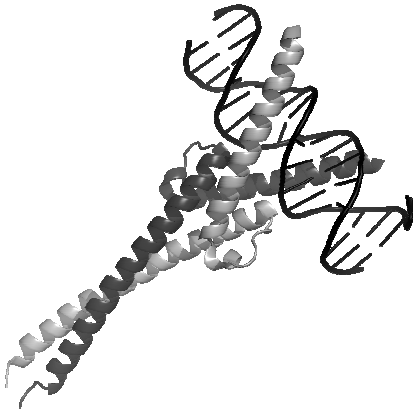

Figure 5. The crystal structure of heterodimer Myc-Max in complex with DNA (pdb id; 1NKP). The c-Myc bHLHLZ motif is colored with light gray, Max bHLHLZ motif with dark gray and the DNA with black.

Max can also interact with other bHLHZ proteins including Mad1 (Ayer *et al.*, 1993) and Mxi1 (Zervos *et al.*, 1993) and also in this formation recognize E-boxes competing for the same target sites as c-Myc. It is suggested that Mad can interfere with the transforming activity of c-Myc and can inhibit cell growth, acting as a transcription repressor. High levels of the Mad protein are found in differentiated cells where c-Myc is not expressed. It is also shown that Mad inhibits cell growth and represses the transforming ability of c-Myc (Hurlin *et al.*, 1994; Larsson *et al.*, 1997; Larsson *et al.*, 1994; McArthur *et al.*, 1998). Furthermore, the level of Myc-Max and Mad-Max might control cell fate, and thereby direct the cell between proliferation, transformation or differentiation, and quiescence.

The N-terminal part of c-Myc (aa 1-143) acts as a transactivation domain (TAD) and interacts with a large variety of proteins including co-activators. Both the C-terminus consisting of the bHLHZ motif (residues 354-434) and N-terminal domain (NTD) are essential for the functions of c-Myc (Oster *et al.*, 2003). From a structure-function point of view, the properties of the c-Myc C-terminal domain (CTD) is fairly well known but the molecular functionality of the N-terminal TAD domain (aa 1-143) is less understood. Although a wealth of TAD interactions with a wide range of proteins including co-activators have been shown (Bin1, Fbw7, Krim1, p21, p107, TBP and TRRAP among other), there is still only one structure of a complex with a small peptide of MBI reviewed by (Oster *et al.*, 2002; Ponzielli *et al.*, 2005). It is also not clear if the two domains are biophysically and/or functionally dependent of each other.

The N-terminal and central domains contains three well conserved regions named myc box1 (MBI), myc box2 (MBII), and myc box3 (MBIII) (Herbst *et al.*, 2005; Oster *et al.*, 2002). Many interactions appear to target these sequences in the protein. Furthermore, almost all mutations leading to cancer tumors are located within or close to these myc boxes. MBI in the N-terminal part of c-Myc TAD (residues 45-63) is crucial for gene activation and interacts with Bin1, a tumor suppressor. MBII (residue 128-143) interacts with many different regulatory proteins such as Snip, TRRAP and TBP and is essential for many cell functions including transformation, proliferation, inhibition of differentiation and gene activation (Boxer & Dang, 2001). MBIII inhibits apoptosis and plays a role in transformation and one additional myc box (MBIV) is found in the

oncoprotein but located in the C-terminal part and induces apoptosis (Boxer & Dang, 2001).

Our group has showed that the TAD domain of c-Myc is molten globule-like or partially disordered (Fladvad & Zhou *& et al.*, 2005), which, as mentioned earlier, is a common feature of proteins related to transactivation in the absence of target protein. The fact that the TAD domain can interact with many binding partners in a specific manner might be due to intrinsic disorder within the domain.

3.2.2 Phosphorylation of c-Myc Ser62 and Thr58

Two well-conserved residues, Thr58 and Ser62, are located in the N-terminal part of c-Myc, and undergo phosphorylation and dephosphorylation after stimulatory signals. Modifications at the two sites affect the protein stability such that an initial phosphorylation of Ser62 stabilizes the protein, whereas a subsequent phosphorylation of Thr58 leads to cellular destabilization (Figure 6) (Escamilla-Powers & Sears, 2007; Sears, 2004). Ser62 is phosphorylated by Ras-activated Extracellular Receptor Kinase (ERK) and Cdk2 (Hydbring *et al.*, 2010) and Thr58 by glycogen synthase kinase (GSK-3β) (Figure 6). Mitogen stimuli initiate c-Myc protein synthesis and activate Ras, a molecular switch. Ras is a monomeric GTPase which are active in complex with GTP and inactive when GDP is bound (Alberts, 2008). Ras signaling is required for proliferation or differentiation, and the stabilizing Ser62 phosphorylation occurs as a response to Ras signaling. At the same time Ras signaling inhibits the GSK-3β, through Akt, which is required for further phosphorylation of Threonine 58. The level of Akt decreases later in the G1 phase promoting activation of GSK-3β, leading to phosphorylation of Thr58. When Thr58 is phosphorylated, the stabilizing phosphate located at Ser62 is removed by protein phosphatase 2A (PP2A) after *cis-trans*-isomerization at the adjacent proline bond catalyzed by the Pin1 isomerase (Sears, 2004). Re-phosphorylation of Ser62 can only be performed by the site specific PP2A when Thr58 is phosphorylated. PP2A recognizes phosphorylated Ser/Thr-Pro sequences in *trans* conformation, enabling the complex formation with c-Myc only after *cis*-to-*trans* conformational change by Pin1, also site specific to the same sequence as PP2A but in the *cis* conformation. The mono-phosphorylated c-Myc can now be targeted for degradation by the

proteasome by recognition of SCF^{Fbw7} for ubiquitination and subsequent rapid turnover.

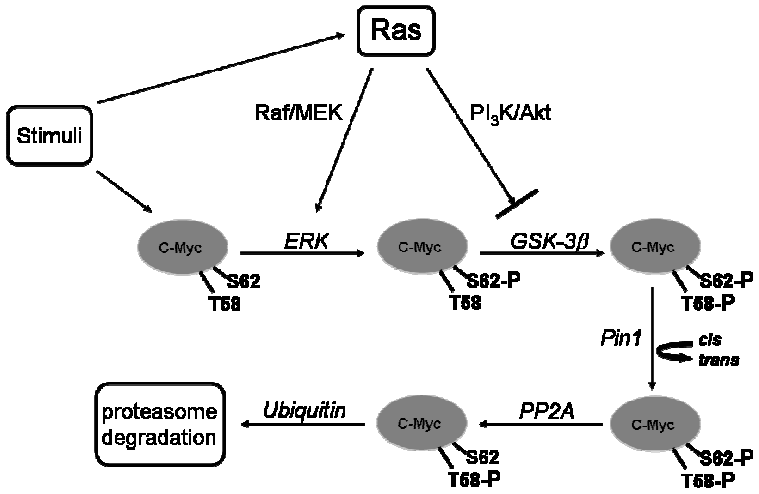

Figure 6. The pathway of c-Myc degradation through phosphorylation.

The initial phosphorylation of Ser62 also promotes c-Myc association with Axin1 (Arnold *et al.*, 2009). The Axin1 protein acts as a scaffold for further complex formation of c-Myc and GSK3β, PP2A or Pin1. Axin1 interacts with the TAD domain of c-Myc and it is shown that the phosphorylation of Ser62 is essential for the complex formation and thereby the degradation pathway of c-Myc.

When c-Myc is unphosphorylated, tumor suppressor Bin1 interacts with MBI. This interaction can be inhibited by phosphorylation at site Ser62 showing the importance of residues that undergo modifications for the function of gene regulation (Pineda-Lucena *et al.*, 2005).

3.2.3 Protein turnover and c-Myc ubiquitination

The c-Myc protein turns over rapidly, with a half-life of approximately 30 minutes, and the degradation of c-Myc is mediated by the proteasome, which recognizes ubiquitinated proteins. All sites of ubiquitination within c-Myc have so far not been identified; however, deletion of lysines within the protein resulted in less extent of ubiquitinylation (Muller & Eilers, 2008).

The turnover of c-Myc is dependent upon three of the myc-boxes, MBI-III located in the N-terminus and in the central region of c-Myc (Figure 4). MBI contains a binding site to ubiquitin ligase Fbw7, and mutations within the binding site of c-Myc leads to decreased ubiquitinylation and thereby more stable c-Myc and affected turnover (Muller & Eilers, 2008). Three ubiquitin ligases are found that are able to ubiquitinylate c-Myc: SCF^{Fbw7}, which associates with c-Myc when phosphorylated in MBI (Yada *et al.*, 2004), Skp2, which can both ubiquinylate and induce degradation of c-Myc (Kim *et al.*, 2003) and finally, the HECT-domain protein HectH9/Huwe1/Arf-Bp1/Mule (Adhikary & Eilers, 2005).

3.2.4 The cell cycle and c-Myc

The role of c-Myc both as being governed by, and governing, the cell cycle is complex and will only be discussed briefly in this thesis. The cell cycle is roughly divided into four stages: the S phase, the G_1 phase, including G_0 resting phase, the G_2 and the M phase (Alberts, 2008). c-Myc is expressed during the entire cell cycle and overexpression of c-Myc can lead to increased cell mass, such as larger lymphocytes, without cell proliferation (Schuhmacher *et al.*, 1999). It is also suggested that c-Myc is involved in metabolic pathways that regulates the cell size due to identification of c-Myc targets as enzymes in glucose and iron metabolism (Dang *et al.*, 2006). c-Myc appears to regulate the protein synthesis machinery, as indicated by the c-Myc dependent acceleration into the S-phase of the cell cycle (Dang *et al.*, 2006). The c-Myc protein has also been found to regulate RNA polymerase I and III involved in the protein synthesis. (Dang *et al.*, 2006; Gomez-Roman *et al.*, 2003).

c-Myc may act both as a inducer or repressor of apoptosis since studies show that tumor cells can respond more or less to apoptotic stimuli when c-Myc is over-expressed (Liao & Dickson, 2000). It has been suggested (Meyer *et al.*, 2006) that proliferating cells are dependent upon c-Myc as a safety check to promote apoptosis if the cells have changed. c-Myc associated apoptosis mechanism can be both dependent and independent of the regulation of p53, a well known tumor suppressor. p53 recognizes cell damage, thus directing the cell for repair during a growth-arrest stage, or to programmed cell death. Depending on if the cells are going for repair or cell death, the tumor suppressor

activates different genes such as *p21*, *GADD45* for growth arrest and *BAX*, *PUMA* and *BIM* for apoptosis. The selection of the type of genes to be expressed is likely regulated by c-Myc. It is also shown (Hermeking & Eick, 1994; Wagner *et al.*, 1994) that deregulation of c-Myc activated p53 leads to apoptosis. Hence, c-Myc can activate p53, which prevents expression genes associated with growth arrest, but includes expression of apoptosis dependent genes instead. It is also likely that the level of both c-Myc and p53 expression is crucial for the selection of whether the cells are going for repair in cell-arrest or to cell-death (Meyer *et al.*, 2006). In the p53 independent apoptosis mechanism, c-Myc induced apoptosis can be related to many overexpressed or inactivated proteins that associates with c-Myc and to mention a few; macrophage migration inhibitory factor, Runx2 and Bin1.

3.3 The role of c-Myc in transcription

The c-Myc protein can act both as a repressor and activator for gene expression, and its interacting properties, such as the recruitment of histone acetylase, chromatin modulating proteins and transcription factors, can be associated with its transcriptional regulation (Dang *et al.*, 2006). The transcription is dependent both on the N-terminal transactivation domain and the C-terminal DNA interacting part of c-Myc. It is shown (Hateboer *et al.*, 1993; Maheswaran *et al.*, 1994) that c-Myc associates with TATA-box binding protein (TBP) and the basal transcriptional machinery, but this association might be modulated by other transcription factors (Dang *et al.*, 2006) including AMY-1 (Taira *et al.*, 1998), Bin1 (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996), Miz1 (Peukert *et al.*, 1997; Schneider *et al.*, 1997), Pam (Guo *et al.*, 1998), YY1 (Shrivastava *et al.*, 1993), p107 (Beijersbergen *et al.*, 1994) and TRRAP (McMahon *et al.*, 1998).

Bridging-Integrator-Protein, also named Bin1, is believed to be involved in endocytosis, transportation of materials from the cell surface into the cell and also in apoptosis. Bin-1 is suggested to be a negative regulator of c-Myc mediated transformation (Ponzielli *et al.*, 2005). It has also been stated that Bin1 may act as a suppressor in many forms of tumors including breast-, liver-, and prostate cancer (Wechsler-Reya *et al.*, 1997), with highest expression in brain and muscle tissues. Bin1 inhibits malignant cell proliferation and this inhibition can be both c-Myc dependent and independent (Elliott *et al.*, 1999). As mentioned earlier, Bin1 interacts with the N-terminal poly-proline containing

part of c-Myc, MBI, through its SH3-domain in the C-terminus. This interaction has been described in structural detail by NMR (Pineda-Lucena *et al.*, 2005). The Bin1 protein binds to an unphosphorylated form of c-Myc and the interaction can be inhibited by phosphorylation at the well conserved c-Myc residue Ser62.

TBP is crucial for the expression of almost all genes, it is highly regulated in the cell, and forms part of a large complex of transcription factors generating the TFIID subunit. This general transcription factor is part of the RNA polymerase II complex, which is sequence specific to the TATA box and initiates the transcription of the gene. The non-DNA binding form of TBP interacts with TBP-associated factors (TAFs) through the concave surface of TBP, (Figure 7), thus modulating the DNA interaction. It has been demonstrated (Mal *et al.*, 2004) that the N-terminal part of *Drosophila* TAF1(11-77) is unstructured in the unbound form but undergoes folding upon binding to a globular DNA mimicking protein.

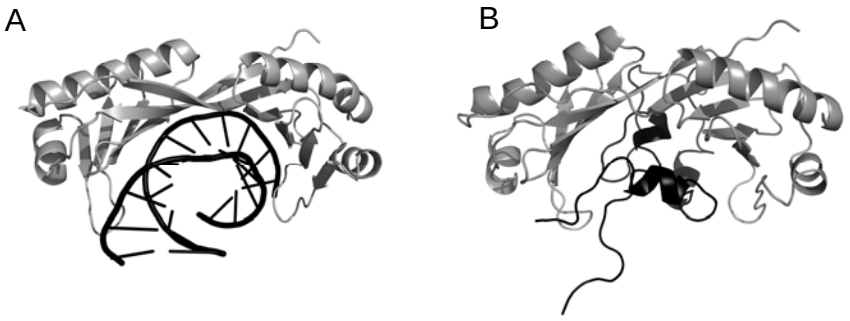

Figure 7. Ribbon representation of **A)** yTBP in complex with DNA. (PDB-id; 1YTB) and **B)** yTBP in complex with dTAF (PDB-id; 1TBA). TBP is colored in gray and the cofactors in black.

The TAFs function as negative or positive regulators for the transcription by providing interaction towards other transcription factors or destabilizing TFIID interaction with the promoter respectively (Liu *et al.*, 1998). The *Saccharomyces cerevisiae* TAFs have also been studied in complex with TBP (Mal *et al.*, 2004). Of the 14 subunit large yTAF, the N-terminal part (yTAND1) binds the concave cleft in TBP, thus inhibiting the association to DNA, and an additional unit

(yTAND2) interacts with the convex surface of TBP, affecting the transcriptional repression. Many regulatory proteins target the TBP such as the NC2 (Chitikila *et al.*, 2002), Brf1 (Juo *et al.*, 2003) and since c-Myc also targets TBP, c-Myc can be an important part of the transcriptional regulation process.

The Transactivation/Transformation Associated Protein (TRRAP) is a 3859 residue large protein and is suggested, together with human histone acetyltransferase (HAT) GCN5, TIP48, TIP49 and BAF53, to be essential for c-Myc oncogenic transcriptional pathway and chromatin remodeling and repair (Liu *et al.*, 2003; Ponzielli *et al.*, 2005). Three regions in TRRAP (aa 1261-1579, 1899-2026, 3402-3828) have been identified to interact both with the C-terminal domain and the N-terminal transactivation domain of c-Myc, and mutations within or deletion of the N-terminal part of c-Myc decreases the TRRAP interaction and are transformation-defective (Cole & McMahon, 1999). TRRAP is a part of the STAGA (SPT3-TAF-GCN5 acetylase) complex. GCN5 and TRRAP cooperate to increase the transcriptional activity of c-Myc TAD domain by increased acetylation of histones H3 and H4 during the transcription. By observing deletion constructs of c-Myc it has been suggested that STAGA complex interacts with residues 1-108 of the c-Myc TAD domain, and residues between 50-108, including parts of the MBI, seem to be important for the binding (Liu *et al.*, 2003).

3.4 Unanswered biophysical questions regarding c-Myc

A plethora of questions regarding the structural, dynamical and biophysical properties of the c-myc TAD domain are still unanswered. A wide range of c-Myc interaction partners have been identified so far but the molecular and biophysical mechanisms for their binding remains to be investigated. Detailed knowledge about these interactions is highly important for an increased understanding of c-Myc functions and the role in human cancers. Today, only the structure of the c-terminal part of c-Myc is determined. Although our work has given more structural insight to the N-terminal 88 residues of the TAD domain, the analysis of the remaining ~200 disordered amino acids, covering at least four additional interaction patches, remain.

4. Regulation of transcription factors and redox processes in the cell

4.1 Thiol based systems

Thioredoxins (TRXs) and glutaredoxins (GRXs) regulate the cellular redox state in many cell systems e.g. growth, proliferation and apoptosis (Berndt *et al.*, 2007). The TRX and GRX proteins are part of the thiol based control system acting as antioxidants, which act as inhibiting or activating signaling molecules in living cells. TRX and GRX proteins were first discovered as proton donors for ribonucleotide reductase, which is essential for DNA replication. Both systems use a cysteine in the active site for reversible reduction of other disulfide containing substrates. The TRX systems consist of NADPH, thioredoxin reductase and thioredoxin whereas a GRX system includes NADPH, glutathione reductase, glutathione and glutaredoxin.

4.1.1 Thioredoxin proteins in the mammalian cell

TRX proteins can be found at two locations in the mammalian cell, the cytosol as TRX1/TRXR1 and in the mitochondria as TRX2/TRXR2 where TRXR is the related thioredoxin reductase (Berndt *et al.*, 2007). Mammalian TRX1 regulates a large range of proteins including transcription factors such as tumor suppressor p53 and glucocorticoid factor. When the TRX1 pathway is active, the protein undergoes small conformational changes mainly in the catalytic site when going from the reduced to the oxidized form, leading to different binding capacity of the reduced form towards other substrates. This means that the protein can act as a catalyst both in oxidized and reduced forms and with different substrates in

both cases. TRX1 can thereby act as a regulator in different cell systems including apoptosis. The TRX2 protein system is related to embryonic development, plays a role in regulation of the apoptotic signaling in the mitochondria and participates in regulating the mitochondrial membrane potential (Koharyova & Kolarova, 2008).

4.1.2 Glutaredoxin proteins in the mammalian cell

Glutaredoxins can be divided into two classes, dithiol- and monothiol glutaredoxins. Dithiol glutaredoxins have similar active site as thioredoxins with residues Cys-X-X-Cys whereas monothiol glutaredoxins lacks the C-terminal cysteine which often is replaced with a serine residue giving Cys-X-X-Ser (Lillig *et al.*, 2008). The monothiol glutaredoxins can consist of a single GRX domain or multiple domains of TRXs and GRXs whereas the dithiol glutaredoxins exhibits only a single domain.

Four different GRX proteins are found in the mammalian cells; GRX1 and Grx3 which are located in the cytosol and GRX2 and GRX5 in the mitochondria. The catalytic sites differ slightly within the systems where GRX1 consists of Cys-Pro-Tyr-Cys and GRX2 of Cys-Ser-Tyr-Cys. In Grx3 and GRX5 which are monothiol glutaredoxins, the active site comprises residues Cys-Gly-Phe-Ser. Monothiol glutaredoxins are present in both eukaryotic and prokaryotic organisms. In human cells, GRX5 contains a single monothiol glutaredoxin domain whereas Grx3/PICOT (Protein kinase C Interacting Cousin of Thioredoxins) consists of an N-terminal TRX-domain and two homologous GRX domains. This arrangement of GRXs domains is also found in monothiol glutaredoxins of other species e.g. *Arabidopsis thaliana* where one of four monothiol glutaredoxins consists of a TRX like domain followed by three GRX domains. Although only a few structures of monothiol glutaredoxins are solved they show similarity of the dithiol glutaredoxin folds and sequence conservation of important residues within different monothiols suggesting that the structure of *E. coli* GRX4 is representative of monothiol glutaredoxins in general (Herrero & de la Torre-Ruiz, 2007). The structures of dithiol and monothiol glutaredoxins are very similar, but their function is distinctly different. For instance, the binding of glutathione (GSH) differ between mono and dithiols due to less conservation degree of an important charge residue within dithiols responsible of the GSH interaction. It is suggested (Rouhier *et al.*, 2010) that some

monothiol glutaredoxins forms iron-sulfur (Fe-S) clusters and can thereby participate in biological processes, act as electron donors/acceptors and radical-generation, all depending of the iron level in the cell. In line with this, it has been shown that the human monothiol Grx3/PICOT forms a iron-sulfur cluster (Haunhorst *et al.*, 2010).

4.1.3 Catalytic mechanism of TRXs and GRXs

During catalysis, the disulfide bond to be reduced is transferred to TRX. The activated N-terminal cysteine attacks the disulfide bond of the substrate protein and a TRX-Substrate complex (a mixed disulfide) is formed. The oxidized form of TRX is more stable and forces thereby formation of an intramolecular disulfide bridge in TRX, causing release of the reduced substrate protein molecule. The oxidized TRX protein is then reduced by TRXR (Collet & Messens, 2010).

Glutaredoxins can reduce disulfides in substrate proteins or mixed disulfides (GSH-proteins) by dithiol or monothiol mechanisms (Fernandes & Holmgren, 2004). Electrons are transferred from NADPH, via glutathione transferase and glutathione to the glutaredoxin protein. Dithiol glutaredoxins, containing a Cys-Pro-Tyr-Cys active site, have a similar catalytic mechanism as the structurally and functionally analogue, thioredoxin. The activated N-terminal cysteine residue in the active site undergoes a nucleophilic attack towards a sulfur atom in the substrate molecule, forming a mixed disulfide between GRX and the substrate. This mixed disulfide formation within the glutaredoxin molecule makes the substrate to leave as a reduced molecule. This intramolecular disulfide can be formed when the N-terminal cysteine sulfur is deprotonated and undergoes a nucleophilic attack on the C-terminal cysteine residue in the active site of glutaredoxin.

For monothiol glutaredoxins, the active site only consists of the N-terminal cysteine which takes part in catalysis. This result in formation of a complex between monothiol glutaredoxin, glutathione (GSH) and the target protein to be reduced. The glutathionylation and deglutathionylation of protein has been discovered to be a redox regulatory mechanism for many proteins including, cell cycle regulators, metabolic enzymes and protein chaperones (Fernandes *et al.*, 2005; Fernandes & Holmgren, 2004).

4.2 Fold and catalytic site of TRXs and GRXs

Thioredoxins and glutaredoxins adopt the characteristic TRX family fold consisting of β-strand in the protein core and a number of helices towards the surface. The first thioredoxin structure was solved by Arne Holmgren and co-workers (Holmgren *et al.*, 1975). The structural fold of thioredoxins are commonly five beta sheets and four helices labeled N-terminal β1α1β2α2β3 and C-terminal β4β5α4 linked together by α3 (Figure 8) (Collet & Messens, 2010). Glutaredoxins usually lacks the β1 strand and the α1 helix. Today, around 20 different dithiol glutaredoxin structures have been published but only four of them are monothiol glutaredoxins.

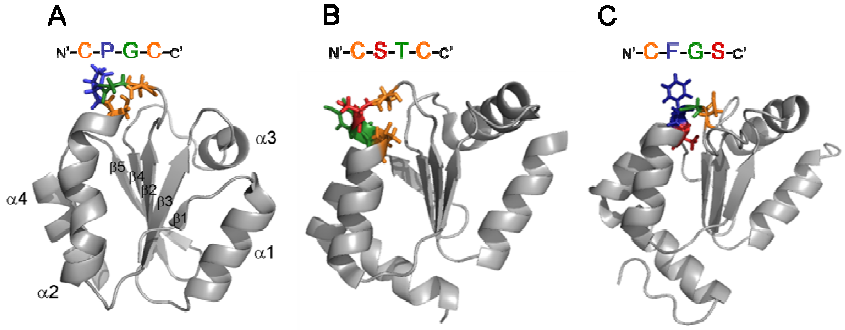

Figure 8 A) The structure of human thioredoxin 1 (PDB-id; TRX3) showing the characteristic TRX-family fold. The active site residues are highlighted where the two cysteines are colored in orange, glycine in green and proline in blue. **B)** The human dithiol glutaredoxin 2 (GRX2) (PDB-id; 2CQ9). The active site residues are two cysteines (orange), serine (red) and threonine (green). **C)** Monothiol glutaredoxin 4 (Grx4) from *E. coli* (PDB-id; 1YKA). The active site residues cysteine (orange), glycine (green), phenylalanine (blue) and serine (red) are located between strand one and helix two.

The catalytic site of TRXs, containing the highly conserved Cys-X-X-Cys motif, is located on the surface, usually in the N-terminal part of α2, (Figure 8). The two cysteines are the active residues providing the sulfhydryl groups involved in the reducing mechanism. The proline is well conserved and important for the redox activity and determines the degree of reducing capacity of the protein. If this proline residue is deleted, the redox activity and protein stability will be affected (Collet & Messens, 2010). Two more prolines in the TRX structure are also conserved; one of those is located close to the active residues. Mutation or deletion of this proline does not lead to changes in the redox activity but affects the protein stability. This proline residue separates the active site residues from

the rest of the α_2 helix. The third proline is found opposite to the catalytic site and is important both for the stability and redox properties of TRX. This proline is always present in *cis*-conformation and a part of the so called *cis*-proline loop where other well conserved residues are found including a WPTXPQL motif. This *cis*-proline loop is thought to be highly important for protein-protein interactions besides protein stability (Martin, 1995). The Cys-Gly-Pro-Cys motif has a high degree of conservation among different species, from prokaryotes to mammals.

4.3 Multidomain monothiol glutaredoxins.

The bacterial monothiol glutaredoxins are always present as single domain protein whereas multidomain monothiol glutaredoxins are found for eukaryote organisms. A thioredoxin domain is located in the N-terminus followed by one (fungi), two (vertebrates) or three (plants) monothiol glutaredoxin domains (Figure 9) (Alves *et al.*, 2009; Herrero & de la Torre-Ruiz, 2007; Rouhier *et al.*, 2010).

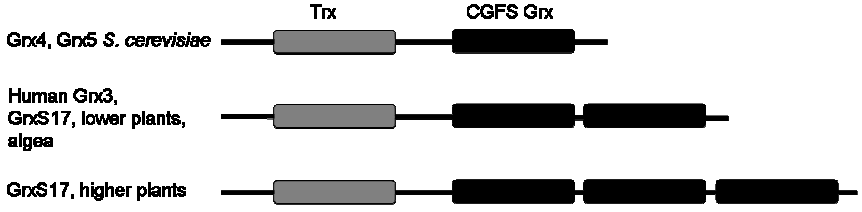

Figure 9. Multidomain monothiol glutaredoxins found in eukaryotic organisms contains an N-terminal thioredoxin like domain (gray) followed by one ore more monothiol glutaredoxin domains (black) with active site residues CGFS.

Although the subdomains show high degree of conservation among different species, the linker region between the TRX and GRX domains is non-conserved. In human cells, both single domain monothiol glutaredoxin (GRX5) and multiple domain monothiols (Grx3) are found.

The functionality of multidomain TRX-GRX proteins is not fully understood. However, for multidomain glutaredoxins Grx3 and GRX4 in *S. cerevisiae* one essential function is the regulation of iron homeostasis. The glutaredoxins harbor the Fe/S cofactor bound to the active site (Herrero & de la Torre-Ruiz, 2007). Both Grx3 and Grx4 regulate the nuclear location of the iron- responsive

transcription factor Aft1 which in turns regulates expression of the iron-uptake regulone. It is shown (Hoffmann *et al.*, 2011) that the multidomain protein did not facilitated the iron transport in absence of the N-terminal TRX-domain although the domain does not participate directly in the interaction with the F2/S co-factor. One suggestion is that the TRX domain functions as a docking-site for interaction partners.

4.4 Human glutaredoxin 3

Human glutaredoxin 3 (Grx3) consists of three domains, an N-terminal thioredoxin like domain and two C-terminal monothiol glutaredoxin domains (PICOT homology domains) (Figure 10). The TRX like domain is separated from the two tandem GRX domains by a non-conserved linker and the domain lacks the active site cysteines found in other TRX domains suggesting that the subunit is devoid of catalytic function. The two homologous glutaredoxin domains contain the active site sequence of Cys-Gly-Phe-Ser commonly found in other monothiol GRXs. The multidomain protein was first discovered as an interaction partner of protein kinase C-θ (PKC-θ) and was originally named Protein Kinase C Interacting Cousin of Thioredoxins (PICOT) (Witte *et al.*, 2000). Members of the PKC family are associated with cellular differentiation and proliferation, immune responses and play an important role in some stimuli such as hormones and growth factors (Newton, 1995).

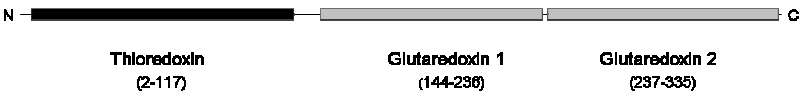

Figure 10. Human Glutaredoxin 3 (Grx3/PICOT) consists of three domains, an N-terminal thioredoxin like domain (black) and two equivalent Glutaredoxin domains (gray). The linker region between the TRX domain and Grx1 consists of residues 117-144.

The TRX domain of Grx3 interacts with Protein kinase C which might have an important role in activation of T lymphocytes (Lillig & Holmgren, 2007) and overexpression of Grx3 in T cells inhibit the activation of two transcription factors, AP-1 and NF-κB (Witte *et al.*, 2000). Human Grx3 is located in the cytoplasm and expressed in heart, testis, and spleen cells. The human Grx3 is an important protein which is suggested to be involved in immune response, cardiac physiology and development of embryos (Haunhorst *et al.*, 2010). It is

also found that Grx3 is overexpressed in lung and colon cancer tissues, which might be due to repressed apoptosis (Cha & Kim, 2009). The Grx3 protein is phosphorylated on tyrosine residues by stimuli with hydrogen peroxide. It has been suggested that the protein forms a homodimeric complex together with two [2Fe-2S] upon the stress signals and thereby functions as a regulatory switch (Haunhorst *et al.*, 2010). The conformation of the domains within the Grx3 protein is of highest interest due to the indications that the dimeric form can form a sulfur-iron cluster during oxidative stress.

4.5 *Escherichia coli* glutaredoxins

In *E. coli*, three dithiol glutaredoxins, Grx1; Grx2; Grx3, and one monothiol glutaredoxin, Grx4, are found. The actual function of these glutaredoxins is poorly understood; however, several substrate proteins which are reduced in *E. coli* have been identified (Fernandes & Holmgren, 2004). The 115 residue Grx4 has high homology with other monothiol glutaredoxins and was discovered by (Fernandes & Holmgren, 2004). Grx4 has a glutaredoxin/thioredoxin-like fold with four β-sheets in the core surrounded by five α-helixes (Fladvad & Bellanda *& et al.*, 2005).

4.6 Unanswered questions regarding human Grx3

Although the structure and function of thioredoxins and glutaredoxins are well characterized, the functional properties within the large family of multidomain monothiol glutaredoxins including Grx3 are yet to be explored. For example, it is highly conceivable that the relative orientation of the glutaredoxin/thioredoxin domains is functionally critical. The N-terminal thioredoxin domain lacks the classical active site residues and thereby the redox activity, but is still very likely to be essential for protein function. It is of interest to evaluate how the conserved thioredoxin fold is related to the mechanism of the protein and also the orientation and dynamics of the different domains.

5. Methodology

The articles and manuscripts in this thesis describe applications using a wide range of biophysical methodology. In this chapter, I will introduce my most important tools: CD spectroscopy, Fluorescence spectroscopy, MALDI-TOF-MS and NMR. For introductory reviews on analytical ultracentrifugation (AUC) and surface plasmon resonance (SPR), please refer to (Homola *et al.*, 1999; Lebowitz *et al.*, 2002; Myszka, 1999; Rich & Myszka, 2010).

5.1 Circular dichroism spectroscopy

CD spectroscopy is the phenomenon where chiral molecules absorb left- or right handed circular polarized light at different levels, resulting in a differential spectra. Two perpendicular plane polarized waves, out of phase by 45 degrees, generates a circulating superposition with the same wavelength. If a sample absorbs less of the left- or right circularly polarized light, the resulting vector oscillates with an elliptical phase. This is called ellipticity and in other words, circular dichroism spectroscopy converts plane polarized light into elliptically polarized when added to a macromolecule (Fasman, 1996).

The angle θ of the ellipse can be measured and related to the differences of absorption in left and right circularly-polarized light in the following way;

$$\theta(rad) = \tan\theta = \left(|E_l| - |E_r|\right)/\left(|E_l| + |E_r|\right) = \left[\exp(-A_l/2) - \exp(-A_r/2)\right]/\left[\exp(-A_l/2) + \exp(-A_r/2)\right] \quad (1)$$

where A is the absorbance and E the extinction coefficient of left and right circularly polarized light, respectively.

CD spectroscopy has a wide range of applications in protein chemistry since the protein backbone contains chiral centers and thereby generates differences in absorption between left and right handed circularly polarized light. The side chains of the amino acids tryptophane, tyrosine and phenylalanine can also be targets for measurements. When analyzing the secondary structure, interactions and stability of proteins, CD is a suitable method.

5.1.1 Secondary structure evaluation

Different types of secondary structure can be analyzed in the far-UV region (190-250 nm) where the protein backbone can generate differential absorption spectra. Three protein states gives different characteristic CD spectra; α-helical, β-sheet and random coil state. These spectra reflect the overall secondary content of the protein molecule, and do not give information of micro-environments such as interactions of specific amino acids. Typically, α-helices give rise to spectra containing a positive peak at 195 nm and two distinct minima at 208 and 222 nm. The β-sheet structure generates a positive signal at 198 nm and negative peak at 216 nm and finally the random coil state is evidenced by a minimum at 198-200 nm (Greenfield, 2006c).

Different algorithms, such as Contin, Selcon3 and CDSSTR which use the data collected in the far-UV region, can be used to evaluate the secondary structure content. Data sets with CD spectra of various types of secondary structure from proteins where the structure has been solved by crystallography are used for the analysis (Kelly *et al.*, 2005). The results from the analysis describe the different fractions of secondary structural elements in the analyzed protein. It should however be remembered that these analysis are only estimations and should be treated with caution.

5.1.2 Tertiary structure evaluation

Changes of the protein structure as reflected by altered aromatic environments can be followed in the near-UV region (250-350 nm), where aromatic amino acids (Trp, Phe, Tyr) and disulfides absorb light. The CD signal is sensitive to the surrounding of the chromophores and gives significant differential spectra only when the chiral residue is structurally constrained, such as in the core of a protein fold, whereas unstructured or molten globular like proteins gives poor signal due to the mobility of the chromophore in these states. In the near-UV region protein interaction and ligand binding can be measured as a result of sensitivity of changes in the local environment around the aromatic amino acids. Thermal denaturation of the protein in this UV range can be compared with that in the far-UV range, to give information on whether the unfolding occurs in one cooperative stage or if additional steps are involved.

5.1.3 Thermal unfolding of proteins

The thermal stability of proteins can also be analyzed by far-UV CD spectroscopy by following the changes in the secondary structure of the protein as a function of temperature as the protein unfolds. This analysis can also determine whether the molecule is loosing all or only parts of the secondary structure and if it undergoes other conformational changes during unfolding, such as entering semi-stable states. The thermal melting midpoint (T_m) of the protein can be calculated from the inflection point in the sigmoidal temperature-dependent response, which is useful when comparing to other proteins and variants (Greenfield, 2006a).

5.2 Fluorescence spectroscopy

Fluorescence is a phenomenon where emission of light occurs from the electronically excited state to the ground state and can be used for many applications in structural biology. For example, the thermal and chemical stability of proteins can be analyzed. Furthermore, measurements of interactions between proteins or protein ligands, molecular motions, distances between important sites in the protein and different conformational states can be pursued (Lakowicz, 2006).

To illustrate fluorescence, Jablonski diagrams are commonly used, showing molecular processes that can occur in the excited state (Figure 11A).

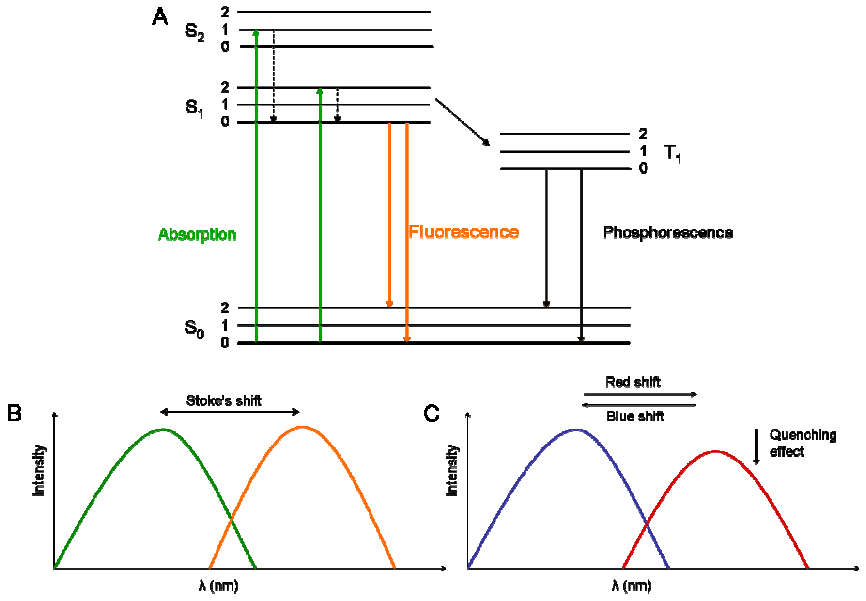

Figure 11. A) Jablonski diagram showing the absorption of energy labeled with green arrows, fluorescence emission highlighted with orange and phosphorescence with black arrows. The internal conversion of photon from higher vibrational energy level to the excited ground state ($S_1(0)$) is marked with dashed arrows. **B)** Stokes shifts represent the difference in wavelength between maximum of absorption (green) and emission (orange). **C)** Blue shift, red shift and quenching effect of the emission spectra.

The fluorophore can be excited from the singlet ground-state (S_0) to either of the two singlet excited states, S_1 or S_2. At these electronic energy levels the fluorophores can exist in a number of vibrational energy levels labeled 0, 1, 2, 3… When a photon is excited to any of the vibrational energy levels in the excited states, S_1 or S_2, energy loss occurs and the photon is relaxing to the excited ground state ($S_1(0)$), followed by emission when the photon is returning to the ground state (S_0) at lower energy and longer wavelength. Emission can occur as fluorescence or phosphorescence where fluorescence emission occurs at higher energy compared to phosphorescence where light is emitted at lower energy and thereby longer wavelength (Figure 11A). In this case the photon undergoes spin conversion to the triplet state of first order (T_1) followed by emission to the ground state. For fluorescence, the lifetime, the average time between the exited state and the return to the ground-state, is shorter, near 10 ns compared to phosphorescence, which occurs at a time scale of milliseconds to seconds. Aromatic compounds often give rise to fluorescence whereas molecules containing heavy atoms such as bromine show phosphorescence after excitation.

Since proteins usually contain the aromatic amino acids Trp, Tyr, and/or Phe, several molecular mechanisms can be followed by fluorescence. The energy of emission is always less and at higher wavelength than absorption (excitation) which is demonstrated in Figure 11A. The difference in energy between absorption and emission, Stokes shift, is due to rapid relaxation to the lowest vibrational level of S_1 and thereby loss of energy (Figure 11B). The extent of the energy decay is influenced by many mechanisms such as quenching by the solvent or energy transfer.

Protein-protein and protein-ligand interaction can be followed by intrinsic tryptophane fluorescence where the local environment of the aromatic amino acids affects the emission spectra or the quantum yield of the fluorophore. If the environment of the tryptophane is hydrophobic, the wavelength of the emission is shorter (blue shifted) than for hydrophilic surroundings (red shifted) (Figure 11C). Several factors can affect the surrounding polarity such as solvent polarity and viscosity, probe conformational changes, internal charge transfer and rigidity of the local environment. For example, if a protein undergoes folding-on-binding, the surroundings of the fluorophore might change from hydrophilic to hydrophobic, while the fluorophore is first exposed to the solvent and then

buried in the structural core. The shift of emission wavelength as well as changes is the quantum yield can be followed by fluorescence spectroscopy during interaction of protein and ligand, folding and unfolding processes and conformational changes.

Structural information regarding proteins can also be obtained from fluorescence spectroscopy using extrinsic probes. For example, to distinguish if the protein has a molten globular structure an external non-covalently attached probe can be used such as 1,8-ANS (1-anilinonaphtalene-8-sulphonic acid). The probe is weakly fluorescent in the absence of protein interactions but shows emission spectra when buried in a hydrophobic environment. The ANS molecule can only bind to hydrophobic pockets of proteins leading to emission with quantum yield around 40 times the unbound molecule.

5.3 MALDI-TOF-MS

For mass analysis of biomolecules MALDI-TOF-MS is an excellent tool because of its wide mass range and high resolution, from small organic compounds up to large proteins (Duncan *et al.*, 2008). The molecule to be analyzed is crystallized together with a matrix, which is an acid or base acting as a proton acceptor or donor. The matrix is often a small conjugated organic compound that absorbs the energy efficiently and thereby transfers it to the analyte in a soft way preventing larger molecules from fragmentation. Examples of matrixes are α-Cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and 2,5-dihydroxybenzoic acid (DHB), where CHCA is used for peptides and smaller proteins, SA for larger macromolecules >10 kDa, and DHB is often used for small organic molecules. An applied laser beam evaporates and ionizes the co-crystallized analyte which is forced by a small applied accelerating voltage to travel against the detector which is relating the time of flight to the mass over charge (m/z) of the biomolecule (Figure 12) (Voyager Biospectrometry Workstation & Users guide, 1999).

MALDI-TOF MS has many advantages: no definite upper mass limit except the inability of the analyte to evaporate, very small amounts of sample is required, (μM concentrations), most physiological buffers can be used and mixtures of analytes can be analyzed in the same spectra. Unfortunately the crystallization of the molecule and the matrix are sensitive towards high concentrations of salt and

detergents. Another disadvantage is that non-covalent complexes are broken because of the high laser energy. This prevents studies of protein-protein and protein-DNA interactions and other ionization methods such as electro-spray ionization must be chosen.

In addition of analysis of molecular weight MALDI-TOF-MS has many applications in particular in the proteomic field. Interactions between two proteins can be followed by enzymatic digestion to generate differences in cleavage pattern between free and bound forms. The extent of site specific labeling or modification of peptides or proteins can also be evaluated if digested with proteases. Furthermore, structural information can also be generated using MALDI where the enzymes digests the protein surface faster then the core during time resolved limited proteolysis.

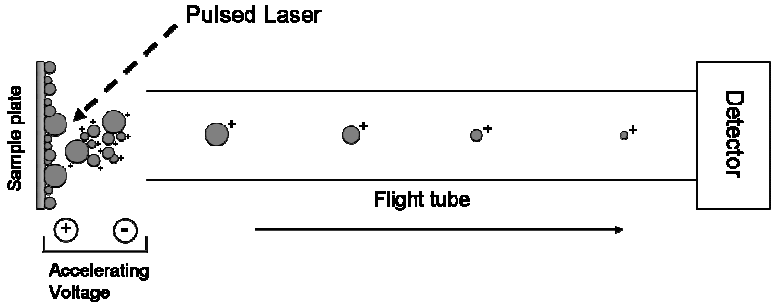

Figure 12. The principle of MALDI-TOF-MS (positive, linear mode); after evaporation of the ionic analyte by a laser pulse, the molecules are travelling in the flight tube where smaller molecules travels faster then larger. The detector relates the time of flight to the mass of the molecules (m/z). The analyte molecule is represented as a grey sphere.

5.4 Nuclear magnetic resonance

NMR has a large number of applications in many fields such as organic chemistry, protein science and medicine. Not only can this method be used to determine the structure of organic compounds and proteins at atomic resolution, but also the dynamics of macromolecules can be analyzed giving important information of biological mechanisms and functions. Furthermore, protein-protein or protein-ligand interactions can also be studied by NMR spectroscopy. NMR has developed rapidly during the last three decades. NMR methodology for determining three-dimensional solution structures of proteins was developed

by Kurt Wüthrich in the early 1980s (Wuthrich *et al.*, 1982). For this achievement he was awarded the Nobel prize in 2002.

5.4.1 Theory

The following section briefly describes NMR methodology and its applications to proteins, based on presentations given in (Cavanagh *et al.*, 2006; Hitchens, 2005; Hore, 1995).

Atomic nuclei exhibit an intrinsic angular momentum, spin, with a magnitude quantified according to Eq. 2.

$$|\mathbf{I}| = (I(I+1))^{1/2}\hbar \qquad (2)$$

where $\hbar$ is the Planck constant divided by 2π. I is the spin quantum number, which mainly is determined by the number of unpaired protons and neutrons in the nucleus. Allowed values for I are integers (0,1,2,…) or half-integers (1/2, 3/2, 5/2,…). Only nuclei for which $I > 0$ are magnetically active e.g. ^{1}H (I=½), ^{2}H (I=1), ^{13}C (I=½) and ^{15}N (I=½). The angular momentum vector **I**, of a spin-I-nucleus, exhibit $2I$+1 projections onto a coordinate axis, for example the z-axis, giving the z-component of the vector, I_z, as

$$I_z = m\hbar \qquad (3)$$

The magnetic quantum number m can have values (I, I-1, I-2…-I+1, -I). Nuclei with spin quantum number $I > 0$ have a nuclear magnetic moment, μ. Its projection onto the z-axis is dependent on the spin state and the gyromagnetic ratio

$$\mu_z = -\gamma I_z = -\gamma\hbar m \qquad (4)$$

When a nucleus with spin I=½, such as ^{1}H or ^{13}C, is placed in a magnetic field, the angular momentum can have two allowed spin states -½ (α) and +½ (β) and the spin can undergo transitions between the levels when excited by application of a radio frequency pulse. The two states are named the Zeeman levels (Figure

13) where generally the β-state magnetic moment is aligned with the external field and thereby has lower energy (ground state), and the α-state has magnetic moment opposed to the external field leading to higher energy. The energies of the spin states are dependent on the gyromagnetic ratio of the nucleus, γ, the external field strength, B_0, and magnetic quantum number (Eq. 5).

$$E = -\gamma\hbar m B_o \tag{5}$$

$$\Delta E = E_2 - E_1 = h\nu \tag{6}$$

$$\nu = \frac{\omega}{2\pi} = \frac{\gamma B_0}{2\pi} \tag{7}$$

The energy difference between the two different states (Eq. 6) and thus also the frequency of electromagnetic radiation that can induce transitions between states, ν, depend on the field strength and the gyromagnetic ratio of the nucleus of interest.

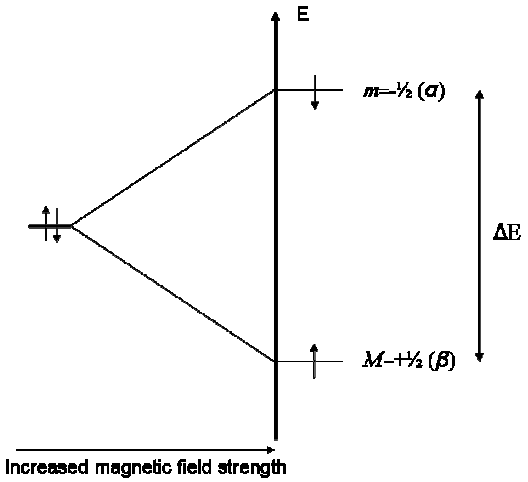

Figure 13. The two possible energy states, α and β of a spin I=½ nucleus after applying an external magnetic field.

The gyromagnetic ratio and the field strength determines the NMR frequency of the nucleus (Eq. 7) but the frequency is also affected by the local environment of the nucleus, more exactly the surrounding electron distribution, leading to a slight difference in resonance frequencies between nuclei in different

environments, which is why a peak in the NMR spectrum at a specific position can be related to the individual chemical environment of that atom. Frequencies are typically reported as chemical shifts, δ, defined in Eq. 8.

$$\delta = 10^6 \frac{\left(\nu - \nu_{ref}\right)}{\nu_{ref}} \tag{8}$$

At equilibrium, the net magnetization vector is located along the z-direction in an x,y,z-coordinate system (Figure 14). After a 90° (excitation) pulse the magnetization is flipped into the transverse plane so that precession about the z-axis with frequency ν can be detected. The precession and return of the magnetization to thermal equilibrium, called the free induction decay (FID), is described using Eq. 9-11.

$$M_x = M_0 \cos(2\pi\nu t) e^{-t/T_2} \tag{9}$$

$$M_y = M_0 \sin(2\pi\nu t) e^{-t/T_2} \tag{10}$$

$$M_z = M_0 \left(1 - e^{-t/T_1}\right) \tag{11}$$

The FID depends on the nuclei and their surroundings and can be converted into frequencies by Fourier transformation, which results in one- or multi-dimensional spectra depending on how the NMR experiment was conducted.

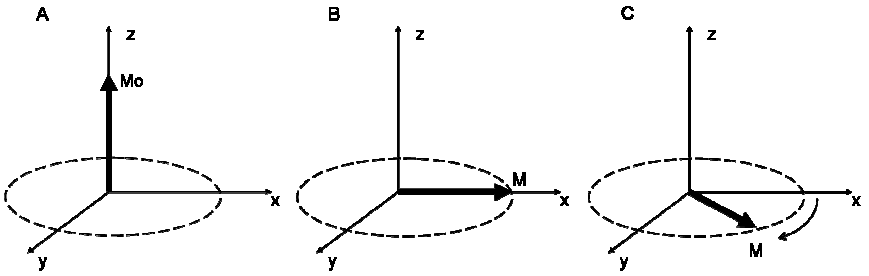

Figure 14. The vector model describing: **A)** Equilibrium; all magnetization (M) is aligned in the z-direction along the external field. **B)** After a 90° pulse the magnetization is flipped into the transverse plane. **C)** The magnetization is evolving in the transverse plane as it relaxation back to equilibrium along the z-axis.

5.4.2 Assignment of NMR spectra

There are several suitable experiments for backbone assignment of proteins, such as HNCA, HN(CO)CA, HNCO, HN(CA)CO, HNCACB and CBCA(CO)NH (Cavanagh *et al.*, 2006). The three-dimensional experiments provide correlations between three nuclei at a time, ^{1}H, ^{15}N and ^{13}C. For example, in an HNCACB experiment (Figure 15D), magnetization is transferred from the amide proton to ^{15}N and further to the Cα and Cβ nuclei of the same and preceding residue. The magnetization is then transferred back to the amide proton for detection. The HNCACB experiment can be combined with CBCA(CO)NH experiment for determination of both intra- (*i*) and inter-residue (*i*-1) correlations, Cα(*i*), Cα(*i*-1), Cβ(*i*) and Cβ(*i*-1) chemical shifts (Figure 15 C, D) (Kanelis *et al.*, 2001). Each amino acid residue shows characteristic chemical shifts and thereby enables the assignment. However, some residues show similar chemical shifts and can thereby be difficult to distinguish. Since the HNCACB and CBCA(CO)NH experiments provide information of both the Cα and Cβ chemical shifts the assignment process becomes much easier compared with experiments only showing the Cα chemical shifts. The backbone chemical shifts are sensitive to secondary structure formations of the protein or peptide and give different values of the chemical shifts compared to the random coil state.

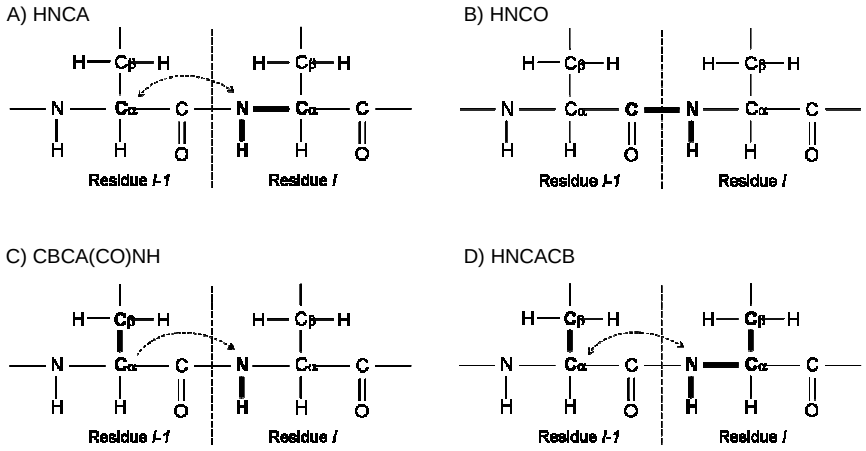

Figure 15. Schematic figure of A) HNCA, B) HNCO, C) CBCA(CO)NH and D) HNCACB. Nuclei (and corresponding bond) participating in magnetization transfer are marked in bold. Residue *i* is the internal and *i*-1 the sequential. Transfer steps involving two covalent bonds are indicated by arrows.

5.4.3 Calculation of protein structures

The secondary shifts $\Delta\delta = \delta_{observed} - \delta_{random\,coil}$ are useful for the calculation of the secondary structure (Wishart and Sykes 1994) where the $\Delta\delta C^{\alpha} - \Delta\delta C^{\beta}$ relation shows positive values for formation of α-helical formation and negative values indicated β-sheet or extended formations. If the secondary structure element is fully developed, values of approximately ±2 ppm are generated. Lower values indicate fractional development of the secondary structures, often seen for intrinsically disordered proteins.

The backbone chemical shifts also contain information about the local environment and position of the nucleus. Thus, the chemical shifts can be used for structural determination by different programs including CS-ROSETTA which combines *de novo* protein structure determinations and chemical shift data (Shen *et al.*, 2008), (Shen *et al.*, 2009). The program uses chemical shift fragments deposited in Biological Magnetic Resonance Bank (BMRB) data base and protein 3D structures from Protein Data Bank (PDB). The experimental input for the calculations are $^{13}C_{\alpha,}$ $^{13}C_{\beta,}$ $^{13}C_{o}$, ^{15}N, $^{1}H_{\alpha}$ and ^{1}H chemical shift usually generated early in the protein assignment process. After the Monte Carlo ROSETTA energy minimization, rescoring of the atomic models is performed, resulting in an ensemble of structures with lowest energies thus best representing the experimental input, which describes the protein structure.

Nuclear Overhauser effect spectroscopy (NOESY) experiments establishes correlations between nuclei close in space (<6Å) (Markwick *et al.*, 2008). Due to the distance dependence, the NOESY experiment provides useful data for structural calculations of proteins. The nuclear Overhauser effect (NOE) is dependent both upon the atomic distance and the angular fluctuations and data can therefore both be used for structural determinations and characterization of protein dynamics. The number of assigned NOEs is highly important for the quality of the structural calculation. Specific NOEs present in secondary structure elements can also be used to analyze structure content in less well-folded structures, such as transiently ordered segments.

Residual dipolar couplings (RDCs) give information about protein structure and dynamics (Bax, 2003; Jensen *et al.*, 2009; Tolman & Ruan, 2006). In contrast to

NOE interactions, RDCs give long range distance structural information and information on dynamics slower than molecular tumbling. The molecules are partially oriented relative to the external field by weak interactions with oriented macroassemblies such as bicelles or the filamentous bacteriophage Pf1 and thereby the dipolar coupling will no longer be averaged to zero. When partially aligning the spins along the external magnetic field, the splitting caused by dipolar coupling will differ as a consequence of the local environment and the molecular tumbling, giving residual dipolar couplings. In folded proteins, the RDC measurements gives information about the orientation of internuclear vectors of two spins (*I* and *S*) referred to a common alignment tensor (Jensen *et al.*, 2009). The RDCs of a folded protein can be used for structural determinations, to analyze protein complexes, and to characterize local dynamics in the molecule.

5.4.4 Interaction analysis using NMR

For analysis of protein-protein or protein-ligand interactions, chemical shift mapping is a suitable method since even a basic protein backbone assignment generates ^{1}H and ^{15}N chemical shifts for identified amino acid fragments. A commonly used experiment for interaction analysis is the HSQC (Heteronuclear Single Quantum Coherence), however, experiments such as HNCO can also be used and are advantageous if the spectral overlap is extensive in the HSQC spectra. The interaction between the protein and ligand can give rise to chemical shifts or altered peak intensity. Chemical shift perturbations (CSPs) can be calculated from the differences in the ^{15}N and ^{1}H chemical shifts by the following expression; $\Delta\delta_{comb} = \sqrt{\Delta\delta_{NH}^2 + \left(\Delta\delta_N / R_{scale}\right)^2}$ (Mulder *et al.*, 1999) where R_{scale} is the scaling factor. The ratio of intensities for bound protein/free protein should be one for unaffected residues and less than one for amino acids participating in the interaction. The chemical shift differences and the intensity show different pattern for slow, intermediate and fast exchange upon interaction. In slow exchange, chemical shift differences are usually large and intensity ratios small, while for rapid exchange, chemical shift differences are usually small and intensity ratios large (Baker *et al.*, 2007; Mittag & Marsh *& et al.*, 2010).

5.4.5 Protein dynamics

Changes in the structural conformation of proteins are often related to the biological function such as protein-DNA interactions or ligand binding. With NMR spectroscopy dynamics can be analyzed on many different timescales and at atomic resolution (Akke, 2002). Protein dynamics is the phenomenon where the populations of conformational states changes. The inter-conversion can be fast (picosecond) or slow (seconds) depending on the nature of the motion. Molecular vibrations typically take place on the picosecond timescale but conformational rearrangements can have motions up to the second timescale (Figure 16, adapted from (Boehr *et al.*, 2006; Jarymowycz & Stone, 2006)).

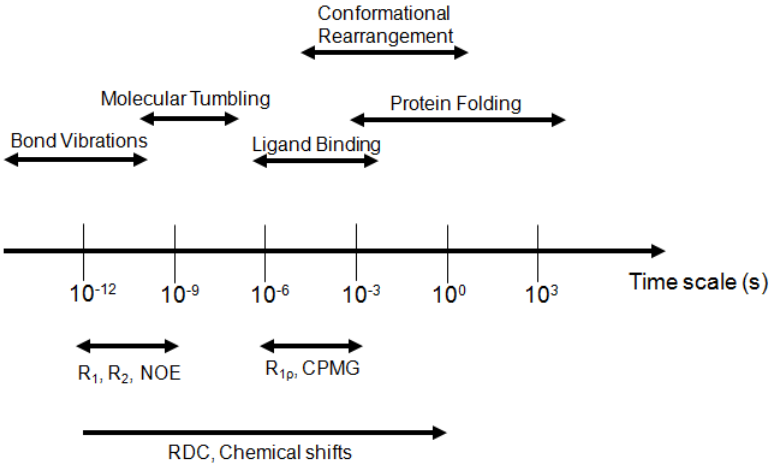

Figure 16. Time scale of dynamic processes within the protein (top) and related NMR experiments (bottom).

NMR experiments related to relaxation are for example backbone correlated measurements of $R_1=(1/T_1)$, $R_2=(1/T_2)$, $\{^1H\}$-^{15}N-NOE probing relaxation on the picosecond to microsecond timescale and CPMG (Carr–Purcell–Meiboom–Gill) used for determination of slower dynamics (ms). The fast relaxation rates depend upon the dipolar coupling and the Chemical Shift Anisotropy (CSA) interactions.

The R_1, R_2 and hetero-nuclear NOE and CPMG relaxation dispersion experiments can be measured in two-dimensional HSQC-type experiments. The

peak-intensity is a function of a relaxation delay and/or of how the experiments were set up (Figure 17C) (Jarymowycz & Stone, 2006).

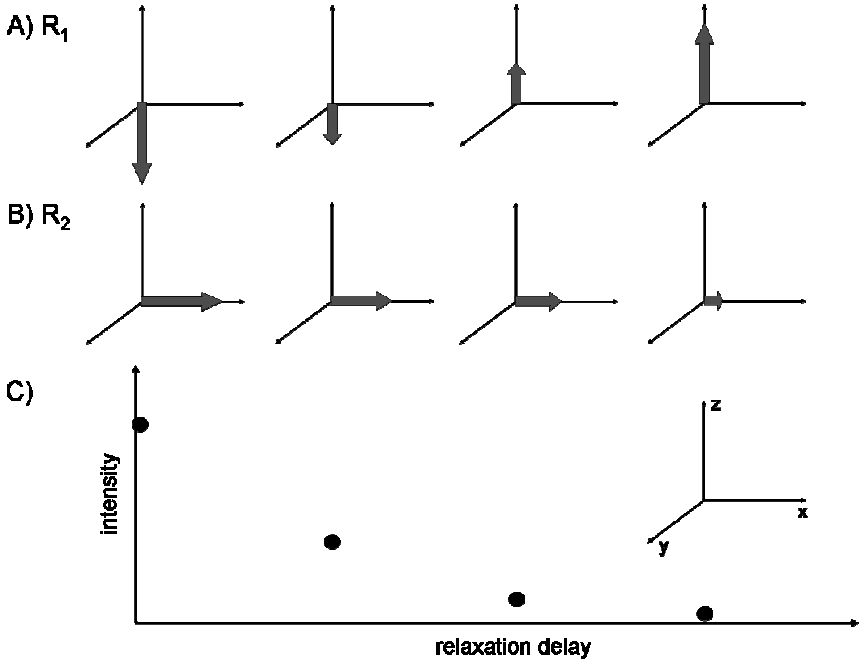

Figure 17. A) and **B)** R_1 and R_2 relaxation where the magnetization in the transverse plane is returning to equilibrium after perturbation by a radio frequency pulse. **C)** Increasing delays causes decreasing peak intensities for the R_2 relaxation experiment.

The R_1 experiment measures the longitudinal relaxation towards equilibrium, also called spin-lattice relaxation (Figure 17A). During the spin-lattice relaxation, the magnetic spins flip between the two available energy states caused by fluctuating local fields. This is the process where the spins relaxes from the exited state with higher energy towards the ground state (lower energy) by releasing energy to the surrounding which can be described by Eq. 13. ΔN is the difference in population between spin-states $m = +½$ and $m = -½$ at time *t*. ΔN_{eq} is this population difference at equilibrium (Eq. 12). This can also be described as the relaxation of the magnetization vector (M) to thermal equilibrium (Eq. 13).

$$\Delta N = \Delta N_{eq}\left[1 - \exp\left(-t / T_1\right)\right] \quad (12)$$

$$M_z(t) = M_0\left(1 - 2e^{-t/T_1}\right) \quad (13)$$

At equilibrium, the total magnetization vector (M_o) is aligned along the external magnetic field (z-axis). Since the R_1 relaxation describes the recovery of the magnetization along the z-axis we only look at the magnetization in z-direction (M_z) during the relaxation process. After a 180° perturbation, the magnetization is flipped to the negative z-axis (M_z=-M_z) and the rate of decay (to equilibrium) can be followed and are unique of each spin and dependent on the surrounding lattice (Figure 17A).

R_2 relaxation, also named transverse relaxation and spin-spin relaxation, (Figure 17B), is like R_1 dependent on the dipolar coupling and the chemical shift anisotropy (CSA) interaction and thereby the molecular tumbling time, also called rotational correlation time, τ_c. Small molecules have faster correlation time then larger. When the molecule is tumbling, or because of internal structural changes, ^{1}H-^{15}N bond vector is re-orientated relative to the external magnetic field, and the dipole interactions of the two magnetic nuclei starts to fluctuate. If the fluctuations in the local magnetic field occur at the appropriate frequency, relaxation of the ^{15}N nucleus will be induced (Jarymowycz & Stone, 2006; Mittermaier & Kay, 2006). The strength of the dipolar coupling is dependent on the internuclear distance, so the dipolar relaxation is mostly affected by the proton nucleus. The CSA relaxation results from shielding of the nucleus of interest from the permanent magnetic field. If no molecular motions occur, the magnetic spins will be refocused along the –x-axis after a perturbation of the magnetization into the transverse-plane followed by a delay (τ), in a spin echo pulse sequence. During the delay the spins evolve with different frequencies due to disturbed coherence. When applying a 180° pulse and another delay time, the magnetization will be in coherence. If the local magnetic fields fluctuate due to variations in the ^{1}H-^{15}N bond vector, the spins will not be refocused after the spin echo experiment.

Two nuclei, I and S, which are not scalar coupled, can still have dipolar interactions that affect the relaxation measurements of the molecule. This is the nuclear Overhauser effect (NOE) (Jarymowycz & Stone, 2006). The peak

intensity of spin I can be affected by spin S. If spin I and S experience local magnetic fluctuations caused by molecular motions (or mutual dipolar interaction) in the same way the phenomenon is called cross-relaxation. Heteronuclear NOE experiments gives information about N-H bond-vector motion when experience the dipolar interaction from neighboring spin. Bond vectors that have faster motions (due to local dynamics) compared to the overall molecular tumbling show a decreased NOE intensity. This is common for the terminal parts and linker region of proteins.

The collected R_1, R_2 and NOE relaxation data can be used for analysis by different methods, for example the Lipari-Szabo model-free (Clore *et al.*, 1990; Lipari & Szabo, 1982) approach. The method is reviewed in (Jarymowycz & Stone, 2006). The model-free method provides intuitive motional parameters such as order parameters (S^2), the effective internal correlation time, τ_e, and the rotational correlation time, τ_c, related to the overall molecular motion (Reddy & Rainey, 2010). Model-free requires that molecular tumbling and internal motions are statistically independent. Order parameters provides a way of characterizing the magnitude of internal motions of bond vectors on the picosecond-nanosecond timescale where a value of zero represents highest disorder of the bond vector and a value of unity describes total restriction. The overall correlation time is the average time it takes for a molecule to tumble through one radian in one direction and is dependent upon the shape and size of the molecule and also the solvent viscosity.

Protein folding, ligand interactions, and catalysis are some biological functions that occur on the microsecond-millisecond timescale. To quantify dynamics on the millisecond timescale, rotating frame spin relaxation ($R_{1\rho}$) or the Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments can be used. Chemical shift exchange caused by different conformations causes excess transverse relaxation rate R_{ex} which can be measured with these NMR experiments. $R_{1\rho}$ relaxation is modulated by a continuous-wave spin-lock field whereas for the CPMG experiment refocusing sequences (Eq. 14) are used (Mittermaier & Kay, 2006).

$$90°_x \left(-\tau_{cp} - 180°_y - \tau_{cp} -\right)_n \qquad (14)$$

If a nucleus experiences chemical shift fluctuations, the magnetization at the end of the sequence is defocused, the peak is broadened and the transverse relaxation rate is large (Figure 18). If a larger number of pulses are applied, thus suppressing dephasing, the magnetization will be less defocused, causing sharper peaks and lower relaxation rates. The CPMG experiment gives information of thermodynamic and kinetic properties of the exchange process as well as structural information of the excited state since the dispersion profile depends on the population of the two states, the exchange rate and the magnitude of the chemical shift difference between the two states (Mittermaier & Kay, 2009).

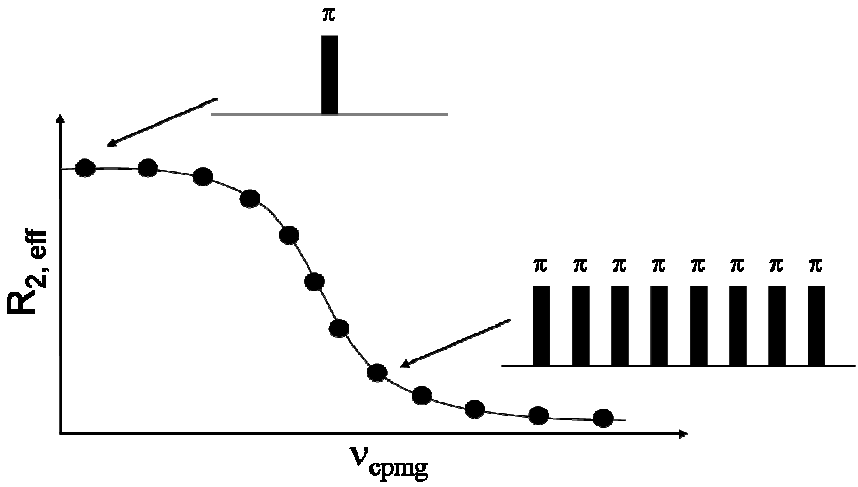

Figure 18. R_2 relaxation rates as a function of the repetition rate of the CPMG refocusing pulses.

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