Tripeptidyl-Peptidase II

Structure, Function and Gene Regulation

ANN-CHRISTIN LINDÅS
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Abstract

The protein degradation process is of vital importance for the cell to maintain cellular functions. An important enzyme in this process is the multimeric tripeptidyl-peptidase II (TPP II). It removes tripeptides from a free N-terminus of the substrates. TPP II has broad substrate specificity and wide-spread distribution, suggesting that the TPP II gene is a house-keeping gene. However, the levels of both mRNA and TPP II protein varies during different conditions and the TPP II gene promoter was therefore identified and characterized. It is a 215 bp fragment just upstream of the coding sequence. This fragment lacks a TATA-box but contains an initiator, two inverted CCAAT-boxes and an E-box. The CCAAT-boxes and the E-box were found to bind the nuclear factor Y (NF-Y) and upstream stimulatory factor-1 (USF-1) respectively. The CCAAT-boxes appear to be most important for the transcriptional activation. Furthermore, several silencer element were identified further upstream of the 215 bp promoter and the octamer binding factor Oct-1 was found to bind one of these fragments.

If Oct-1 is responsible for the inhibition of the transcription of the TPP II gene remains to be investigated. In addition, the substrate specificity was investigated. For this purpose an expression system using Pichia pastoris was developed. The purified recombinant TPP II was found to have the same enzymatic properties as the native enzyme. In order to identify the amino acids involved in the binding of the N-terminus of the substrate, wild-type murine TPP II and four mutants E305Q, E305K, E331Q and E331K were purified. Steady-state kinetic analysis clearly demonstrated that both Glu-305 and Glu-331 are important for this binding as the K_m is more than 10^3 higher for the mutants than wild-type. Finally, the pH-dependence for cleavage of two chromogenic substrates was compared for TPP II from different species.

Keywords: gene regulation, protein expression, enzyme kinetics, tripeptidyl-peptidase II, proteasome, intracellular protein degradation, exopeptidase

Ann-Christin Lindås, Department of Biochemistry and Organic Chemistry, BMC, Box 576, Uppsala University, SE-75123 Uppsala, Sweden

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Papers included in the thesis

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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Abbreviations

AAA-pNA \(\text{Ala-Ala-Ala-paranitroanilide}\)
AAF-pNA \(\text{Ala-Ala-Phe-paranitroanilide}\)
CMA \(\text{Chaperone mediated autophagy}\)
CP \(\text{Core particle (i.e. the 20S proteasome)}\)
EMSA \(\text{Electrophoretic mobility shift assay}\)
HEK cells \(\text{Human embryonic kidney cells}\)
IMAC \(\text{Immunolised metal ion affinity chromatography}\)
Inr \(\text{Initiator}\)
NF-Y \(\text{Nuclear factor Y}\)
NRE \(\text{Negative regulatory element}\)
Oct-1 \(\text{Octamer binding factor}\)
PCR \(\text{Polymerase chain reaction}\)
RP \(\text{Regulatory particle (i.e. the 19S subunit of the 26S proteasome)}\)
TAF \(\text{TBP-associated factor}\)
TBP \(\text{TATA-box binding protein}\)
TPP II \(\text{Tripeptidyl-peptidase II}\)
TPP2 \(\text{The gene encoding tripeptidyl-peptidase II}\)
USF-1 \(\text{Upstream stimulatory factor-1}\)
UTR \(\text{Untranslated region}\)
wild \(\text{Wild-type}\)
Preface

Tripeptidyl-peptidase II (TPP II) is an enzyme involved in the protein turnover in the cytosol of almost all eukaryotic cells. TPP II sequentially removes tripeptides from a free N-terminus of oligopeptides, which are mainly generated by the proteasome. There are several reports about TPP II being upregulated, but also downregulated, during different conditions in the cell i.e. sepsis, cancer and starvation. Besides the broad substrate specificity, TPP II also seems to have more specific substrates. The generation of certain epitopes for antigen presentation and the inactivation of cholecystokinin-8 are dependent on TPP II. One of the aims of the investigation presented in this thesis was therefore to identify the promoter of the TPP II gene and in addition identify sequence elements and/or transcription factors involved in the regulation of the transcription of the gene. The other aim was to identify amino acids involved in the substrate binding thereby responsible for the specific tripeptidyl-peptidase specificity of TPP II.
Introduction

Proteins - Macromolecules with diverse functions

Proteins can be found in all living cells and in all biological tissues. In the human body there are between 30,000 to 50,000 different proteins of which the largest part of the total protein mass is muscle proteins. The word protein was introduced by Jöns Jakob Berzelius (1779-1848) and referred to proteins being a necessary part of the diet (Hartley, 1951).

Today there is much more known about proteins. The protein molecule is built up from one or more chains of amino acids, so called polypeptides. There are 21 amino acids with different properties available for the protein synthesis. The combination of the amino acids in the polypeptide chain, which is determined by the DNA sequence in the corresponding gene, determines the properties of the protein. However, the properties may also be dependent on integration of other molecules and/or atoms into the structure. The proteins have several functions, which are reflected in their different structures. They may function as structural elements for example in cartilage, tendon, skeleton, and skin, others function as transport proteins, antibodies, transcription factors, proteins involved in signalling, enzymes etc. (Mathews et al., 2000). The enzymes, which are the catalysts in the cell, are necessary for the proceeding of the hundreds of reactions taking place in the living cell. The enzyme tripeptidyl-peptidase II (TPP II) is the subject for the investigations presented in this thesis, which will be referred to in more detail later in the thesis.

Enzyme catalysis

Enzymes are amongst the most efficient and specific of all catalysts known. Their function is to increase the rate of the reactions inside or outside the cell. Without the participation of enzymes, these reactions would be very slow under the physiological conditions in the cell, however, different enzymes affect the reaction rate to various extents. In extreme cases the rate may be increased million-fold or even billion-fold (Mathews et al., 2000).
The enzyme binds the substrate to the active site where the reaction takes place. This binding can be achieved according to two different models; "lock-and-key" or "induced-fit". In the lock-and-key model, the enzyme has a pocket or a cleft shaped to fit and bind the substrate, whereas in the induce-fit model the enzyme has to change conformation in order to bind (Koshland, 1958).

For TPP II, which hydrolyses peptide bonds, the conversion of a substrate S to the product P via enzyme-substrate complexes by the enzyme E may be schematically described as:

\[ E + S \xrightleftharpoons[k_{-1}]{k_1} ES \rightarrow^k_{2} ES' \rightarrow^{k_3} E + P \]

where lower case k denotes rate constants for formation (k₁) and dissociation (k⁻¹) of the enzyme-substrate complex, or for chemical steps. k₂ is the rate of formation of an acylenzyme intermediate and k₃ the rate of its hydrolysis. Enzyme activities are usually measured during "steady-state" conditions, i.e. when the concentrations of intermediates are constant (Fersht, 1999). All activity measurements in this work were done according to steady-state kinetics.

The rate of a reaction is proportional to the enzyme concentration. When the substrate concentration increases, the reaction rate increases linearly at low substrate concentrations and at sufficiently high concentrations of the substrate, the change in reaction rate will decrease as the enzyme becomes saturated (Fig. 1).

Figure 1. Substrate saturation curve for an enzyme following Michaelis-Menten kinetic.
This change in the reaction rate with substrate concentration can be described by the Michaelis-Menten equation

\[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} \quad \text{(Equation 1)} \]

\( V_{\text{max}} \) is the maximal reaction rate that can be achieved for a certain enzyme concentration. \( K_M \) (the Michaelis constant) is an apparent dissociation constant for all enzyme-bound species and the substrate concentration when the reaction rate is \( V_{\text{max}}/2 \) (Fig. 1) (Fersht, 1999).

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E]_0} \quad \text{(Equation 2)} \]

The turnover number, \( k_{\text{cat}} \), represents the total number of substrate molecules that can be turned into product per active site and time unit. This is a measure of the efficiency of the enzyme towards a certain substrate. Another kinetic parameter is the specificity constant, \( k_{\text{cat}}/K_M \), which is obtained from the reaction rate at very low substrate concentrations (Fig. 1). This constant can be used for comparing specificity of competing substrates (Fersht, 1999).

The efficiency of an enzyme to catalyse a reaction is also dependent on the pH in the cell. It is usual for amino acids involved in catalysis to have acidic or basic side chains, e.g. Asp, Glu, His, Lys etc. These are protonated or deprotonated in a pH depending manner and usually only one ionic form is enzymatically active. Therefore, the overall activity of the enzyme is dependent on the ratio between the protonated and deprotonated form of the residues (Fersht, 1999). This is also reflected in the kinetic parameters described previously.

From DNA to protein

The human genome, which has recently been sequenced, is comprised of approximately \( 6 \times 10^9 \) nucleotides encompassing about 30 000 genes (Venter et al., 2001). The amino acid sequence for a certain polypeptide is determined by the nucleotide sequence for its corresponding gene. In the eukaryotic cell there are three RNA polymerases, pol I, pol II and pol III respectively, which are responsible for the transcription of the genes. Pol I synthesizes large ribosomal RNA (rRNA), pol II synthesizes messenger RNA (mRNA) and small nuclear RNA (snRNA) and pol III synthesizes a variety of RNAs, for example transfer RNA (tRNA) (Ogbourne and Antalis,
The most diverse set of genes is transcribed by pol II. RNAs from pol II are synthesized as primary RNAs containing exons (coding sequences) and introns (noncoding sequences) but also 5'- and 3'-UTR (untranslated region). The introns are spliced off and the resulting messenger RNA (mRNA) functions as a template for the protein synthesis. The mRNA binds to the ribosome where the assembly of amino acid into a polypeptide takes place. It is the amino acid sequence that determines the folding and thereby the three-dimensional structure of the protein (Mathews et al., 2000). To be active, the protein may have to be further modified by e.g. phosphorylation, glucosylation, methylation etc.

To be able to characterize a protein with respect to structure and function and equally the relationship between these, it is necessary for a large amount of protein to be present. Previously, the only method to obtain the protein of interest was to purify it from that tissue expressing the protein, whereas today, it is possibly to use a host cell, usually bacteria. The complementary DNA (cDNA), which corresponds to the already spliced mRNA, is inserted in the cells. In the cell, the cDNA is transcribed directly into mRNA, which is then translated to a polypeptide. The protein synthesized in this way is called a recombinant protein, and the process heterologous gene expression. There are several advantages with this method, i.e. the growth rate of bacteria is relatively fast (they duplicate about every 30 min compared with about 24 h for mammalian cells) and a large amount of protein can therefore be obtained. Other advantages are that mutations can be introduced in the gene, and the protein of interest can be synthesized with a tag (e.g. a polyhistidine tail) fused to either the N- or C-terminus. The latter facilitates the purification of the protein, by increasing the selectivity and reducing the number of purification steps. To be able to perform the structure-function studies of TPP II presented in this thesis, an expression and purification system using the yeast cell *Pichia pastoris* was developed.

**Transcriptional regulation of gene expression**

Because of the diverse functions of the proteins, not all proteins are needed at the same time, or to be constantly present in the cell. Therefore, the pathway from DNA to protein is strictly regulated and the regulation may occur at different levels of the process. One of the first steps involves the regulation of the transcription of the gene. As mentioned earlier, the RNA polymerases are responsible for the transcription of the genes, however, the polymerases themselves are not able to recognize and distinguish between different genes to be transcribed (White, 2001). Consequently, for this purpose the transcription factors are needed. The mammalian genome, may encode as many as 10 000 transcription factors (White, 2001). These are proteins that recognize and bind to a certain DNA sequence with high affinity and selectivity. This paragraph will focus on the pol II transcription.
A pre-requisite for the positioning and assembly of the preinitiation complex or basal transcription machinery, is the recognition of the core promoter (Hahn, 2004 and references therein). The sequence elements found in eukaryotic core promoters are the TATA-box, initiator (Inr), DPE (downstream promoter element) and BRE (TFIIB-recognition element). One or more of these elements are found in most of the promoters. The TATA-box and BRE are located about 25-30 bp upstream of the transcription start site, whereas the Inr element overlaps this site and DPE is found downstream thereof (Hahn, 2004). Besides the RNA polymerase, the pre-initiation complex consists of six different proteins: TFIIA; TFIIB; TFIID; TFIIE; TFIIF; and TFIIFH known as the general transcription factors (GTFs) (Weis and Reinberg, 1997). The TFIID is a multisubunit protein complex, which consists of the TATA-box binding protein (TBP) and TBP-associated factors (TAFs) (Weis and Reinberg, 1997). The TBP recognizes and binds to the TATA sequence element, which initiates the assembly of the pre-initiation complex. In TATA-less promoters, which instead contain an Inr, the TAFs seem to be responsible for the recognition and binding to this sequence element. In addition, the TAFs appear to be targets for negative and positive regulation of the transcription (Hahn, 2004). Studies have shown that the preinitiation complex formed at an Inr element is sufficient for transcription initiation, however, the presence of upstream elements can drastically increase the level of transcription (Weis and Reinberg, 1997). Examples of such sequence elements are the GC-box and the CCAAT-box recognized by for example the specificity protein 1 (Sp-1) and nuclear factor Y (NF-Y) respectively. The CCAAT-box is present in approximately 30% of eukaryotic promoters and is therefore one of the most ubiquitous sequence elements (Mantovani, 1999). Furthermore, NF-Y is the major protein recognizing this sequence and one of the functions of NF-Y is to associate with TBP and TAFs to recruit the RNA polymerase to the transcription start site (Kabe et al., 2005). In one of the projects presented in this thesis, NF-Y has been identified as one of the transcription factors binding to the TPP2 promoter and will therefore be discussed further.

Regulation of the transcription is achieved through enhancers or silencers. An enhancer is a sequence element that can stimulate the transcription from the promoter. This sequence element is independent of orientation and can be located several kilobases downstream or upstream of the promoter (White, 2001). Usually the enhancer contains binding sites for many different transcription factors, which can affect the activation level to various extents (White, 2001). The main function of the activators is to interact direct or indirect via co-factors with the basal transcription machinery (White, 2001). In contrast to the enhancers, the silencers are sequence elements that contribute to the inhibition of transcription. These elements are also position and orientation independent and bind transcription factors/repressors that usually interfere with the formation of the preinitiation complex (Ogbourne...
and Antalis, 1998). Position dependent silencers (NREs) are found in a large number of promoters. The repressors binding to the NREs inhibit the binding of transcription factors to their specific binding sites (Ogbourne and Antalis, 1998).

Several transcription factors have been found able to both activate and repress transcription of different genes. One example is Oct-1, which can adopt different conformations depending on the actual DNA-sequence present (Ogbourne and Antalis, 1998). The different conformations bind different cofactors, which results in either activation or repression of transcription (Ogbourne and Antalis, 1998). Another example is the transcription factor GATA-1, which represses the transcription of the GATA-2 gene but activates the transcription of the β-globin gene (Rodriguez et al., 2005). Some activators, repressors and co-factors may only be present or function in certain cell types, which results in a tissue-specific regulation. Furthermore, transcription factors interact with each other and so do the co-factors to regulate the transcription, which brings this process to an even higher complexity.

Methods for investigation of transcriptional regulation

There are several methods available for investigating the regulation of gene expression, however, two methods have been used for the study presented in this thesis: reporter assays and electrophoretic mobility shift assays (EMSA).

For reporter assays, reporter vectors and some form of detection system are used. Usually the reporter vector contains a gene which when transcribed results in a protein, which is easy to detect or catalyses a reaction easy to measure and in both cases, with high sensitivity. The reporter vector has no promoter, instead a potential promoter fragment from the investigated gene is inserted upstream of the reporter gene in the vector. If the inserted fragment contains a functional promoter, the reporter gene will be transcribed and the resulting protein can be detected. The reporter vector used for the investigations presented, contains the luciferase gene. Luciferase is a protein found in fireflies and catalyses the reaction of luciferine under emission of light. For a certain concentration interval of the reporter vector, there is a linear relationship between how strong the particular promoter is, the level of transcription and the amount of protein expressed. Therefore, the intensity of the light emitted in the reaction is proportional to the ability for the potential promoter to activate transcription.

When a promoter or other regulatory elements have been identified, it is interesting to find out if these fragments are able to bind transcription factors. One method that can be used for this purpose is EMSA. The DNA sequence of interest is labelled with $^{32}$P and incubated with nuclear proteins purified from cells used in the investigation. If any of the proteins present recognize and bind to the DNA, there will be one or more DNA-protein complexes of different sizes formed. These are separated from each other
and from the labelled unbound DNA on a native polyacrylamide gel and detected by autoradiography (Fig. 2).

Figure 2. Illustration of the detection of DNA-protein complexes formed in EMSA. The sample in lane A contains only the labelled probe, whereas in lane B the probe has been incubated with nuclear proteins. Complexes formed between DNA and proteins migrate slower in the gel compared with the free probe due to differences in size.

To further identify the protein or proteins bound to the DNA, competition experiments can be done using an excess of a consensus oligonucleotide for the transcription factor of interest. A complement is to use antibodies against the transcription factor. The antibodies can affect the complex formation in two ways, provided that they bind to a protein either by competing with the DNA for binding or by participating in the complex formation. Consequently, the former abolish the complex formation and the later results in a so called super-shift due to the even larger complex formed.

Post-transcriptional regulation of gene expression

There are a large number of events preceding the obtaining of the mRNA to be translated, such as mRNA transport, translocation, mRNA stabilization etc. The mRNA stability may vary between a few minutes to many hours and the major degradation pathway is initiated by shortening of the polyA tail followed by decapping of the 5'-end (Day and Tuite, 1998 and references therein). Furthermore, the assembly of the ribosomal subunits, mRNA and the Met-tRNA is regulated by different initiation factors (IFs) (Day and Tuite, 1998). Once the assembly of the different components are accomplished, the initiation of translation is regulated by nucleotide sequences present in both the 5'- and 3'-UTR of the mRNA (Day and Tuite, 1998).
After translation the resulting peptide may be further processed to be active. This processing involves for example phosphorylation, methylation, glucosylation, proteolytic cleavage etc. Regulation at protein level is very fast, but it is costly for the cell to synthesise proteins and therefore, unnecessary if the proteins are not required. Consequently, it is less demanding for the cell if the regulation occurs more closely to or at level of transcription.

**Protein degradation**

Up to the early 1940's, proteins of the body were considered as stable molecules that only went through minor changes. The dietary proteins were then thought only to function as a source of fuel (Ciechanover, 2005). Today it is known, from the work of Schoenheimer, that an extensive protein degradation, but also synthesis, are constantly going on in the cell (Ciechanover, 2005). The ability for a cell to degrade proteins is necessary for its survival. This process is essential for preservation of normal cell function by degrading damaged proteins. It also guarantees the replacement of cellular components before they lose their functionality (Cuervo, 2004 and references therein). Up to 30% of all newly synthesized peptides do not pass the quality control and are therefore degraded (Princiotta et al., 2003). Furthermore, the intracellular protein degradation has an important regulatory role (Cuervo, 2004; Ciechanover, 2005). In the eukaryotic cell, the degradation occurs mainly in the cytosol and the lysosomes.

**Degradation in the lysosomes**

Lysosomes are vesicles containing about 40 different hydrolytic enzymes where their role is to degrade intracellular macromolecules (Alberts et al., 2002). The lysosomal membrane functions as a barrier between these hydrolases and the cytosolic components. Furthermore, the lysosomal lumen has a pH about 5.0 and the lysosomal hydrolases are therefore as most active in an acidic environment. The main regulation step in the lysosomal degradation is the uptake of material into the lysosome. Once inside the lysosome, the substrates are degraded within 5-10 min (Cuervo, 2004). The material to be degraded can be delivered to the lysosomes by three different pathways: endocytosis, autophagy and phagocytosis (Alberts et al. 2002). Autophagy can be further divided into macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Cuervo, 2004). Macroautophagy is the ingestion of cytosolic components, such as soluble proteins and whole organelles. This pathway is activated for instance during starvation and is the major source for amino acids, lipids, sugars, nucleotides etc. during this condition (Cuervo, 2004). Microautophagy involves the same substrates as macroauto-
phagy but one of the differences is that this process is not strictly regulated. In CMA, the proteins to be degraded are transported directly into the lysosomal lumen, via receptors on the surface of the lysosome (Cuervo, 2004). To be able to cross through the membrane, the proteins have to be unfolded which is achieved by the chaperone. One common feature of the proteins degraded by CMA is an amino acid sequence related to KFERQ, which is recognized by the chaperone (i.e. the heat-shock protein hsc 73) (Cuervo, 2004). This sequence is found in approximately 30% of all proteins in the cytosol (Cuervo, 2004). As for macroautophagy, the degradation via CMA is activated in response to nutrient deprivation, however, the activation of CMA, which is tissue-dependent is particularly pronounced in liver, heart, kidney and spleen during starvation (Cuervo, 2004).

Degradation in the cytosol

In contrast to the lysosomal protein degradation, the degradation of proteins in the cytosol has to be strictly controlled, otherwise, it would be devastating for the cell and in addition it has to be very specific. The proteins to be degraded are marked with polyubiquitin. It has been discovered that the human genome encodes hundreds of different ubiquitin protein ligases. These are responsible for the differences in the polyubiquination of the proteins, which leads to the high specificity of the degradation process (Ciechanover, 2005). The ubiquitin-proteasome system is the major proteolytic system in the cytosol and involved in a large number of different processes in the cell as illustrated in Fig. 3 (Ciechanover, 2005).

![Figure 3. The ubiquitin-proteasome system and its biological functions. (The cartoon of the proteasome is from Tomkinson, 1999).](image)
The 26S proteasome is a 2.5 MDa enzyme complex consisting of the 20S core particle (CP) and two regulatory particle (RP) denoted 19S (Groll et al., 2005 and references therein). The 20S CP has a cylinder shaped structure assembled from four stacked rings with 7 subunits per ring. The outer ring in each end of the cylinder consists of α-subunits and the two inner rings consist of β-subunits. There are only three enzymatically active subunits, i.e. β1, β2 and β5, located inside the cylinder (Groll et al., 2005). Bound to the entrances of the 20S CP, the 19S RP are responsible for substrate recognition, substrate unfolding and translocation of the substrate into the 20S tunnel (Groll et al., 2005).

In mammalian cells, the three active β-subunits can be replaced by β1i, β2i and β5i respectively in response to activation of the immune system. The newly formed immunoproteasome is responsible for the generation and control of peptides transported to the cell surface by major histocompatibility complex class I molecules for antigen presentation (Groll et al., 2005). Another role for the ubiquitin-proteasome system is in the control of the cell cycle. It has been shown that the degradation of cyclin is necessary for the cell to exit mitosis (Glotzer et al., 1991).

The substrates of the 26S proteasome are a large variety of proteins and the length of the peptides generated by the proteasome is 3-25 amino acids with an average length distribution of 7-9 amino acids (Groll et al., 2005). These are further degraded into free amino acids by different proteases including tripeptidyl-peptidase II, which is the subject for the investigations presented in this thesis.

**Tripeptidyl-peptidase II**

**Gene structure**

The human TPP II gene (*TPP2*) is 82 kb divided into 30 exons from which exon 24 is subject for alternative splicing (Tomkinson, 1994). Experiments have revealed that the presence of exon 24 results in an enzyme complex of at least twice the size compared with the complex formed when this exon is spliced off (Tomkinson et al., 1997). A comparison between the coding parts of the murine and human DNA revealed a 90% identity. At amino acid level, this corresponds to a 96% identity and 98% similarity (Tomkinson, 1994).

**Protein structure**

TPP II is a large multimeric enzyme complex. It consists of two dimerized strands, twisted around each other to form a spindle-shaped structure (Fig. 4) (Macpherson et al., 1987; Rockel et al., 2002).
The three-dimensional structure of TPP II from *Drosophila melanogaster*, has been determined by electron microscopy at 2.2 nm resolution (Rockel et al., 2005). From this structure, it could be revealed that each strand is built up from 10 dimers, which in turn are composed of monomers arranged in a head-to-head fashion (Rockel et al., 2005). With a molecular mass of 150 kDa (138 kDa for the human and murine enzyme) per subunit, the whole complex reaches a total mass of >6 MDa (Rockel et al., 2005). The N-terminal part of the subunits appears to be involved in the interactions between the two strands forming the spindle (Rockel et al., 2005). Furthermore, the assembly of the monomers results in formation of an internal tunnel through the strand (Rockel et al., 2005). This may suggest that TPP II is a self-compartmentalizing enzyme (Tomkinson, 2000; Seyit et al., 2006). The complex is able to spontaneously dissociate and reassociate and the association is facilitated in the presence of substrates or competitive inhibitors (Tomkinson, 2000). For full enzymatic activity, the whole complex is required and dissociation into dimers results in a 90% reduction of the enzyme activity (Tomkinson, 2000; Seyit et al., 2006).

Enzymatic properties
Tripeptidyl-peptidase II (TPP II) was discovered in 1983 by Örjan Zetterqvist and his co-workers, when they were searching, in rat liver, for a peptidase that was able to cleave phosphorylated proteins (Bälöw et al., 1983). TPP II was found to be an extra-lysosomal exopeptidase that removes tripeptides from a free N-terminus of oligopeptides (Bälöw et al., 1983).
However, different tripeptides are cleaved off with different rates and TPP II cannot cleave N- or C-terminal of proline (Bålöw et al., 1986). Besides the predominant exopeptidase activity, the enzyme also displays a low endopeptidase activity (Geier et al., 1999). TPP II can be found in a large number of different eukaryotic cells from different species (Bålöw et al., 1986; Bålöw and Eriksson, 1987). Investigations of the active site of the enzyme could establish that TPP II is a serine peptidase of subtilisin type (Bålöw et al., 1986; Tomkinson et al., 1987). In mammalian cells, there is only one other peptidase which can be ascribed tripeptidyl-peptidase activity and that is the lysosomal tripeptidyl-peptidase I. In contrast to TPP II, which has a neutral pH-optimum, the lysosomal enzyme is active at acidic pH (Vines and Warburton, 1998).

Function

The main function of TPP II is probably in the general protein turn-over in the cell, together with the proteasome and other proteases (Fig. 5) (Tomkinson and Lindås, 2005). This assumption is based on the broad substrate specificity and wide-spread distribution of the enzyme.

Figure 5. Illustration of the cytosolic protein degradation. Different pools of proteins or polypeptides are degraded in the cytosol to generate amino acids for protein synthesis. Some of the peptides are rescued and used for antigen presentation. (DRiP stands for defective ribosomal products.) The role of TPP II in this process is discussed in the text. (This figure is a reproduction from Tomkinson and Lindås, 2005).
The degradation of oligopeptides longer than 14 amino acids appears to be dependent on TPP II (Reits et al., 2004; York et al., 2006). In addition, TPP II appears to have more specific substrates such as cholecystokinin-8 (CCK-8). Cholecystokinin is a family of hormonal and neuronal peptides, which participates in the regulation of food digestion processes (Ganellin et al., 2000). A membrane-bound form of TPP II cleaves CCK-8 and thereby inactivates this peptide (Rose et al., 1996). In vivo experiments showed that mice, which intravenously have been given the specific TPP II inhibitor butabindide, had a decreased food intake compared with the control group (Rose et al., 1996).

Antigen presentation
Some of the peptides generated by the proteasome are rescued for transport to the cell surface after they have been trimmed by different proteases including TPP II. The formation of the HIV Nef(73-82) epitope is dependent on TPP II (Seifert et al., 2003). In addition, TPP II appears to be important for the formation of the RU134-42 epitope (Lévy et al., 2002). Inhibition of the proteasome and TPP II respectively, decreases the expression of peptides on the cell surface to almost the same extent (Kloetzel, 2004). Simultaneously inhibition of the two proteases does not lead to an additive effect, which therefore indicates that both the proteasome and TPP II acts on the same pool of peptides (Kloetzel, 2004). This is however not supported by York et al. whom have demonstrated that TPP II knockdown does not affect the antigen presentation (York et al., 2006).

Muscle wasting
Upregulation of TPP II has been observed in rat muscle cells during sepsis. A consequence of sepsis, AIDS, severe injury, cancer etc., is increased muscle degradation (Hasselgren et al., 2002). Both TPP II protein and mRNA levels are increased in white, fast-twitch skeletal muscle during sepsis, but decreased in liver under the same conditions (Wray et al., 2002). The enhanced expression of TPP II protein and mRNA can also be seen during cancer induced muscle degradation (Chand et al., 2005). The main factor regulating muscle degradation during sepsis is glucocorticoids (Hasselgren et al., 2002) whereas during cancer proteolysis appears to be regulated by the proteolysis-induced factor (PIF) (Chand et al., 2005). In both cases the regulation of TPP II and the proteasome seems to occur in parallel, rather than in tandem (Wray et al., 2002; Chand et al., 2005).

Cancer and apoptosis
The importance of the proteasome and TPP II has also been studied in tumour cells. EL4 lymphoma cells adapted to grow in presence of proteasome inhibitors show an increased expression of TPP II (Glas et al., 1998; Geier et al., 1999). Furthermore, EL4 cells overexpressing TPP II are able to survive
exposure to proteasome inhibitors at concentrations otherwise lethal for the cell (Wang et al., 2000). This suggests that TPP II can overtake some functions of the proteasome. Burkitt's lymphoma cells have impaired proteasome function and additionally these cells display an increased TPP II expression, together with increased levels of the oncoprotein c-Myc, upon exposure to proteasome inhibitors (Gavioli et al., 2001). Inhibition of TPP II in these cells induced apoptosis and accumulation of ubiquitin conjugates (Gavioli et al., 2001), which further supports that TPP II can, to some extent, compensate for the loss of proteasome function. Princiotta and co-workers maintain that the proteasomes in the EL4 cells are not completely inhibited and the remaining activity is sufficient for the degradation of ubiquitin conjugates (Princiotta et al., 2001). Furthermore, the accumulation of ubiquitin conjugates in cells exposed to TPP II inhibitors is probably due to an increased amount of substrates that have to be handled by the proteasome (Princiotta et al., 2001).

It has also been demonstrated that TPP II can protect macrophages from *Shigella flexneri* induced apoptosis where in these cells TPP II seems to inhibit the maturation of caspase-1 (Hilbi et al., 2000). Furthermore, investigations have shown that overexpression of TPP II in EL4 cells may inhibit the degradation of IAPs (inhibitor of apoptosis protein) and thereby inhibiting the cells to progress into apoptosis (Hong et al., 2003). Recent investigations have proposed a role for TPP II in cell cycle progression. Overexpression of the enzyme resulted in accelerated cell proliferation, centrosome abnormalities, multipolar mitosis and genetic instability (Stavropoulou et al., 2005). Using RNA interference to lower TPP II concentrations significantly slowed down the cell growth and prevented the cells to progress through mitosis (Stavropoulou et al., 2005). Together these investigations suggest that TPP II could be important in tumour progression (Stavropoulou et al., 2005, 2006).

The number of reports about the involvement of TPP II in different cellular processes under different conditions in the cell constantly increases. This makes TPP II a highly interesting enzyme for further investigations. Furthermore, TPP II may also be a potential drug target (e.g. as a CCK-8 inactivating enzyme) and different research groups are therefore trying to develop inhibitors of TPP II (Ganellin et al., 2000, 2005; De Winter et al., 2005). For this purpose the knowledge of structure, function and physiological role of TPP II is crucial.
Present investigation

Background and Aims

Tripeptidyl-peptidase II is involved in the intracellular protein turn-over, which is an essential process within a cell. A broad substrate specificity and ubiquitous expression of the enzyme suggest that the TPP II gene (*TPP2*) is a house-keeping gene. These are genes constitutively expressed to maintain the basal functions within a cell. Several reports, however, indicate that both the TPP II protein and the mRNA levels are altered during different conditions in the cell as described in the introduction. To our knowledge there were nothing known about the regulation of the expression of TPP II before this thesis. Thus, one of the aims of the projects was to identify the *TPP2* promoter and further identify sequence elements and transcription factors involved in the regulation of this gene. Hopefully, this will contribute to an increased knowledge of the physiological role for TPP II in the cell under different conditions.

The ability to release tripeptides from oligopeptides is quite a unique property among proteases. In mammalian cells there are TPP II in the cytosol and TPP I in the lysosomes. Homologues of TPP I belonging to the sedolisin family have recently been discovered in the fungi *Aspergillus fumigatus* (Reichard et al., 2006). The tricorn protease can be found in some archaea and eubacteria. It is an exopeptidase with preferential di- and tripeptidase activity (Groll et al., 2005). In bacteria there are several exopeptidases present which can release tripeptides (e.g. prolyl tripeptidyl aminopeptidase) (Ito et al., 2006). This indicates that the generation of tripeptides is an important step in the degradation process of proteins. The other aim was therefore to investigate the substrate specificity of TPP II in particular how it achieves its tripeptidyl-peptidase specificity by identifying amino acids involved in this process.
Regulation of *TPP2* transcription (papers I and II)

**Identification and characterization of the *TPP2* promoter**

When this project was initiated, the human genome had just been sequenced (Venter et al., 2001). Thus, a BAC (bacterial artificial chromosome) clone containing the human TPP II gene could be obtained from the Sanger Centre. For the identification of the *TPP2* promoter (paper I) the reporter vectors pGL3-basic and pGL3-control, containing the luciferase gene were used. An 8 kb Nco I fragment, just upstream of the start of the coding sequence of the TPP II gene was inserted in the pGL3-basic vector. This long fragment was expected to contain the sequences required for activation of the basal transcription of the TPP II gene. To obtain shorter fragments, 5'-deletions of the 8 kb construction were performed and the resulting fragments are schematically illustrated in Fig. 6.

![Figure 6. Potential promoter fragments obtained by deletions. The positions of the fragments are shown in relation to the start codon of *TPP2*. This also agrees with the position of the fragments in relation to the luciferase gene in the reporter vector.](image)

The shortest fragment obtained by deletion was 215 bp. To be able to investigate even shorter fragments (i.e. 60 and 130 bp) PCR was used for the amplification. Both HEK-293 and NIH3T3 cells were transfected with the different constructions, containing the potential promoter fragments. Approximately 24 hours after transfection the cells were lysed and the luciferase activity was measured. Since the transfection efficiency and the amount of cells may vary between different wells, values for the luciferase activity were corrected on the basis of these parameters. From the luciferase assays it could be concluded that the shortest functional promoter was the 215 bp fragment (Fig. 7). However, fragments up to 4.6 kb were able to activate transcription to the same extent as the 215 bp fragment. Surprisingly, the 8 kb fragment could only promote a very low luciferase activity (paper I).
may indicate the presence of a potential silencer between 4.6 and 8 kb that inhibits the transcription (investigated in paper II). These results were the same for both cell lines tested.

Figure 7. Identification of the TPP2 promoter. Luciferase activity promoted by different reporter constructs in HEK-293 cells. The activities were related to the negative control, the empty pGL3-basic vector. The values represent means±S.D. from six transfections.

The 215 bp sequence is GC-rich and lacks a TATA-box but contains two inverted CCAAT-boxes, an E-box (CACGTG) and an initiator (Inr) (Fig. 8). Both CCAAT-boxes are conserved between man, mouse and rat whereas the E-box is present only in the sequence upstream of the human gene (paper I).

Figure 8. The 215 bp TPP2 promoter. A schematic illustration of the arrangement of the two CCAAT-boxes, the E-box and the Inr in the TPP2 promoter.

The CCAAT-box is a common sequence element in a large variety of promoters. Promoters of house-keeping genes, however, require an intact
CCAAT-box (Mantovani, 1999). The main factor recognizing the CCAAT-box motif is the nuclear factor Y (NF-Y) (Mantovani, 1999). It is a ubiquitously expressed transcription factor, which is not able to activate transcription by itself to any larger extent. Instead NF-Y recruits other transcription factors and facilitates the binding of the basal transcription machinery to the transcription start site and by that increasing the transcription level (Kabe et al., 2005). In paper I it has been clearly demonstrated, using EMSA, that the 215 bp promoter gives rise to at least four DNA-protein complexes of different sizes when incubated with nuclear proteins from HEK-293 cells (Fig. 9). Competition experiments using a CCAAT-consensus oligonucleotide reveal that this sequence element is necessary for the formation of the two largest DNA-protein complexes. Furthermore, super-shift assays confirm that NF-Y is involved in the complex formation since antibodies against NF-Y abolished the formation of the two largest complexes (Fig. 9A and paper I).

Since TPP II is upregulated in cells overexpressing c-Myc (Gavioli et al., 2001) it was interesting to see if c-Myc could bind the E-box (paper II). Another transcription factor also recognizing the E-box sequence is the ubiquitously expressed upstream stimulatory factor (USF) (Corre and Galibert, 2005). Competition experiments, using EMSA, with an E-box containing oligonucleotide revealed that the formation of the two smaller DNA-protein complexes was competed out by the E-box sequence. To identify proteins involved in the formation of these complexes, antibodies against c-Myc and USF-1 respectively, were allowed to incubate with labelled DNA and nuclear proteins. The antibodies against USF-1 resulted in a super-shift whereas the antibodies against c-Myc did not affect any of the complexes formed (Fig. 9B and paper II).
The transcription factor USF-1 is ubiquitously expressed and activates transcription in the same way as NF-Y by directing RNA polymerase II to the transcriptional start site. In addition, USF-1 is able to recognize and bind the Inr sequence element (Corre and Galibert, 2005).

To investigate the contribution of the CCAAT-boxes and the E-box in transcriptional activation, mutations were made in these sequences (paper II). The respective sequence element was mutated one at a time and also in combinations. The mutated sequences were inserted into the pGL3-basic vector and HEK-293 cells were transfected with respective construction. The results from the reporter assay indicated that the upstream CCAAT-box is the most important sequence element for the transcriptional activation. Mutations in this element alone reduced the transcription level with approximately 50% while mutations in the downstream CCAAT-box and E-box respectively did not significantly affect the transcription. However, mutations in both CCAAT-boxes reduced the transcription with 75% and there was no additional effect when the E-box was also mutated. This proposes that both CCAAT-boxes, but not the E-box, contribute to increase the transcription of the luciferase gene by the 215 bp TPP2 promoter in HEK-293 cells (paper II).
Identification of upstream silencer elements

During the identification of the TPP2 promoter it appeared that the 5'-flanking sequence of the 8 kb fragment (denoted the 3.3 kb fragment) inhibited the transcription promoted by the 215 bp fragment (paper I). Therefore, this region was further investigated to identify potential silencer elements (paper II). The 3.3 kb fragment was inserted into the empty pGL3-basic vector and also upstream of the 215 bp promoter in the same vector. It was confirmed that this fragment inhibited the transcription and only promoted a very low transcription by itself (paper II). Restriction digest with Hind III resulted in 7 shorter fragments. In the reporter assay all fragments inhibited the transcription although to differing extents. The inhibition by two of the fragments appeared to be specific for the TPP2 promoter. These two fragments 440 bp and 1300 bp, constituting the 5' end of the 3.3 kb fragment were chosen for further investigations. Using PCR, shorter parts of these fragments were amplified. None of the shorter fragments were able to inhibit the transcription by themselves. This suggests that there may be more than one sequence element required to achieve the inhibition. The Match program was used to predict binding sequences for potential transcription factors. According to how good the sequence match was and what have been reported previously about how the particular transcription factor affects the transcription, seven different transcription factors AREB6, Gfi-1, Oct-1, TGIF, GATA, E4BP4 and YY-1 were chosen for further investigations. Consensus sequences for these factors were used for competition experiments in EMSA. In addition, consensus oligonucleotides for c-Myc (E-box) and NF-Y (CCAAT-box) were also tested since regions with sequences similar to these elements can be found in the investigated fragments. From these experiments and super-shift assays with antibodies, the transcription factor Oct-1 could be identified as one of the proteins binding to the 1300 bp fragment. The other consensus oligonucleotides tested could not abolish the binding of nuclear proteins even at 100-fold molar excess (paper II).

Oct-1 is a transcription factor present in almost all cell types. It can both activate and repress transcription depending on the DNA sequence and co-factors present. The DNA sequence affects the conformation of Oct-1 and thereby which co-factor that will be recruited (Ogbourne and Antalis, 1998). However, the exact role of Oct-1 in the regulation of the transcription of the TPP II gene remains to be further investigated.
Investigation of substrate specificity of TPP II (papers III and IV)

Development of a new expression and purification system

For the investigation of enzymatic properties and structure of TPP II, it is crucial to be able to express and purify the enzyme in large amount. Previously, wild-type (wt) TPP II has been overexpressed 6-8 fold in HEK-293 cells (Tomkinson et al., 1997). These cells were therefore used as hosts during the initial attempts to overexpress and purify the murine wt TPP II (mTPP II) (paper III). To facilitate the purification, a histidine tag was fused with the N-terminus of the enzyme. The purification of his-tagged mTPP II was performed on a Ni-IMAC (immobilized metal ion affinity chromatography) column eluted with imidazol at different concentrations. Western blot analysis of the fractions revealed that complexes containing both recombinant (145 kDa) and endogenously (138 kDa) expressed subunits are formed (paper III).

Due to the difficulties to separate recombinant expressed enzyme from the endogenously expressed in the HEK-293 cells, another system using the yeast *Pichia pastoris* was developed (paper III). The cDNA for the wt mTPP II was inserted into a vector for intracellular protein expression induced by methanol. The enzyme was expressed with a histidine tag fused to the N-terminus. Both Western blot analysis and activity measurements revealed a high amount of mTPP II expressed in this system. The specific activity for the enzyme in lysates from the yeast cells was 15-fold higher compared with lysates from HEK-293 cells. Furthermore, lysates from cells transfected with the expression vector without insert did not contain any TPP II protein and showed no TPP II activity. This indicates that these cells did not have any endogenously expressed TPP II, which could interfere with the complex formation (paper III).

The enzyme activity was analysed using the chromogenic substrate Ala-Ala-Phe-para-nitroanilide (AAF-pNA) at pH 7.5 and 37 °C. Glycerol and dithiothreitol (DTT) were added to the assay mixture to stabilize TPP II, and bestatin was added for inhibition of aminopeptidases, others than TPP II, in the crude lysates (paper III).

For the purification of his-tagged enzyme, a Ni sepharose 6 fast flow gel was used. This gel allows DTT in the lysate and has a reduced leakage of Ni²⁺. Unfortunately, the yield from this purification was very low with only 15% at its highest. The major reason for this appears to be a low affinity of the his-tagged enzyme for the gel, with more than 50% of the enzyme activity eluted in the flow-through. Experiments have been performed with increased gel volume with similar results, which rules out the possibility that the poor binding is due to overloading of the gel (unpublished data). In addi-
tion, there was a decrease in the total activity eluted in the fractions from the IMAC gel compared with the amount of activity loaded (paper III). This suggests a dissociation of the enzyme complex during this purification step or inhibition of the enzyme by Ni^{2+} and/or imidazol. Additional experiments have been performed with other metal ions (i.e. Zn^{2+} and Co^{2+}) without any significant change in the yield (unpublished data). Nevertheless, the 200 mM imidazol fractions from the IMAC purification contain 8-fold purified mTPP II of the right molecular size (paper III).

Despite the low yield, the expression system with *Pichia pastoris* has made it possible to purify enough recombinant TPP II for the initial characterizations. However, the system will be further optimized in terms of yield and purity.

Characterization of recombinant murine wt TPP II (paper IV)

With the expression system using *Pichia pastoris* it is now possible to express and purify recombinant TPP II. The primary aim of this investigation was to compare the recombinant expressed mTPP II with a native homologue (paper IV). For this purpose the human enzyme (hTPP II) purified from erythrocytes was chosen. There is 96% identity and 98% similarity in the amino acid sequences between the human and murine TPP II (Renn et al., 1998). In earlier studies of the kinetic properties of the enzyme from man and rat there were no differences detected (Bålöw et al., 1986). Taken together, this makes the endogenously expressed human enzyme a good model for the recombinant expressed mTPP II.

The major difference between the two enzymes is that the recombinant mTPP II is expressed with a histidine tag fused to the N-terminus. Experiments have demonstrated that the N-terminus is involved in the formation of the spindle and that a maltose-binding protein tag prevents the formation of this structure (Rockel et al., 2005). Therefore, it was important to investigate if the histidine tag in the recombinant mTPP II affects the kinetic properties of the enzyme.

The pH-optimum was determined for cleavage of the substrates AAF-pNA and Ala-Ala-Ala-pNA (AAA-pNA) respectively, in a pH interval between 6.0 and 8.8 (paper IV). Both the human and the recombinant murine enzyme showed a pH-optimum at 7.5 with AAF-pNA, but for AAA-pNA the pH-optimum was less pronounced (Fig. 10).
Figure 10. Determination of pH-optimum. The pH-optimum was determined for the cleavage of the substrates AAF-pNA A) and AAA-pNA B). These pH-profiles are obtained with the murine enzyme and agree with those obtained with the human TPP II. The ● and ■ represents the activity measured in phosphate buffer and Tris-acetate buffer, respectively.

The results obtained with the native hTPP II agree with earlier reported results (Bålöw et al., 1983) and therefore it can be suggested that the recombinant expressed mTPP II have the same pH-profile as the native enzyme for the cleavage of the two substrates tested (paper IV).

Steady-state kinetic analysis was performed for a further comparison. The $V_{\text{max}}$ and $K_M$ were determined at different values of pH (i.e. 6.5, 7.5 and 8.5) using the substrates AAF-pNA and AAA-pNA respectively (paper IV). A comparison of the apparent $K_M$ ($K_M^{\text{app}}$) values for AAF-pNA at pH 7.5 for the mTPP II and hTPP II showed that these were of the same magnitude, 15 and 45 µM respectively. The $K_M^{\text{app}}$ for hTPP II with this substrate has previously been determined to 20 µM (Tomkinson, 2000). For AAA-pNA the $K_M^{\text{app}}$ values for mTPP II and hTPP II at pH 7.5 were of the same magnitude, 6 and 11 µM respectively (paper IV). These results indicate that the his-tag present in the recombinant expresses mTPP II does not interfere with the substrate binding.

The $V_{\text{max}}^{\text{app}}$ values determined for the murine and human enzymes cannot be compared with each other since the preparation of these enzymes have different purities, and the exact amount of TPP II present cannot be determined. However, the change in $V_{\text{max}}^{\text{app}}$ from pH 6.5 to 7.5 and 7.5 to 8.5 respectively, could be calculated for each enzyme and substrate and compared. As for the $K_M^{\text{app}}$, the changes in $V_{\text{max}}^{\text{app}}$ between the different pH values were the same for the recombinant murine enzyme compared with the human enzyme (paper IV). Interesting however, was that the $V_{\text{max}}^{\text{app}}$ values obtained with AAA-pNA were approximately 3 times lower than with AAF-pNA for both enzymes.
Identification of amino acids involved in binding of the free N-terminus of the substrate (paper III)

To our knowledge there were nothing known about how TPP II, or other homologues, achieve their tripeptidyl-peptidase specificity before this investigation. Our hypothesis was that there might be a "molecular ruler" in the enzyme, which involves the binding of the free N-terminus of the substrate in the S3-site of the enzyme. The aim of this project was therefore to identify amino acids involved in this binding and propose a mechanism for how TPP II achieves its tripeptidyl-peptidase specificity (paper III).

**Alignments, computer models and site-directed mutagenesis**

The subtilisin-like N-terminus of TPP II from a large variety of species was aligned and compared with subtilisin. Since the free amino terminus of the substrate is positively charged the amino acids in the enzyme binding this part of the substrate should be negatively charged. Two negatively charged amino acids that were conserved between all TPP II sequences could be found, Glu-305 and Glu-331. To get an idea of the positions of these amino acids in the three-dimensional structure of the enzyme and also in relation to each other, a homology model of TPP II was generated with subtilisin as the template. In this model the two residues were situated opposite each other at a distance from the cleavage site, which would correspond to the S3-site in subtilisin (Fig. 11)

![Figure 11. Homology model of the subtilisin-like N-terminal part of TPP II. The catalytically important residues (Asp-44, His-264, Ser-449 and Asn-362) and Glu-305 and Glu-331 are indicated by ball-and-stick. Amino residues 55-237 were excluded from the model since these are not present in subtilisin used as template.](image-url)
Thus, Glu-305 and Glu-331 were chosen for site-directed mutagenesis. Four different mutants were generated E305Q, E305K, E331Q and E331K. The change to a glutamine (Q) results in a change from a negatively charged residue to an uncharged amide, which should lead to a weaker binding of the free N-terminus of the substrate if the hypothesis is correct. Furthermore, the change to a lysine (K) results in a change in charge from negative to positive, which should lead to an even weaker binding or repulsion of the substrate (paper III).

Characterization of TPP II mutants

The wt mTPP II and the four mutants thereof were expressed and purified from *Pichia pastoris*. Determination of the specific activity for the different enzymes proved the importance of the Glu-305 and Glu-331 residue for the enzyme activity. Compared with the wt, the mutants showed a 200 to 100 000-fold lower specific activity (paper III). The E305 mutations appear to have a larger effect on the activity than the E331 mutations and this was more pronounced when Glu was changed to Lys. It is important to note that the activities from all mutants except E331Q were extremely low and therefore there is a large uncertainty in the values obtained.

To investigate whether the differences in specific activities were an effect on substrate binding (K_M) or the reaction rate (V_max), these parameters were determined. For the wt enzyme with the substrate AAF-pNA, K_M^app was determined to 12 ± 5 µM at pH 7.5. For the E331Q and E331K mutants, there was an almost linear increase of the reaction rate up to at least 1 mM AAF-pNA. At 2 mM substrate concentration the enzyme was not yet saturated and at even higher concentrations the substrate precipitated. Therefore, a K_M^app could not be determined, but the measurements indicated that this value should be well above 1 mM (paper III). Instead, (V_max/K_M)^app was determined for comparison of the wt and mutants. The results showed that the catalytic efficiency with AAF-pNA for E331Q was approximately 4000 times lower compared with the wt enzyme, and even lower for the other mutants (i.e. E331K, E305Q and E305K) (paper III).

The effect of different inhibitors on the enzyme activity of the wt, E331Q and E331K was also investigated (paper III). Butabindide is a specific competitive inhibitor of TPP II, designed to bind the active site (Rose et al., 1996). For the wt the K_i^app was calculated, from IC_{50}, to 40 nM and 110 nM at 200 and 20 µM AAF-pNA, respectively. Assuming a K_M^app of 1 mM for E331Q, the K_i^app was estimated to over 140 µM. These results revealed that butabindide cannot bind to E331Q as efficiently as to the wt. To test if this is due to the binding of the free amino group present in butabindide (Rose et al., 1996), or an effect on other interactions in the binding pocket, another inhibitor was investigated.

The octapeptide Val-Leu-Arg-Arg-Ala-Ser-Val-Ala is an efficient competitive inhibitor of TPP II (Bålòw et al., 1986). The corresponding peptide
without a free N-terminus, isovaleryl-Leu-Arg-Arg-Ala-Ser-Val-Ala does not inhibit the enzyme to any larger extent (Bålöw et al., 1986). As expected, the octapeptide efficiently inhibited the wt TPP II, whereas there was no detectable inhibition from the octapeptide without a free N-terminus at 200 µM AAF-pNA. With 20 µM AAF-pNA, inhibition could be seen but the IC50 value exceeded 1 mM. This demonstrates the importance of a free N-terminus of the inhibitor for binding to the enzyme. In contrast to this, both peptides inhibited E331Q to the same extent. The K_i values were calculated to 1 µM for the octapeptide with wt and >2 mM with E331Q, which clearly shows a role for the E331 residue in binding of the amino terminus.

The inhibition of E331K was measured at one inhibitor concentration, 100 µM for butabindide and 2 mM for the peptides. No inhibition was detected with either butabindide or the octapeptide without a free N-terminus, but the octapeptide reduces the enzyme activity with about 20% at the inhibitor concentration 2 mM.

All results confirm that a free N-terminus of the substrate or inhibitor is important for the binding to the active site of the enzyme. Moreover, it has been demonstrated that at least Glu-331 is involved in the binding of the free N-terminus. Since E305Q and E305K are even less active than the E331 mutants, the E305 residue would be expected to be more important for the substrate binding. However, because of the difficulties to detect any enzyme activity for the E305 mutants with the assay used, it cannot be ruled out that other factors (e.g., dissociation of the complex) may affect the enzyme activity negatively.

Comparison of substrate specificity for TPP II from different species (paper IV)

The different pH-profiles obtained for the cleavage of AAF-pNA and AAA-pNA with mTPP II and hTPP II was unexpected. To investigate whether it was specific for the murine and human enzymes or include all homologues, the TPP II from Drosophila melanogaster (dTPP II) was tested. The amino acid sequence for this enzyme is only 38% identical to the human enzyme (Renn et al., 1998). As mentioned earlier, the subunit of dTPP II is also larger, 150 kDa compared with 138 kDa for the human and murine TPP II. Despite these differences the pH-profile for dTPP II was the same as for the murine and human homologues for both substrates (paper IV).

The determination of steady-state kinetic parameters was performed with mTPP II, hTPP II and dTPP II. Consistent with previous reports, the K_M values with AAF-pNA for dTPP II was almost 10 times higher at pH 7.5 compared with the two other enzymes (Renn et al., 1998; Seyit et al., 2006). Also for dTPP II, the K_M values with AAA-pNA were considerably lower than with AAF-pNA.
Since both the $K_M^{\text{app}}$ values and the $V_{\text{max}}^{\text{app}}$ values are decreased with AAA-pNA compared with AAF-pNA this results in almost the same $(V_{\text{max}}/K_M)^{\text{app}}$ values for both substrates with each enzyme (i.e. mTPP II, hTPP II and dTPP II) (paper IV). A comparison of the change of each parameter with pH for the two substrates and the three enzymes indicates that the pH-profiles obtained for the cleavage of respective substrate are affected by both $K_M$ and $V_{\text{max}}$ (paper IV).

Discussion

Transcriptional regulation of $TPP2$ (papers I and II)

Mutations of the CCAAT-boxes and the E-box, one at a time or in combination, in the 215 bp promoter demonstrated that the CCAAT-boxes were the most important sequence elements for transcriptional activation and additional mutations in the E-box did not further affect the transcription level (paper II). However, even though both CCAAT-boxes and the E-box were mutated at the same time, the 215 bp promoter could activate transcription approximately 50-fold compared with the reporter vector without insert. Using EMSA, no DNA-protein complexes could be detected with the 215 bp fragment with the triple mutations (paper II). The remaining promoter function may be due to the binding of the preinitiation complex to the Inr sequence element. In the absence of a TATA-box or other upstream elements, the general transcription factor TFIID can bind to the Inr via the TAF-subunits and initiate the assembly of the preinitiation complex. This has been shown to be sufficient for activation of the transcription (Weis and Reinberg, 1997). Experiments have also demonstrated that in the absence of an upstream TATA-box this DNA-protein complex cannot be detected using EMSA (Smale, 1997). Another explanation for the remaining promoter activity may be that the 215 bp promoter can still bind NF-Y and/or USF-1, despite the mutations albeit with very low affinity. If this is the case, a DNA-protein complex would presumably not be detected with EMSA.

The role of USF-1 in the transcriptional regulation of $TPP2$ is still unclear. It cannot be ruled out that USF-1 may be involved in regulation in other cell types or under other conditions than tested. Furthermore, there may also be sequence elements downstream or upstream of the 215 bp promoter, which are crucial for the function of USF-1.

The transcription factor Oct-1 was found to bind one of the upstream fragments that inhibit the transcription of the TPP II gene (paper II). Amongst the other consensus sequences tested, the consensus oligonucleotide for TGIF, GATA and NF-Y respectively, could decrease the formation of DNA-protein complex when added in 100-fold molar excess for competi-
tion in EMSA. This may suggest that these transcription factors bind, albeit a weak binding to the 5'-end of the 8 kb fragment.

Oct-1 is able to both activate and repress transcription. Depending on the recognition sequence Oct-1 can adopt different conformations (Ogbourne and Antalis, 1998). This results in recruitment of different co-factors and thereby different effects on the transcription of the particular gene (Ogbourne and Antalis, 1998). It has been demonstrated that Oct-1 protein levels are increased due to cellular stress and serum withdrawal (Tantin et al., 2005), which are conditions where TPP II also has been reported to be upregulated (Wang et al., 2000; Hong et al., 2003). Also the transcription factors NF-Y (i.e. NF-YA) upregulates during genotoxic stress and both NF-Y and Oct-1 are necessary for the activation of the GADD45 promoter in response to DNA damage (Jin et al., 2001). Even though Oct-1 activates the GADD45 promoter during stress, it is possible that Oct-1 can repress the transcription of *TPP2* under the same conditions.

Many genes coding for proteins involved in the cell cycle are regulated by NF-Y (Frontini et al., 2004). NF-Y consists of three subunits, NF-YA, NF-YB and NFYC, which are all required for protein function. Both NF-YA and NF-YB are present in the nucleus whereas NF-YC is present also in the cytosol. During cell cycle progression, the levels of NF-YC in the nucleus changes and thereby alter the effect of NF-Y on different promoters (Frontini et al., 2004). Since overexpression of TPP II appears to promote the cells to progress through mitosis (Stavropoulou et al., 2006), *TPP2* may be regulated by NF-Y in a cell cycle dependent manner.

Similar to Oct-1 and NF-Y, USF-1 is upregulated in response to stress but also involved in cell cycle regulation as NF-Y (Corre and Galibert, 2005). However, the function of these transcription factors in the regulation of *TPP2* needs to be further investigated.

The overexpression of c-Myc in BL-like cells resulted in increased levels of TPP II (Gavioli et al., 2001). An investigation of the effect of c-Myc on the transcription of *TPP2* was therefore started together with Maria Masucci and her co-workers at the Karolinska Institute in Stockholm. Furthermore, in collaboration with Rickard Glas at the Karolinska Institute in Stockholm, we investigated the regulation of *TPP2* during starvation. Finally, in collaboration with Per-Olof Hasselgren and his co-workers at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston we investigated the regulation of *TPP2* in muscle cells treated with glucocorticoids. However, these projects have not been successful so far, primarily due to difficulties in setting up proper cell systems to use as models.
Investigation of substrate specificity for TPP II (papers III and IV)

The expression and purification system developed using *Pichia pastoris* have enabled us to purify recombinant TPP II without any interference from endogenously expressed enzyme (paper III). It could be demonstrated that the recombinant expressed His-tagged wt mTPP II had the same enzymatic properties as the native enzyme with respect to pH-optimum for the cleavage of AAF-βNA and AAA-βNA and *K_m* for the same substrates (paper IV).

It has clearly been demonstrated that Glu-305 and Glu-331 are involved in the binding of the free N-terminus of the substrate. The four mutants used for the investigation, E305Q, E305K, E331Q, and E331K, had a considerably lower (i.e. 200-100 000 fold) specific activity with AAF-βNA compared with wt TPP II (paper III). The degree of dissociation of the enzyme complex varied between the different preparations. It has previously been shown that the dissociated complex (i.e. dimers) has 1/10 of the activity compared with the whole complex (Tomkinson, 2000; Seyit et al., 2006). Furthermore, *K_m* has also been determined for the dissociated dTPP II with a two-fold decrease as maximum (Seyit et al., 2006). From this it could be concluded that, even if the degree of dissociation varies between the preparations, the 

\[
\frac{V_{\text{max}}}{K_m}^{\text{app}}
\]

can only be affected with a factor twenty as highest. The extremely low activity of the mutants, and particular the E305 mutants, have made it difficult to determine any kinetic parameters without large uncertainties. However, since the differences in kinetic properties between the wt and mutants are so large, the effect of dissociation will not change the main conclusions.

The inhibition studies were performed with the competitive inhibitor Val-Leu-Arg-Arg-Ala-Ser-Val-Ala for the wt enzyme and E331Q with the corresponding octapeptide without an N-terminus, isovaleryl-Leu-Arg-Arg-Ala-Ser-Val-Ala as a control (paper III). The results clearly demonstrate the importance of a free amino terminus of the substrate or competitive inhibitor.

The specific TPP II inhibitor butabindide was used in experiments with wt, E331Q and E331K and *K_i^{\text{app}}* was more than three orders of magnitude higher for E331Q compared with wt. For E331K there was no detectable inhibition. This suggests that butabindide binds with its free amino group in a position corresponding to the P3-site of a substrate, which also was suggested by Rose et al. (1996). However, these results speak against the interaction model suggested by De Winter and co-workers which propose interaction between the free amino group of butabindide and Asp-44 in the catalytic triad (De Winter et al., 2005). If their hypothesis was correct there would be no differences in inhibition of the mutants compared with wt since the catalytic triad was unchanged.

Our results suggest that Glu-305 and Glu-331 may be responsible for the tripeptidyl-peptidase specificity of TPP II. They could be positioned at a
distance from the active site corresponding to a tripeptide, thus function as a "molecular ruler". A similar mechanism has recently been proposed for the substrate binding in prolyl tripeptidyl aminopeptidase, based on determination of the crystal structure for the enzyme (Ito et al., 2006).

All three enzymes (i.e. recombinant mTPP II, hTPP II and dTPP II) investigated showed different pH profiles for the hydrolysis of AAF-pNA and AAA-pNA respectively. There was a clear pH-optimum at 7.5 for the cleavage of AAF-pNA whereas the cleavage of AAA-pNA displayed a more flat pH profile with no distinct pH-optimum (paper IV). By determining $K_M$, $V_{max}$ and $V_{max}/K_M$ it could be seen that differences in both $K_M$ and $V_{max}$ affect the pH profile. For AAA-pNA both $K_M$ and $V_{max}$ were lower compared with AAF-pNA for all enzymes but the $V_{max}/K_M$ was therefore unaffected with the same value as for AAF-pNA. This may be explained by a non-productive binding of AAA-pNA to the enzyme. However, this will be subject to further investigations.
Conclusions and future perspectives

From the investigation of the transcriptional regulation of *TPP2*, it can be concluded that the 215 bp promoter contains two inverted CCAAT-boxes, to which the transcription factor NF-Y binds that are crucial for increasing the transcription of *TPP2* from the promoter. There is also an E-box, which binds USF-1, present in the promoter but the function of this sequence element is unclear so far. Further upstream of the 215 bp promoter several silencer elements have been identified. The transcription factor Oct-1 has been found to bind one of these elements. All three transcription factors identified are involved in cellular processes in which TPP II has been reported to be upregulated. However, the specific role of these factors in the regulation of *TPP2* remains to be investigated.

Furthermore, the regulation of *TPP2* in different cell model systems may be further investigated through the already initiated collaborations. Hopefully, better cellular system will be identified. So far, only the 215 bp and 3 kb reporter constructs were tested in the muscle cells treated with glucocorticoids. There are a large number of other reporter constructions that may be interesting to investigate, especially since there seem to be an altered regulation in these cells compared with both HEK-293 cells and NIH3T3 cells. The upregulation of TPP II during starvation in EL4 lymphoma cells may also be further investigated. Here it would be interesting to investigate the regulation on different levels also downstream the gene transcription. It would also be interesting to continue the investigation of the role of c-Myc in the transcriptional regulation of *TPP2*.

In the second part of this thesis, an expression and purification system using *Pichia pastoris* has been developed. The recombinant His-tagged wt mTPP II purified from this system was shown to have the same kinetic properties as the native enzyme. Thus, the yeast cell appears to be a god host for expression of recombinant TPP II. Seyit and co-workers have developed a method for expression of recombinant dTPP II in *E. coli* (Seyit et al., 2006), where they dissociate the enzyme complex prior to purification. This will be considered in further optimization of the yeast system.

The expression system enabled us to obtain enough of the mTPP II and four mutants thereof to investigate the mechanism behind the tripeptidyl-peptidase specificity of the enzyme. It was demonstrated that Glu-305 and Glu-331 are involved in binding of the free N-terminus of substrates. This
substrate binding could therefore function as a "molecular ruler" and ensure that the third peptide bond is cleaved and a tripeptide is removed.

Before this thesis there have not been any reports, to our knowledge, about the transcriptional regulation of the TPP II gene, neither about the mechanism behind the tripeptidyl-peptidase specificity of the enzyme. Therefore, the results presented have contributed to an increased and important knowledge about TPP II. Through the work to characterize the TPP2 promoter, a foundation has been laid for further investigations of the transcriptional regulation of the TPP II gene. Another important project was the development of the yeast expression system. This makes it possible to purify recombinant TPP II for structure-function investigations. After further optimizations, the yield may be increased to such an extent that enough enzyme for crystallization experiments can be obtained. A crystal-structure would be invaluable for continued structure-function studies. The increased knowledge about the function and regulation of TPP II may also result in a better understanding of the physiological role of the enzyme.
Summary in Swedish

Tripeptidylpeptidas II – struktur, funktion och genreglering

Den här avhandlingen handlar om enzymet tripeptidylpeptidas II (TPP II). Jag har dels tittat på vad som styr hur mycket av enzymet som bildas i cellerna, dels har jag tittat på hur enzymet arbetar, framför allt varför det alltid frisätter tre aminosyror i taget när det bryter ner peptider (proteinbitar).

Inledning

Förmågan att kunna bryta ner proteiner är livsviktig för en cell. Gamla och skadade proteiner måste hela tiden bytas ut mot nya, innan de förlorar sin funktion. Genom nedbrytningen frigörs de byggstenar, aminosyror, som används vid syntesen av de nya proteinererna. Det är inte bara gamla och skadade proteiner som bryts ner utan även proteiner vars funktion inte behövs i cellen längre. Detta gäller till exempel proteiner som ingår i cellcykeln och som bara behövs vid vissa steg i processen och det kan till och med vara skadligt för cellen om dessa finns kvar.

I cellen sker proteindubbrytningen huvudsakligen i cellens lysosomer och i cytosolen. Lysosomer är vesiklar som innehåller ett 40-tal olika hydrolytiska enzymer, s.k. hydrolaser, som har till uppgift att bryta ner andra proteiner. Inne i lysosomerna är pH-värdet omkring 5, vilket bland annat hjälper till att denaturera de proteiner som ska brytas ner. Nedbrytningen i lysosomerna regleras främst genom upptaget av proteiner. Väl inne i lysosomen bryts proteinererna ner inom 5-10 min.

I cytosolen är inte proteaserna inkapslade i vesiklar och därmed inte skilda från cellens organeller eller andra proteiner. Detta kräver att den cytosoliska proteindubbrytningen är strikt reglerad och mycket specifik. Proteiner som ska brytas ner märks med polyubiquitin och blir därmed ett mål för 26S proteasomen, vilket är det huvudsakliga proteaset i cytosolen. Det är ett stort enzymkomplex som består av en cylinderformad 20S proteasom och en regulatorisk enhet, 19S, i var ände av cylindern. Den regulatoriska enheten känner igen de proteiner som märks med ubiquitin och binder dessa för att
sedan veckla ut dem och mata in dem i cylindern för nedbrytning. Proteiner-
na bryts ner till oligopeptider med en genomsnittslängd på 7-9 aminosyror.
Dessa bryts sedan vidare ner till fria aminosyror av olika andra proteaser,
däribland tripeptidylpeptidas II, som har studerats i den här uppsatsen.

Tripeptidylpeptidas II

Tripeptidylpeptidas II (TPP II) är, liksom proteasomen, ett stort enzymkom-
plex där varje subenhet väger 138 kDa och hela komplexet mer än 4 MDa.
TPP II är ett serinproteas av subtilisintyp och bryter ner oligopeptider, från
den fria N-terminalen, till tripeptider. Enzymet har en mycket bred substrat-
specificitet och finns i många olika eukaryota celler från olika organismer.
Förutom den nog så viktiga funktionen att bryta ner gamla och skadade pro-
teiner för att ersätta dessa med nya, så verkar TPP II vara inblandat i mer
specifika processer. Cholesystokinin-8 (CCK-8) är en neuropeptid som bland
annat påverkar mättnadskänslan. Inaktiveringen av CCK-8 har visat sig vara
beroende av TPP II. Den N-terminala trimningen av vissa peptider för anti-
gen presentation verkar bara kunna utföras av TPP II. Det har också visat sig
att mängden protein kan variera i vissa celler under vissa förhållanden. TPP
II är uppreglerat i muskelceller vid sepsis då det i dessa också sker en ökad
nedbrytning av muskelproteiner. Denna ökade muskelnedbrytning och ök-
ning av TPP II har också observerats vid cancer. Vissa tumörceller, som har
en nedsatt proteasom funktion, har ett ökat uttryck av TPP II vilket ser ut att
gå dessa celler en överlevnadsfördel jämfört med de celler som inte överut-
trycker enzymet. Rapporter som nyligen kommit ut visar att TPP II troligtvis
också, om det överuttrycks, kan leda till genetiska skador.

TPP II har en viktig funktion i den intracellulära proteinnefyrkningen
och dess roll i inaktiveringen av CCK-8 har gjort att enzymet blivit ett mål
för läkemedelsdesign. Både Ganellin och De Winter med medarbetare arbe-
tar för att ta fram inhibitorer för TPP II. För detta arbete är det speciellt vik-
tigt att känna till enzymets struktur, funktion och dess fysiologiska roll i
cellen vilket gör att fortsatt forskning om TPP II är viktig.

Mitt arbete

Transkriptionsreglering av TPP II genen (delarbete I och II)
Eftersom både mRNA- och proteinnivåerna av TPP II ökar i vissa celler
under vissa förhållanden är det intressant att studera vad denna ökning beror
på och vilken funktion TPP II har i cellen under dessa förhållanden. Därför
påbörjades arbetet med att identifiera TPP II genens promotör. Med hjälp av
e en luciferas-reportervektor kunde promotorn identifieras inom 215 bp upp-
ströms om den kodande sekvensen. Promotorn saknar en TATA-box men
innehåller i stället en(initiator (Inr) och dessutom två inverterade CCAAT-
boxar och en E-box. Med hjälp av gel-skiftförsök har jag visat att transkrip-

**Undersökning av TPP IIIs substratspecificitet (delarbete III och IV)**

Det finns endast ett fåtal proteaser, förutom TPP II, som uppvisar tripeptidylpeptidas-specificitet. För att kunna studera mekanismen bakom denna specificitet behövdes först ett expressionssystem för rekombinant TPP II. Ett system med jästcellen *Pichia pastoris* utvecklades därför. En karaktärisering gjordes av rekombinant vildtyps-enzym från mus (wt mTPP II) som renats fram från jästcellerna. Undersökningen visade att det rekombinanta mTPP II kan antas ha samma enzymatiska egenskaper som det nativa enzymet.


Den enzymatiska karaktäriseringen av det rekombinanta wt mTPP II omfattade bland annat bestämning av pH-optimum för hydrolysen av både AAF-pNA och AAA-pNA. Det visade sig att pH-profilerna för de båda substraten skilde sig åt. För klyvningen av AAF-pNA var det ett tydligt pH-optimum omkring 7.5 medan klyvningen av AAA-pNA upphävade en mer flack pH-profil. Både $K_M$ (substratbindningen) och $V_{max}$ (reaktionshastighe-
ten) för AAA-pNA är lägre jämfört med AAF-pNA vilket kan bero på en icke-produktiv bindning av det substratet till enzymet. Detta behöver dock undersökas vidare.

Slutsatser


Alla tre transkriptionsfaktorerna, NF-Y, USF-1 och Oct-1, är inblandade i regleringen av gener i celler som utsätts för stress av olika slag och/eller i regleringen av cellcykeln som också är sådana situationer där TPP II mängden ökar. Exakt hur det går till måste dock undersökas vidare.

Ett expressionssystem med Pichia pastoris har också utvecklats för att renna fram rekombinant TPP II. Det rekombinanta enzymet framenrat från detta system verkar uppvisa samma enzymatiska egenskaper som det nativa. Med hjälp av detta system renades fyra mutanter av mTPP II fram, E305Q, E305K, E331Q och E331K för att undersöka dessa två aminosyraresters funktion i substratbindningen. Undersökningen visade tydligt att båda resterna är viktiga för inbindningen av den fria amino-terminalen hos substratet eller en konkurrensämmare och bestämmer därmed också tripeptidylpeptidases-specificiteten för enzymet.

Möjligheten att nu kunna uttrycka och renna fram rekombinant TPP II öppnar många vägar för vidare studier av enzymets struktur och funktion och samhänd därmed.
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References


Hartley H. (1951) Origin of the Word 'Protein'. Nature 168, 244.


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