

Linköping University
Medical Dissertations
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PEPTIDYL-PROLYL *CIS-TRANS* ISOMERASES IN THE CHLOROPLAST THYLAKOID LUMEN

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Linköping 2007

Cover

Graphically manipulated picture of *Arabidopsis thaliana* cultivated by the author.
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ISBN 978-91-85715-76-3

ISSN 0345-0082

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Printed in Sweden by LTAB Linköpings Tryckeri AB, 2007.907

Till min familj

ABSTRACT

The Sun is the ultimate energy source on Earth. Photosynthetic organisms are able to catalyze the conversion of solar energy to chemical energy by a reaction called photosynthesis. In plants, this process occurs inside a green organelle called the chloroplast. The protein complexes involved in the photosynthetic light reactions are situated in the thylakoid membrane, which encloses a tiny space called lumen. The Peptidyl-Prolyl *cis-trans* Isomerase (PPIase) family is the most abundant protein family in the thylakoid lumen. The three PPIase subfamilies, cyclophilins, FKBP (FK506 binding proteins) and parvulins form a group by their enzymatic activity despite lack of sequence similarity between the subfamilies. Cyclophilins and FKBP, collectively called immunophilins, were originally discovered as the targets of the immunosuppressive drugs cyclosporine A and FK506, respectively. By suppressing the immune response in humans, these immunophilin-drug complexes revolutionized the field of organ transplantation by preventing graft rejection. *Cis-trans* isomerization of peptide bonds preceding the amino acid proline is the rate-limiting step of protein folding and several immunophilins have been shown to be important for catalysis of protein folding *in vivo*. PPIases have been found to be part of large protein complexes as well as in functions such as signalling, protein secretion, RNA processing and cell cycle control. A picture is therefore emerging in which the actual interaction between the PPIase and its target is perhaps more important than the PPIase activity.

In the present work, PPIases have been characterized in the chloroplast thylakoid lumen of *Spinacia oleracea* (spinach) and *Arabidopsis thaliana* (Arabidopsis). The most active PPIase in the spinach lumen was identified as the cyclophilin TLP20. AtCYP20-2, the Arabidopsis homologue of TLP20, was found to be upregulated at high light and attached to the thylakoid membrane, more precisely to the outer regions of photosystem II supercomplexes. In Arabidopsis, up to 5 cyclophilins and 11 FKBP were predicted to reside in the lumen. Of these 16 immunophilins, only 2 were identified as active PPIases and significant differences were observed between the two plant species. AtCYP20-2, like TLP20, is an active isomerase although AtFKBP13 is the most active PPIase in the lumen of Arabidopsis. Mutant Arabidopsis plants deficient in AtCYP20-2 displayed no phenotypical changes or decrease in total luminal PPIase activity. Being the only active PPIase in the mutants, the redox sensitive AtFKBP13 is proposed to compensate for the lack of AtCYP20-2 by oxidative activation. In agreement with the experimental data, the sequence analyses of catalytic domains of luminal immunophilins demonstrate that only AtCYP20-2 and AtFKBP13 possess the amino acids found essential for PPIase activity in earlier studies of human cyclophilin A and FKBP12. It is concluded that with

the exception of AtCYP20-2 and AtFKBP13 most immunophilins in the lumen of Arabidopsis lost their PPIase activity on peptide substrates and developed other specialized functions.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Solen är den ultimata energikällan för livet på jorden. Vi kan utnyttja denna energi tack vare att organismer som växter, alger och cyanobakterier kan omvandla solenergin till kemisk energi via en process som kallas fotosyntes. I växter sker fotosyntesen i kloroplasten, vilken innesluter den lösliga stroman och det s.k. tylakoidmembranet. De proteinkomplex (bl.a. fotosystem I och II) som är involverade i fotosyntesen sitter i tylakoidmembranet vilket i sin tur omsluter ett litet utrymme som kallas lumen.

Förutom att fotosyntesen tillvaratar solens energi så frisätts även syre som en biprodukt. Den bundna energin, i form av energirika molekyler, används för att fixera koldioxid från luften och producera kolhydrater. Forskning kring dessa grundläggande processer har, förutom mer insikt om hur reaktionerna fungerar och regleras, även praktiska och ekonomiska aspekter. Med ökad kunskap och bättre tekniker skulle t.ex. tillväxten av viktiga grödor kunna ökas samt effektiviteten för artificiell fotosyntes förbättras.

I växtcellen, och alla andra celler, är det i själva verket proteiner som utför de flesta reaktionerna. Proteiner består av mindre byggstenar, s.k. aminosyror, som sätts ihop till långa kedjor. Stället där två aminosyror sitter ihop kallas för peptidbindning och genom att vrida på dessa bindningar kan kedjan veckas ihop som ett litet nystan och proteinet får sin tredimensionella struktur. Beroende på hur två aminosyror är arrangerade runt peptidbindningen säger man att de är i *cis* eller *trans* konformation. Rotation (*cis-trans* isomerisering) kring en peptidbindning framför aminosyran prolin (peptidyl-prolyl) är det hastighetsbegränsande steget när proteiner veckas till sin tredimensionella struktur.

Peptidyl-Prolyl *cis-trans* Isomeraserna (PPIaser) är en proteinfamilj som kan katalysera denna reaktion och är därför viktiga vid av proteinveckning i cellen. Det finns tre underfamiljer av PPIaser: cyklofiliner, FKBP (FK506 bindande proteiner) och parvuliner. Trots samma enzymatiska (katalytiska) aktivitet och liknande aminosyrasekvens inom varje underfamilj skiljer sig sekvensen mycket mellan de olika familjerna. Cyklofiliner och FKBP som tillsammans kallas för immunofiliner upptäcktes ursprungligen som receptorer (mottagare) för de immunsuppressiva läkemedlen cyclosporin A och FK506. Dessa läkemedel revolutionerade transplantationskirurgin genom nedsättning av immunsystemets funktion vilket förhindrar avstötning av transplanterade organ. PPIaser är också del av större proteinkomplex samt involverade i andra funktioner som t.ex.: signalering i cellen; bearbetning av RNA samt kontroll av cellcykeln. En bild börjar därför formos där kanske det viktigaste är själva interaktionen mellan PPIaset och dess partner och inte den enzymatiska PPIas aktiviteten.

Tidigare studier har visat att PPIaserna är den största proteinfamiljen i lumen. I detta avhandlingsarbete har både spenat och *Arabidopsis thaliana* (Arabidopsis) använts för att studera dessa lumen PPIaser. Arabidopsis, backtrav på svenska, är egentligen ett ogräs men används ofta som modell när man vill studera växter. En cyklofilin som döptes till TLP20 visade sig vara det mest aktiva PPIaset i spenatlumen. Vidare karaktärisering av homologen (motsvarande protein) till TLP20 i Arabidopsis, AtCYP20-2, visade på uppreglering av proteinet vid ökad ljusstyrka. Dessutom interagerar AtCYP20-2 med lumensidan av tylakoidmembranet, mer specifikt vid de yttre regionerna av fotosystem II. Baserat på tidigare studier av lumen samt jämförelse av aminosyrasekvensen hos alla PPIaser i Arabidopsis tror man att det finns så många som 5 cyklofiliner och 11 FKBP i lumen. Trots det stora antalet är endast två av dessa aktiva isomeraser och skillnader observerades mellan de båda växterna. AtCYP20-2, liksom TLP20, är ett aktivt isomeras även om det redox-reglerade enzymet AtFKBP13 är det mest aktiva PPIaset i lumen på Arabidopsis. Muterade Arabidopsisplantor som saknar AtCYP20-2 visade ingen fenotypisk (utseende) skillnad eller minskning av den totala PPIas aktiviteten. En hypotes är därför att AtFKBP13, det enda aktiva PPIaset i dessa plantor, kompenserar för avsaknaden av AtCYP20-2 genom oxidativ aktivering. Analys av aminosyrasekvensen i de katalytiska domänerna visar att endast AtCYP20-2 och AtFKBP13 har de aminosyror som studier av de mänskliga immunofilinerna, cyklofilin A och FKBP12, visat vara nödvändiga för PPIas aktivitet. Detta stöder de experimentella resultaten och slutsatsen blir att, med undantag av AtCYP20-2 och AtFKBP13, har immunofilinerna i lumen på Arabidopsis förlorat sin PPIas aktivitet mot peptidsubstrat och utvecklat andra specifika funktioner.

ORIGINAL PUBLICATIONS

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

- I. **Edvardsson, A., Eshaghi, S., Vener, A.V. and Andersson, B. (2003)**
The major peptidyl-prolyl isomerase activity in thylakoid lumen of plant chloroplasts belongs to a novel cyclophilin TLP20
FEBS Letters, 542(1-3): 137-41
- II. **Romano, P.G.N., Edvardsson, A., Ruban, A.V., Andersson, B., Vener, A.V., Gray, J.E., and Horton, P. (2004)**
Arabidopsis AtCYP20-2 is a light-regulated cyclophilin-type peptidyl-prolyl *cis-trans* isomerase associated with the photosynthetic membranes
Plant Physiology, 134(4): 1244-1247
- III. **Shapiguzov, A., Edvardsson, A. and Vener, A.V. (2006)**
Profound redox sensitivity of peptidyl-prolyl isomerase activity in Arabidopsis thylakoid lumen.
FEBS Letters, 580(15): 3671-3676
- IV. **Edvardsson, A., Shapiguzov, A., Petersson, U.A., Schröder, W.P. and Vener, A.V. (2007)**
Knockout of AtCYP20-2 confirms degeneration of peptidyl-prolyl isomerase activity of immunophilins in the thylakoid lumen of *Arabidopsis thaliana*
Submitted

Other publications

Andersson, A., Eshaghi, S., Weber, P., Herrmann, R.G., Vener, A.V. and Andersson, B. (2001) Biochemical characterization of two thylakoid luminal cyclophilins – TLP20 and TLP40. *Proceedings of the 12th International Congress on Photosynthesis, S24-016*, CSIRO Publishing, Melbourne, Australia

ABBREVIATIONS

A-substrate	Succinyl-Alanine-Alanine-Proline-Phenylalanine-4-Nitroanilide
ATP	Adenosine 5'-triphosphate
CaM	Calmodullin
CN	Calcineurin
CsA	Cyclosporin A
CyPA	Human cyclophilin A
Cyt b ₆ f	Cythochrome b ₆ f complex
<i>E. coli</i>	<i>Escherichia coli</i>
FKBP	FK506 Binding Protein
L-substrate	Succinyl-Alanine-Leucine-Proline-Phenylalanine-4-Nitroanilide
LHCII	Light Harvesting Complex of photosystem II
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate (oxidized)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NFAT	Nuclear Factor of Activated T cells
PC	Plastocyanin
PSI	Photosystem I
PSII	Photosystem II
PPIase	Peptidyl-Prolyl <i>cis-trans</i> Isomerase
TPR	Tetratrico Peptide Repeat
Trx	Thioredoxin

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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FROM ORGAN TRANSPLANTATION TO REGULATION OF PHOTOSYNTHESIS

Introducing the peptidyl-prolyl *cis-trans* isomerase (PPIase) family

The story about this protein family began in the early 1970s when a cyclic undecapeptide named cyclosporin A (CsA) was discovered. It was produced in the fungus *Tolypocladium inflatum* and showed a mild antifungal and antibiotic activity. Later it was found that CsA also reduced the immune response in humans and it was approved as a drug for prevention of transplant rejection. In 1984 Handschumacher and co-workers identified the cellular target of CsA from bovine thymus and named the protein Cyclophilin (Handschumacher et al., 1984). In the same year, a protein purified from porcine kidney showed ability to catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds (Fischer et al., 1984). This protein was classified as the first member of the peptidyl-prolyl *cis-trans* isomerase (PPIase) enzyme class (EC 5.1.2.8.). The isomerization of a peptidyl-prolyl bond is illustrated in Figure 1. It was not until 1989 that two independent groups realized that Cyclophilin also possessed isomerase activity, which could be inhibited by CsA, and that PPIase and cyclophilin in fact were the identical protein (Fischer et al., 1989; Takahashi et al., 1989). At the same time a functionally related protein was found to interact with another immunosuppressive drug, FK506 extracted from *Streptomyces tsukubaensis*, and was named FK506-binding protein (FKBP; Harding et al., 1989; Siekierka et al., 1989). The FK506 related substance rapamycin also displays immunosuppressive actions in complex with FKBP (Schreiber, 1991) and inhibits the PPIase activity. Cyclophilins and FKBP are collectively called immunophilins due to their ability to suppress the immune response in the presence of corresponding inhibitors.

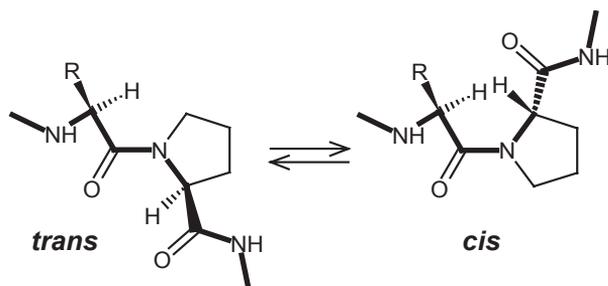


Figure 1. Schematic illustration of *cis-trans* isomerization of a peptidyl-prolyl bond. The peptide backbone is indicated with thick lines.

The Calcineurin/NFAT pathway and mechanism of immunosuppression

The discovery of human cyclophilin A (CyPA), FKBP12 and their respective immunosuppressive ligands triggered intensive research into their cellular functions. Even though the PPIase activity of each immunophilin is inhibited by its specific drug, the inhibition of PPIase activity is unrelated to the immunosuppressive action of these drugs. The mechanism by which FKBP12-FK506 and CyPA-CsA complexes, but not their separate components (Liu et al., 1991), exert immunosuppressive actions on T-cells have been described in a number of reviews (Schreiber and Crabtree, 1992; Rao et al., 1997; Martinez-Martinez and Redondo, 2004) and a simplified summary of this mechanism is described below (see also Figure 2).

Antigen recognition by T-cell surface receptors triggers a complex signal cascade that results in activation of a tyrosine kinase, which in turn phosphorylates and activates phospholipase C. Phospholipase C then cleaves phosphatidylinositol 4,5-bisphosphate into the second messengers inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Binding of inositol 1,4,5-triphosphate to a receptor localized in the endoplasmic reticulum results, via several steps, in influx of extracellular Ca^{2+} . The increased levels of Ca^{2+} activate calcineurin (CN), a Ca^{2+} /calmodulin (CaM) dependent serine-threonine phosphatase, composed of the catalytic subunit calcineurin A and a regulatory subunit termed calcineurin B.

One of the best characterised substrates of CN is the NFAT (nuclear factor of activated T-cells) family. In the cytoplasm of unstimulated cells, these transcription factors are present in an inactive, extensively phosphorylated, state. Activated CN dephosphorylates serine/proline repeats of NFAT proteins, which unmask nuclear localization sequences (NLS) necessary for translocation of NFATs to the nucleus. Although CN is mainly situated in the cytoplasm, it may be transported into the nucleus in association with NFAT (Shibasaki et al., 1996).

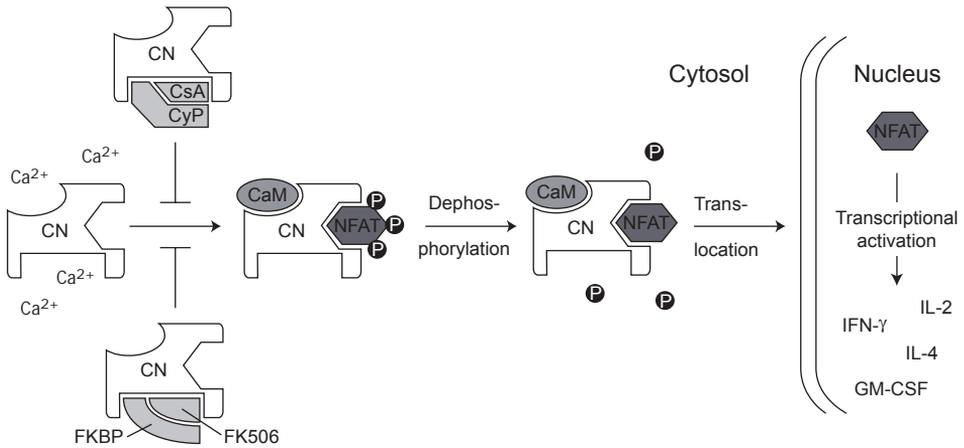


Figure 2. Simplified model of the immunosuppression mechanism. Recognition of an antigen on the T-cell surface triggers a complex signal cascade that results in increased intracellular levels of Ca^{2+} . This activates calcineurin (CN), a Ca^{2+} /calmodulin (CaM) dependent phosphatase, which in turn dephosphorylates NFAT (nuclear factor of activated T-cells). Dephosphorylation unmasks nuclear localization sequences necessary for translocation of NFAT to the nucleus. When NFAT reaches the nucleus the transcription of several early T cell activation genes such as interleukin 2 and 4 (IL-2, IL-4), granulocyte-macrophage colony-stimulating factor (GM-CSF), and γ -interferon (INF- γ) is upregulated. Binding of FK506-FKBP12 or CsA-CyPA complex to CN inhibits the phosphatase activity. NFAT is neither dephosphorylated nor translocated to the nucleus, which interrupts the formation of a proper immune response. (Modified from Göthel and Marahiel, 1999).

The calcineurin/NFAT pathway is used for cell signalling and a variety of functions in different cell types. When NFATs reach the nucleus of immune cells they upregulate the transcription of several early T-cell activation genes such as interleukin 2 and 4, granulocyte-macrophage colony-stimulating factor and γ -interferon. This signalling pathway is essential for activation of the immune response with differentiation and proliferation of lymphocytes.

The hydrophobic nature of CsA and FK506 allow these compounds to diffuse across the plasma membrane. Once in the cytoplasm CsA and FK506 bind to their respective receptors. The FK506-FKBP12 or CsA-CyPA complex then bind to a site formed between the two subunits of CN, as confirmed by the crystal structures of CN-FK506-FKBP12 (Griffith et al., 1995; Kissinger et al., 1995) and CN-CsA-CyPA (Huai et al., 2002; Jin and Harrison, 2002) complexes.

Since the phosphatase activity is inhibited by the immunophilin-drug complex, NFAT is neither dephosphorylated nor translocated to the nucleus. This interrupts the induction of genes essential for triggering of an immune response and the calcium-dependent signal cascade is abolished.

The complex between FKBP and rapamycin acts within T lymphocytes in a somewhat different way. The FKBP-rapamycin complex binds to FRAP (FKBP-rapamycin associated protein; Brown et al., 1994), RAFT1 (rapamycin and FKBP

targets; Sabatini et al., 1994) or mTOR (Target Of Rapamycin; Sabers et al., 1995). mTOR is the mammalian homologue of the yeast protein TOR, which is required for the G1-S progression in the cell cycle (Kunz et al., 1993).

PPIases, a ubiquitous protein family

Grouped together according to their enzymatic activity the PPIase family consists of three subfamilies: cyclophilins, FKBP and parvulins. The latter is the most recently identified family possessing PPIase activity. The first parvulin was originally discovered in *Escherichia coli* (*E. coli*) as a novel PPIase consisting of only 92 amino acids, hence named parvulin after the Latin word *parvus* meaning small (Rahfeld et al., 1994). The enzymatic activity of parvulins is irreversibly inhibited by juglone (Hennig et al., 1998), although this inhibitor-protein complex does not exert any immunosuppressive actions. A proposed fourth family, called Trigger factor, is now generally considered part of the FKBP family, as determined by substrate specificity and a distantly related central FKBP domain (Callebaut and Mornon, 1995). PPIases have been identified in all completely sequenced genomes to present date and although they share the same enzymatic activity, the sequence similarity is low between the three subfamilies.

During protein biosynthesis most peptide bonds are connected in the *trans* conformation, and this is also the most abundant arrangement in native structures (Weiss et al., 1998). *Cis* peptide bonds are not as frequently seen, most likely due to steric hindrance and unfavourable interactions between neighbouring residues. However, the cyclic structure of Pro introduces specific properties and this amino acid is more often found in *cis* conformation (Stewart et al., 1990; Weiss et al., 1998). Due to the resonance stabilizing effect of the imide bond, the rotation of Xaa-Pro peptide bonds was proposed to be the rate limiting step in protein folding already in the 1970s (Brandts et al., 1975) and the reaction catalyzed by PPIases is therefore important to improve folding rates.

While few *in vivo* functions of PPIases have been proven, a wide variety of functions has been suggested for these proteins both *in vivo* and *in vitro*. As expected, many PPIases are involved in protein folding (Freskgård et al., 1992; Matouschek et al., 1995; Hesterkamp et al., 1996; Dartigalongue and Raina, 1998). They have also been reported to be members of large complexes such as chaperone complexes (Kandror et al., 1994; Lazar and Kolter, 1996; Pratt, 1998) as well as receptors, channels, and pores (Nicolli et al., 1996; Marks, 1997; Marx et al., 2000). PPIases also participate in and regulate functions such as: signalling and trafficking (Shieh et al., 1989; Maleszka et al., 1997; Yurchenko et al., 2002), protein secretion (Kontinen and Sarvas, 1993), RNA processing (Mi et al., 1996; Krzywicka et al., 2001), apoptosis (Lin and Lechleiter, 2002), cell cycle control (Lu et al., 1996; Shen et al., 1998), developmental regulation (Dornan et

al., 1999; Patterson et al., 2000), and pathogen-host interactions (Wintermeyer et al., 1995; Hoerauf et al., 1997; Deng et al., 1998). Furthermore, these proteins are involved in human diseases such as HIV (Luban et al., 1993; Braaten et al., 1997) and Alzheimer (Butterfield et al., 2006). Several of these proteins and their functions are described in reviews regarding PPIases in general (Göthel and Marahiel, 1999; Ivery, 2000; Fischer and Aumüller, 2003; Galat, 2003) and in relation to photosynthesis (Vener, 2001; He et al., 2004; Romano et al., 2005; Vallon, 2005).

Even though several PPIases have been identified and isolated, their cellular functions in the absence of inhibitor are poorly understood. Four of the in depth characterized functions of PPIases are briefly described below.

Human FKBP12 associates with and affects the activity of intracellular Ca^{2+} release channels including ryanodine receptors (RyR) and the inositol-1,4,5-triphosphate receptor (Marks, 1997). FKBP12 regulates coupled gating between neighbouring RyR channels (Marx et al., 1998), and a direct functional proof of its importance was shown when mice lacking FKBP12 displayed altered channel functions resulting in severe cardiac defects (Shou et al., 1998). This is explained by RyR hyperphosphorylation, leading to altered channel properties, abnormal Ca^{2+} leakage and ultimately to heart failure (Wehrens et al., 2005) in the absence of FKBP12.

The Hsp90 chaperone complex present in the cytosol of eukaryotic cells consists of Hsp90 associated with Hsp70 and one copy of either FKBP51, FKBP52 or CYP40 together with some additional cofactors (reviewed in Pratt and Toft, 2003). All three immunophilins listed above contain three tetratricopeptide repeats (TPRs), which mediate protein-protein interactions, required for interaction with Hsp90. Which of the immunophilins, FKBP51, FKBP52 or CYP40, that binds to the Hsp90 complex depends mainly on the target substrate, e.g. both the progesterone receptor and the glucocorticoid receptor prefer FKBP51 as part of the chaperone complex in favour of FKBP52 and CYP40 (Nair et al., 1997; Barent et al., 1998). In general, however, FKBP52 shows the strongest affinity for Hsp90 while the Cyp40 interaction is the weakest (Pirkl and Buchner, 2001). Both FKBP52 and Cyp40 have been shown to display chaperone activity independent of their PPIase activity (Bose et al., 1996; Freeman et al., 1996) although association of FKBP52 with interferon regulatory factor-4 (IRF-4) was dependent on functional PPIase activity that resulted in a structural modification of IRF-4 (Mamane et al., 2000). Cytoplasmic steroid receptors exist mainly as receptor-Hsp90-immunophilin complexes and the immunophilins have been proposed both to affect hormone affinity to steroid receptors and to facilitate the translocation of receptors to the nucleus (reviewed in Ratajczak et al., 2003).

Two essential parvulins, the human Pin1 and its yeast homologue Ess1, regulate the mitotic cell cycle (Lu et al., 1996). A proposed model for this regulation includes a specific serine or threonine phosphorylation on residues

preceding proline, which creates the binding site for Pin1 (Yaffe et al., 1997). The following isomerization induces conformational changes that may alter the activity of the phosphorylated protein. Depletion of Pin1 induces mitotic arrest and apoptosis and this pathway might be a potential target for cancer therapy (Lu et al., 1996).

One of the first characterized cyclophilins is the fruit fly (*Drosophila melanogaster*) protein NinaA (Schneuwly et al., 1989). Expressed only in the eye, NinaA possesses one transmembrane domain and is localized within the endoplasmatic reticulum. There, NinaA and rhodopsin form a complex required for proper maturation and localization of rhodopsin. Mutant flies lacking NinaA accumulate rhodopsin in ER, resulting in visual defects (Stamnes et al., 1991), suggesting a chaperone function of NinaA in the fly (Baker et al., 1994).

Before continuing with a closer description of plant PPIases and the luminal immunophilins examined in this thesis, an introduction to photosynthesis will be presented.

Introduction to photosynthesis

Most life forms on Earth depend on the Sun as the ultimate energy source, but not all of them are able to use the energy directly from the Sun. For instance, animals and humans depend on the ability of photosynthetic organisms like plants, algae and cyanobacteria to absorb solar energy and to convert a simple molecule like carbon dioxide into energy-rich carbohydrates. Moreover, we are so dependent of the photosynthetic reactions that even the photosynthetic “waste product” oxygen, is crucial to us.

There are also practical and economical aspects of photosynthetic research, which can be divided into two main areas: (i) knowledge and techniques to increase crop productivity (Horton, 2000), and (ii) artificial photosynthesis for energy conversion (Hammarström, 2003; Alstrum-Acevedo et al., 2005). It is therefore important to understand how the photosynthetic mechanisms are regulated and how environmental changes such as light, temperature and nutrient availability affect plants. Spinach (*Spinacia oleracea*) was for a long time the biochemists’ choice in photosynthetic research of higher plants due to its fast growth and moderate size. However, in recent years *Arabidopsis* (*Arabidopsis thaliana*), a weed belonging to the mustard family (*Brassicaceae*) also including cultivated species such as cabbage and radish, has become the model organism of choice in plant research. The main breakthrough for *Arabidopsis* as a model plant came in 2000 with the complete genome sequence (*Arabidopsis* Genome Initiative, 2000) revealing a genome of approximately 125 million base

pairs (125 Mbp). This can be compared with the genomes of maize (2400 Mbp) and wheat (16000 Mbp – 128 times larger than *Arabidopsis* and 5 times larger than human). Even though *Arabidopsis* is not considered to be of any economic significance, it offers several benefits for genetic research such as short generation time, large seed production, ease of transformation and a small genome. The goal of using a simple model organism is then to reveal how the molecular mechanisms function within the cell and whole organism, and subsequently use this knowledge on plants of more agricultural and economical importance. The *Arabidopsis* genome is divided in five chromosomes with more than 25 000 protein encoding genes. In addition, the chloroplasts and mitochondria contain genomes encoding approximately 60 and 80 proteins, respectively. The most apparent drawbacks with *Arabidopsis* in biochemical analyses are difficulties to obtain intact organelles (Kunst, 1998) and large material quantities often needed. However, the latter problem is to some extent overcome by hydroponical growth (Norén et al., 2004). During this thesis work, both spinach (Paper I) and *Arabidopsis* (Papers II, III and IV) have been used as experimental model systems.

The chloroplast

Chloroplasts are semiautonomous and carry their own genome and protein translation machinery. As shown in the schematic model in Figure 3, the chloroplast consists of six different compartments: three soluble fractions and three membranes. Two membranes, the outer and inner envelope, enclose the soluble intermembrane space and separate the chloroplast from the surrounding cytosol. Situated within the chloroplast is the soluble stroma together with the thylakoid membrane that in turn encloses the soluble lumen. The thylakoid membrane forms a continuous network with appressed regions called grana stacks interconnected by non-stacked stroma lamellae, which form right-handed helices wound around the grana stacks (Mustardy and Garab, 2003).

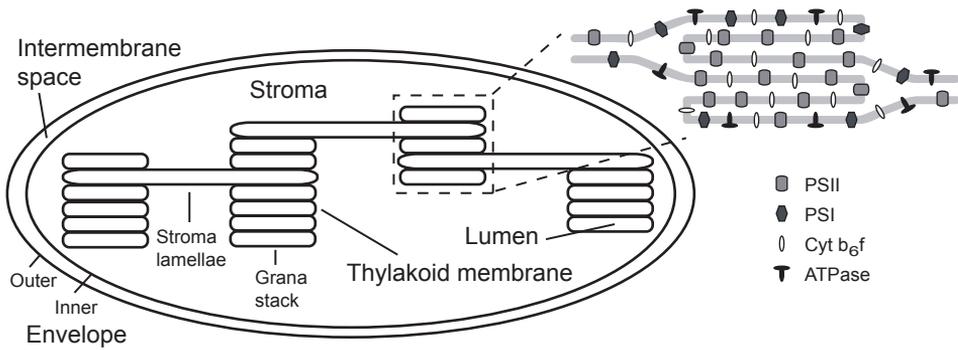


Figure 3. Schematic organisation of the chloroplast. The inner and outer envelopes, enclosing the intermembrane space, separate the chloroplast from the cytosol. The thylakoid membrane, divided in grana stacks and stroma lamellae, surround an inner space called lumen. The Calvin cycle reactions take place in the stroma while the thylakoid membrane harbours the photosynthetic complexes involved in the electron transport chain. The expanded part of a grana stack illustrates the heterogeneous distribution of photosynthetic complexes in the thylakoid membrane.

Photosynthetic reactions

The photosynthetic process consists of two different types of reactions: (i) The light reactions in which light energy is captured and converted into chemical energy in the form of reducing power and ATP. (ii) The carbon fixation reactions, also called Calvin cycle, in which the reducing power and ATP produced in the light reactions are used to fixate carbon dioxide and produce carbohydrates. The enzymatic reactions of carbon fixation take place in the stroma, while four multi-subunit protein complexes embedded in the thylakoid membrane (see Figure 4) carry out the light reactions. These four complexes, namely photosystem I and II (PSI, PSII), cytochrome b_6f complex (Cyt b_6f) and ATP synthase (ATPase), carry pigments and other cofactors. The distribution of these protein-pigment complexes in the thylakoid membranes is heterogeneous (see Figure 3; Andersson and Anderson, 1980; Albertsson, 2001). The majority of PSII is located in grana while PSI and ATPase are mainly found in the stroma-exposed regions. Cyt b_6f , which links the electron flow between the two photosystems, is evenly distributed between both membrane regions.

The structural knowledge about the four protein complexes involved in the light reaction has been greatly improved during the last decade (reviewed in Nelson and Yocum, 2006). The first structural model of PSI was presented in 1996 (Krauss et al., 1996), and a fully active PSII complex from a cyanobacterium was purified and crystallized in 2001 (Zouni et al., 2001). The first structure representing a eukaryotic integral membrane electron transport

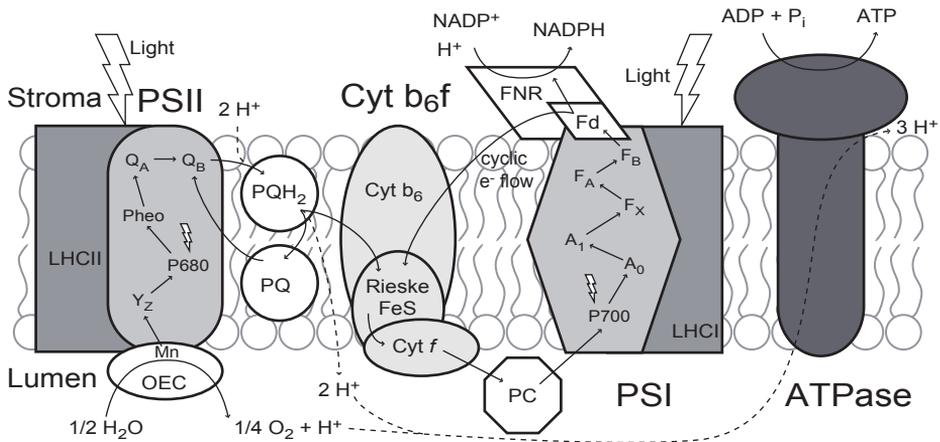


Figure 4. Schematic presentation of the photosynthetic electron transport chain. The four main membrane complexes involved in photosynthesis are illustrated. Sunlight is shown by flashes while the flow of electrons (e^-) and protons (H^+) are illustrated with solid and dashed arrows, respectively.

complex was the Cyt b_6f from the green alga *Chlamydomonas reinhardtii* (Stroebel et al., 2003).

As seen in Figure 4, PSII catalyzes the first step in photosynthetic electron transport (Nield and Barber, 2006) providing electrons for the electron transport chain by photooxidation of water, a reaction that also produces oxygen. Absorption of light energy by carotenoids and chlorophyll molecules in the light harvesting antenna complexes of PSII (LHCII) initiates the reaction. From LHCII, organized in trimers (Hobe et al., 1994), the energy is funnelled to three minor antenna proteins: CP24, CP26, and CP29 (Jansson, 1999), and then to the PSII reaction center. Situated in the middle of PSII are the two reaction center proteins D1 and D2. They are surrounded by two large proteins named CP43 and CP47, which serve as inner antennae accepting the energy from the peripheral antennae. These four subunits, together with a number of small subunits (PsbE-F, PsbH-N, PsbR-T, PsbW-Z; Shi and Schröder, 2004) build the PSII core. The proteins of the oxygen evolving complex (OEC), PsbO, PsbP and PsbQ, are located on the luminal side of PSII (Ferreira et al., 2004) and stabilize the manganese cluster essential for the water splitting reaction. When the energy of the absorbed light reaches the special chlorophyll pair P680 in the reaction center a charge separation occurs and an electron is extracted. The oxidized P680 is reduced again, by a tyrosine residue (Yz), with electrons withdrawn from the manganese cluster and subsequently from water. This is called the water splitting reaction, and it releases oxygen and deposits protons in the lumen. The electron extracted from P680 is translocated, *via* several steps, to a loosely bound plastoquinone (PQ) at the Q_B site. After receiving two electrons, PQ is reduced to plastoquininol (PQH_2) and released from PSII. PQH_2 is

reoxidized upon the encounter and delivery of electrons to Cyt b_6f , a dimeric membrane complex with the iron-sulfur containing Rieske subunit (Cramer et al., 2005). Simultaneously, protons are released into the lumen. Electrons from Cyt b_6f are transferred to PSI via plastocyanin (PC), a soluble 11 kDa copper-containing protein localized in the lumen. PSI core in plants consists of two large subunits (PsaA-B), four extrinsic subunits (PsaC-E, PsaN) and a number of intrinsic subunits (PsaF-L, PsaO-P; reviewed in Nelson and Yocum, 2006). In a light mediated reaction, similar as in PSII, the primary electron donor P700 is excited and an electron is transferred to ferredoxin (Fd), a 12 kDa stroma localized protein containing an iron-sulfur cluster. P700 is connected to the electron transport chain by reduction with an electron transferred from PC. The high reduction potential of Fd is used for reduction of NADP^+ to NADPH, a reaction catalyzed by ferredoxin-NADP reductase (FNR). The NADPH produced is then utilized in the carbon fixation reactions. As electrons flow from PSII to PSI, and by the splitting of water, a proton gradient is formed across the thylakoid membrane. This proton force is used to drive the ATPase in the energy dependent formation of ATP (McCarty, 2005). An alternative electron path is the cyclic electron flow (see Figure 4; Bendall and Manasse, 1995). In this case, only ATP is produced since the electron passes back from Fd to Cyt b_6f with no production of NADPH or oxygen.

The ATP and NADPH produced during the light reactions are used in fixation of carbon dioxide (Calvin cycle), occurring in the stroma. The actual fixation of CO_2 (Figure 5) takes place in the first step where ribulose biphosphate carboxylase/oxygenase (Rubisco) catalyses the reaction between 3 CO_2 and 3 ribulose-1,5 biphosphate (RuBP) to produce 6 molecules of 3-phosphoglycerate. A reaction so vital that Rubisco is the most abundant protein in plants and probably the most abundant enzyme on Earth. In the two following reactions, which require 6 ATP and 6 NADPH, respectively, the 6 molecules of 3-phosphoglycerate are converted to 6 glyceraldehyde 3-phosphate molecules. Only 1 of these proceeds to form glucose while 5 are required for the ATP demanding regeneration of the first 3 RuBP. Only three enzymes are unique to the Calvin cycle while the other ten are also found in other carbohydrate pathways. This important process requires good regulatory mechanisms for optimal function and several enzymes are regulated through changes in light, redox status as well as availability of ATP (reviewed in Bukhov, 2004).

Responding to environmental conditions

On a sunny day, we can move ourselves into the shade and likewise seek shelter for changes such as rain and wind. This is a luxury the sessile plants do

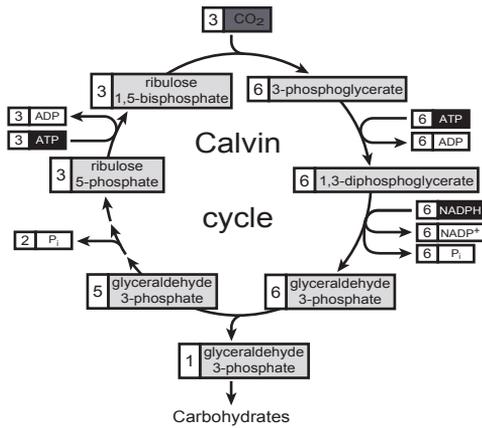


Figure 5. Model of the Calvin cycle. The fixation of three CO₂ give a net yield of one molecule of glyceraldehyde 3-phosphate at the cost of nine ATP and six NADPH.

not have. Instead, they have had to evolve molecular mechanisms to adapt to both short and long-term stress conditions in the environment, such as change in light and temperature, drought, or attacks from herbivores. If unable to cope with these conditions, the plant will die, but protecting mechanisms help it to survive. The photosynthetic reactions are essential for photoautotrophic growth and therefore both long-term regulation, e.g. altering protein expression and transport, and short-term regulation occur in order to optimize these reactions in the cell. Plants have evolved several short-term protection mechanisms to cope with light fluctuations and to withhold optimal photosynthetic capacity. (i) Posttranslational modifications, such as protein phosphorylation, regulate the activity of many enzymes (Aro and Ohad, 2003; Vener, 2005). (ii) State transitions, where LHCII is able to migrate from PSII (state 1) to PSI (state 2) and back (Mullineaux and Emlyn-Jones, 2005). In this way, the distribution of absorbed light energy is balanced between the two photosystems in an optimal way. (iii) Non-photochemical quenching, in which excess energy is dissipated as heat through the xanthophyll cycle (Cogdell, 2006). (iv) Scavenging of reactive oxygen species by nonenzymatic antioxidants such as carotenoids, ascorbate, tocopherols, glutathione and the scavenging enzymes namely, superoxide dismutase, ascorbate peroxidases and glutathione peroxidases (Edreva, 2005). (v) Rapid repair, mainly targeted towards PSII, in which damaged D1 protein is selectively degraded and replaced by a new copy (Andersson and Aro, 2001). These protective mechanisms are affected not only by light but also by additional environmental factors such as temperature and access to water. Redox sensitive molecules and proteins can therefore serve as regulators, recognizing and regulating the status of the photosynthetic electron transport (Buchanan and Balmer, 2005). In this respect, the mapping of an entire luminal proteome less than a decade ago (Kieselbach et al., 1998; reviewed in Kieselbach and Schröder, 2003) offered scientists the possibility to elucidate these regulatory mechanisms in more detail. Originally thought to contain only a few soluble and membrane associated proteins necessary for the photosynthetic

reactions, e.g. PC (Kato and Takamiya, 1961), PsbO-Q (Kuwabara and Murata, 1979; Åkerlund and Jansson, 1981; Åkerlund et al., 1982; Ettinger and Theg, 1991) and violaxanthin de-epoxidase (Hager and Holocher, 1994), proteomic studies of the lumen revealed the presence of around 80 proteins (Peltier et al., 2000; Peltier et al., 2002; Schubert et al., 2002) of which probably all are involved in regulation of the photosynthetic processes in one way or another.

Overview of plant PPIases

The first plant PPIases were identified in 1990 with the isolation of cyclophilin cDNA sequences from tomato (*Lycopersicon esculentum*), maize (*Zea mays*), and oilseed rape (*Brassica napus*; (Gasser et al., 1990)). The genome of Arabidopsis encodes the largest number of prolyl isomerases identified today, with 29 cyclophilins, 23 FKBP's including one trigger factor, and three parvulins (reviewed in He et al., 2004; Romano et al., 2004; Romano et al., 2005)). The genome of the unicellular green alga *Chlamydomonas reinhardtii* also encodes 52 immunophilins and the dramatic increase in the number of PPIases is suggested to have occurred after divergence between green algae and cyanobacteria, (Vallon, 2005) since the *Synechocystis* genome, for example, only encodes less than ten immunophilins.

Although not experimentally confirmed for all, nine of the 21 single domain cyclophilins in Arabidopsis are predicted to be localized in the cytosol, five targeted to the secretory pathway, five to the chloroplast and two to the mitochondria. Four of the eight multidomain cyclophilins are predicted to be located in the cytosol, three in the nucleus and one in the chloroplast lumen.

The multidomain cyclophilins AtCYP57, AtCYP59, AtCYP63 and AtCYP95 all possess additional RNA interaction motifs (He et al., 2004; Romano et al., 2004), suggesting involvement in RNA processing in both nucleus and cytosol, whereas the cytosolic AtCYP65 might play a role in protein degradation, which is indicated by the presence of a U-box motif.

Some clues about the putative functions in plants can be drawn from plants lacking AtCYP40. This cytosolic cyclophilin consists of an N-terminal cyclophilin domain and a C-terminal domain composed of a nuclear targeting signal, as well as three tetratricopeptide repeats (TPRs) involved in assembly of multiprotein complexes. Plants deficient in AtCYP40 display defects in vegetative development, especially in the juvenile to adult transition (Berardini et al., 2001). Furthermore, the cyclophilins AtCYP18-3, AtCYP18-4 and AtCYP20-3 were found to associate with the VirD protein and were proposed to be involved in the T-DNA infection of *Agrobacterium tumefaciens* (Deng et al., 1998). However, the involvement of AtCYP20-3 is questionable due to its

stromal localization in the chloroplast, which makes it unlikely to be involved in DNA transfer.

The Arabidopsis FKBP family is one of the largest FKBP families identified to date, consisting of 16 single domain and seven multidomain proteins (He et al., 2004). Remarkable is also the fact that half of these (11 of 23) are predicted to be located in such a tiny space as the chloroplast lumen. Among the non-luminal single domain FKBP, two contain a nuclear targeting signal, two are located in the endoplasmic reticulum, while AtFKBP12 is the only one found in the cytosol. Similar to AtCYP40, some multidomain FKBP such as the nuclear AtFKBP72 and the cytosolic AtFKBP42, AtFKBP62 and AtFKBP65 possess three TPR regions as well as a calmodulin (CaM) binding motif. These FKBP, with the exception of AtFKBP42, also have three repeats of the FKBP domain, a feature not found within the cyclophilin family. The nuclear localization signal and an arginine-lysine rich region of AtFKBP43 and AtFKBP53 suggest an involvement in the RNA processing machinery. The poorly conserved central FKBP domain of the Arabidopsis trigger factor, AtTIG, is flanked by ribosome binding motifs (He et al., 2004), and based on its predicted stromal localization it might be involved in the chloroplast translation process.

Additional hints regarding the functions of Arabidopsis FKBP came with the *pasticcino* (*pas*) mutants displaying severe phenotypical changes throughout the growth stages (Faure et al., 1998). A mutation of the *AtFKBP72* (*pas1*) gene causes altered response towards both exogenous cytokinin (Vittorioso et al., 1998) and auxin (Harrar et al., 2003). Recently it was shown that its C-terminus (TPR region) was required for proper subcellular localization and interaction with a transcription factor (Smyczynski et al., 2006).

Mutants with a disruption in the *AtFKBP42* gene, known as *twisted dwarf 1* (*TWD*; Kamphausen et al., 2002) or *ultracurvata 2* (*UCU2*; Perez-Perez et al., 2001), show extreme developmental abnormalities with pleiotropic phenotype including dwarfism, circinate leaves, helical rotation of a number of organs and reduced fertility. AtFKBP42 is a membrane-anchored protein, with an N-terminal FKBP domain and three TPRs in the C-terminus, found in both plasma and vacuolar membranes (Kamphausen et al., 2002; Geisler et al., 2003; Geisler et al., 2004). Even though AtFKBP42 is an inactive PPIase (Kamphausen et al., 2002) it interacts with a number of multidrug-resistance ABC transporters through both its FKBP domain and its C-terminal TPR and CaM domains (Geisler et al., 2003; Geisler et al., 2004). AtFKBP42 is suggested to regulate auxin signalling (Perez-Perez et al., 2004; Bouchard et al., 2006).

The interaction between the smallest immunophilin AtFKBP12 and a protein called AtFIP37 (FKBP interacting protein; Faure et al., 1998) seems to be crucial for Arabidopsis development by regulation of the cell cycle, similar to what was shown for FKBP12 and TOR (target of rapamycin) in mammals (Vespa et al., 2004).

The Arabidopsis genome encodes three parvulins, two located in the cytosol (AtPIN1 and AtPIN2) while one resides in the chloroplast stroma (AtPIN3). Missing in all Arabidopsis parvulins is the N-terminal WW domain present in the human parvulin Pin1, which first was thought to be required for binding of phosphorylated substrates (Lu et al., 1999). Nevertheless, AtPIN1 retains phosphoprotein specificity and can complement a yeast strain lacking the essential ESS1 parvulin (Landrieu et al., 2000; Metzner et al., 2001; Yao et al., 2001). Structural studies of AtPIN1 (Landrieu et al., 2002) show that its structure is similar to the catalytical domain of Pin1 (Ranganathan et al., 1997). In Pin1 the residues L122, M130, F134 define the proline binding pocket while the three basic residues: K63, R68 and R69 create the binding site for the amino acid preceding proline, preferentially a phosphorylated residue (Ranganathan et al., 1997). All these residues are present in the Arabidopsis parvulins with the exception of AtPIN2 and AtPIN3 that lack two or three of the phosphobinding residues. Although their precise functions are still unknown, the rhodanese domain in stromal AtPIN3 may suggest a function in the formation of iron-sulfur complexes. Furthermore, the parvulin inhibitor juglone inhibits auxin response in a cell-free system suggesting the involvement of a parvulin in auxin signalling (Dharmasiri et al., 2003).

The work on this thesis begun just after the revealing of a complex proteome in the chloroplast thylakoid lumen of spinach (Kieselbach et al., 1998). The following sequencing of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000) established that immunophilins, known as potential prolyl isomerases, represent the most abundant protein family in the thylakoid lumen. At that time only one luminal PPIase, the spinach cyclophilin TLP40, was identified (Fulgosi et al., 1998).

This thesis work was aimed at characterization of PPIases in the chloroplast thylakoid lumen and specifically focused on:

- Identification of a previously unknown PPIase in the chloroplast thylakoid lumen of spinach (Paper I).
- Biochemical characterization of the newly identified luminal cyclophilin in Arabidopsis (Paper II).
- Distribution of PPIase activity between different enzymes in the thylakoid lumen of Arabidopsis (Paper III).
- Characterization of mutant Arabidopsis plants lacking the only cyclophilin possessing PPIase activity (Paper IV).

THE PPIASE ACTIVITY ASSAY

The classic PPIase activity assay was first described by Fischer and co-workers in 1984 (Fischer et al., 1984). In this simple but elegant assay a synthetic peptide substrate, with the general structure of succinyl-alanine-X-proline-phenylalanine-4-nitroanilide (X = any natural amino acid), is mixed together with the PPIase to be assayed. Proteases disturb the *cis-trans* equilibrium around the peptidyl-prolyl bond and in this case, α -chymotrypsin is used in excess as a helper protease that can only cleave the *trans* conformer of the substrate. In the first step, all natural occurring *trans* prolyl bonds are cleaved in a rapid burst reaction to release the chromophore 4-nitroaniline (pNA) that can be monitored at 390 nm (Figure 6). The relatively slow *cis* to *trans* isomerization of peptidyl-prolyl bonds is then monitored at 390 nm by the immediate proteolysis of appearing *trans* conformers (Figure 6). In the presence of an active PPIase, the isomerization speed is increased compared to the uncatalyzed reaction. To suppress the speed of spontaneous thermal isomerization of the peptidyl-prolyl bond the assay is normally conducted at 10°C.

Drawbacks of this method are the low solubility of the peptide and low signal-to-noise ratio since only about 10% of the substrate is in the *cis* form, which is actually monitored during analysis. These problems were partially overcome by dissolving the peptide substrate in trifluorethanol supplemented by LiCl (Kofron et al., 1991). Addition of LiCl improves solubility of peptides in organic solvents (Seebach et al., 1989) and the content of *cis* isomers is also increased, in some cases up to 70%.

This coupled irreversible assay still has several limitations: (i) Only chromogenic peptides with the X-proline-phenylalanine-4-nitroanilide sequence can be analyzed (using other proteases it is possible to investigate sequences with amino acids other than phenylalanine). (ii) The kinetic analysis is only possible under nonequilibrium conditions. (iii) Only the *cis* to *trans* and not the *trans* to *cis* reaction is characterized. (iv) Proteolytic products or the helper protease could influence the PPIase activity. (v) The determination of microscopic rate constants is not possible. All these problems can be overcome by the use of dynamic nuclear magnetic resonance (NMR) spectroscopy

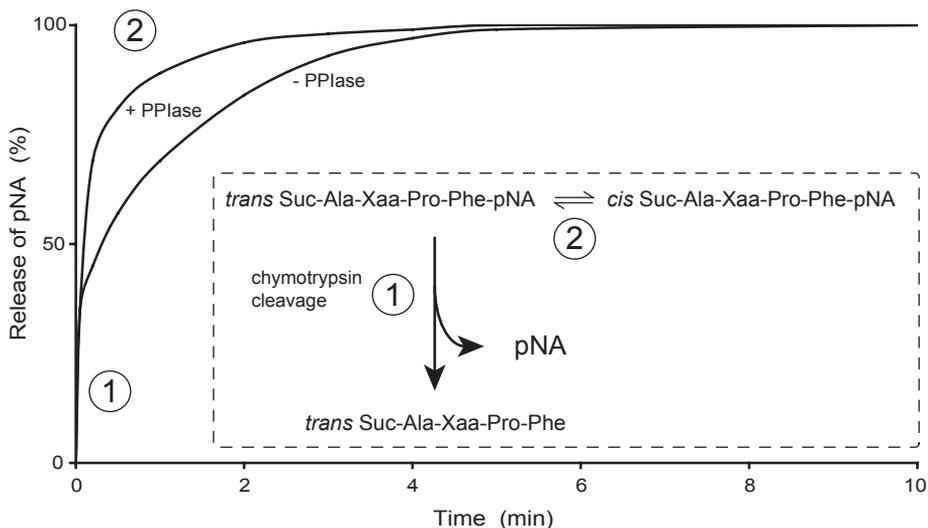


Figure 6. Peptidyl-prolyl *cis-trans* Isomerase assay. When the reaction is initiated a mixture of *trans* and *cis* peptide substrate is present. 1) The initial *trans* isoform is cleaved by chymotrypsin in a rapid burst reaction. Release of the chromophore pNA is monitored at 390 nm. 2) The slower *cis-trans* isomerization of the remaining *cis* peptide is monitored by immediate cleavage of formed *trans* peptides and release of pNA. In the presence of an active PPIase the isomerization speed is increased and reaches completion faster.

(Kern et al., 1995) in which distinct signals occur for the *trans* and *cis* isomers in the one-dimensional ^1H NMR spectrum. With this method, for the first time, all microscopic rate constants were determined for both the *cis* and *trans* isomer under reversible conditions. However, this method also has drawbacks since it requires very high concentrations of PPIase, which in the case of non-overexpressed or low abundant PPIases might be hard to obtain. It also demands advanced technical equipment and the analysis of the ^1H NMR spectra is quite time-consuming.

An uncoupled protease-free assay is a further development of the simpler spectrophotometric assay, which allows the use of standard peptide substrates and determination of rate constants for both *cis* and *trans* isomerization (Janowski et al., 1997). The isomerization is assayed under the same experimental conditions as before but without the protease. The small difference in absorption coefficients determined for *cis* and *trans* conformers is then used to calculate the first-order rate constants. These rate constants represent the sum of the spontaneous *cis* to *trans* and *trans* to *cis* prolyl isomerizations. Hence, the difference of the observed constants obtained from the protease-free and the proteolytically coupled assay represent the rate constant for *trans* to *cis* isomerization.

Substrate specificity of PPlases

The most commonly used substrate peptide is the succinyl-alanine-alanine-proline-phenylalanine-4-nitroanilide (X = alanine; A-substrate). However, when assayed for substrate specificity, the FKBP12 activity dramatically changes depending on the residue preceding proline. The k_{cat}/K_M of FKBP12 increased over 1000 times with a preference for large bulky hydrophobic amino acids such as leucine, isoleucine and phenylalanine (Albers et al., 1990; Harrison and Stein, 1990). Cyclophilins on the other hand showed a low preference regarding the residue preceding proline with only 5-times difference between the highest (alanine/ valine) and the lowest (histidine; Harrison and Stein, 1990). Although cyclophilins usually have higher k_{cat}/K_M -values and are considered to be quite substrate unspecific, some minor variations are seen. For example, both human and *E. coli* Cyp18 show the highest preference for valine, alanine and leucine, but the order between them is different (Liu et al., 1991).

These general findings are however not entirely supported by this thesis work. Both TLP20 and AtCYP20-2, the homologous proteins in spinach and Arabidopsis, displayed high activity towards the A-substrate, while very low activity was found with X = leucine (L-substrate; Paper I and III; Edvardsson, Shapiguzov, Andersson and Vener, unpublished results). On the contrary, AtFKBP13 was the most active luminal immunophilin in Arabidopsis on both substrates, with only about 30% lower activity when the A-substrate was used (Paper III).

The substrate specificities above were based on analyses with small peptides, however if this also holds true for the protein substrates *in vivo* is not known.

SEQUENCE ANALYSES OF LUMENAL IMMUNOPHILINS

Luminal cyclophilins

Cyclophilins fold as β -barrels with eight strands of antiparallel β -sheet and three α -helices. The first three-dimensional structure of an unligated recombinant human CyPA was determined at 2.5 Å in 1991 (Ke et al., 1991) and refined to 1.63 Å one year later (Ke, 1992). In contrast to most β -barrel structures, in which the hydrophobic core is open for ligation, the hydrophobic centre of cyclophilins is sealed off by two α -helices, one on top and one at the bottom. This suggests that CyPA is probably neither functionally nor evolutionally related to other β -barrel structures (Ke, 1992). Structures of CyPA in complex with both CsA or peptide substrates suggest the hydrophobic pocket, creating the binding site, to be formed by the residues: H54, R55, I57, F60, M61, Q63, G72, A101, N102, A103, Q111, F113, W121, L122, H126 and R148 (all following numbers referring to the human CyPA unless otherwise noted; Kallen and Walkinshaw, 1992; Spitzfaden et al., 1992; Mikol et al., 1993). The crystal structures also reveal distinct but overlapping sites for CsA binding and the PPIase active site. Site-directed mutagenesis has confirmed the importance for many of these residues. For instance, point mutations have proven H54, R55, F60, Q111, F113, W121 and H126 to be the most important residues for PPIase activity (Zydowsky et al., 1992) and R55, F60 and H126 being the most crucial ones with less than 1% remaining activity in the mutated protein. This study also experimentally confirmed the distinct binding sites of isomerase activity and inhibition since all mutants (except the W121A) were still able to inhibit calcineurin in the presence of CsA.

In Figure 7, all luminal Arabidopsis cyclophilins are aligned with human CyPA. In addition the stromal AtCYP20-3 and the spinach TLP40 (homologue of AtCYP38) are included. The most conserved proteins are the stromal AtCYP20-3 and the luminal AtCYP20-2. Both enzymes possess all seven residues important for isomerase activity (indicated in Figure 7) conserved and

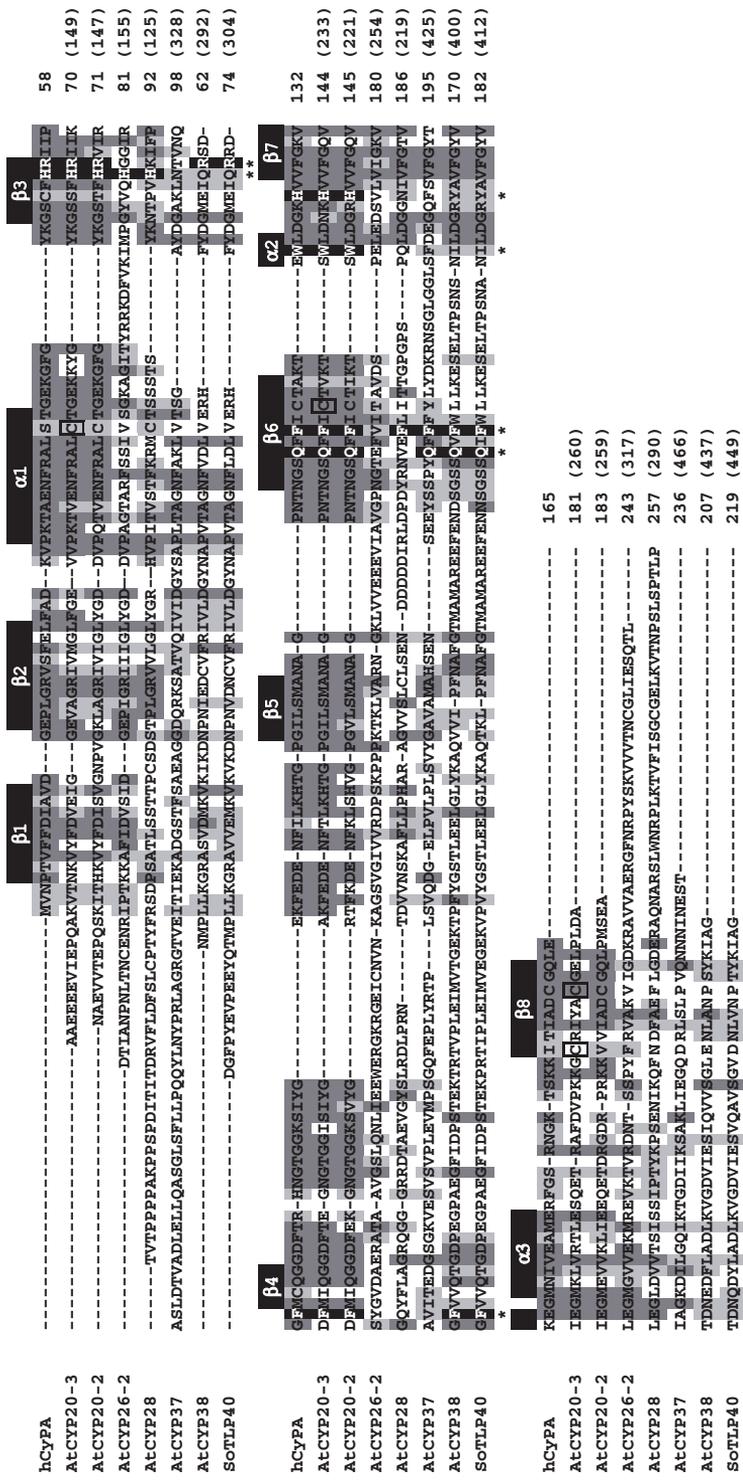


Figure 7. Alignment of human cyclophilin A with Arabidopsis chloroplast cyclophilins and spinach TLP40. Secondary structure elements of hCypA are indicated above the alignment. Important amino acids for PPIase activity, determined by site-directed mutagenesis of hCypA (Zydowsky et al., 1992), are labelled with asterisks below the alignment and with black background when identical. Other residues, identical or similar to hCypA are shaded dark and light grey, respectively. The four cysteine residues involved in redox control of AtCYP20-3 are boxed. Numbers indicate amino acid positions in mature and (precursor) form of the protein.

are highly active (Paper III, Lippuner et al., 1994). The most active PPIase in the spinach lumen is TLP20 (Paper I), and based on the 41 amino acids known from its sequence (Paper I) it seems very similar to AtCYP20-2. Therefore, one might assume that all seven residues are also conserved in TLP20.

In both AtCYP38 and its spinach homolog TLP40, four of the seven residues are present. When assayed with the A-substrate, no activity was found in fractions containing AtCYP38 (Paper III) in contrast to what was found in TLP40 fractions (Paper I) and for the purified protein (Fulgosi et al., 1998). This discrepancy cannot be explained by any difference in the conserved catalytic residues. However, a closer look at the two amino acids following R55 might give some clues. In human CyPA, R55 is followed by two isoleucines, i.e. two uncharged amino acids. In the active TLP40, these two residues are substituted by arginine and aspartic acid, also resulting in a zero net charge, while in AtCYP38 a serine and aspartic acid create a negative charge that might alter the binding properties of the peptide substrate.

Based on the sequence information, neither AtCYP26-2, AtCYP28 nor AtCYP37 would be active since they lack most of the needed residues, especially the three most important ones. The finding that none of these proteins were identified in the chromatographic separations with subsequent activity measurements (Paper III) supports this idea.

W121 is not only important for PPIase activity but also the most crucial residue for interaction with CsA. Point mutations to phenylalanine or alanine decreased the PPIase sensitivity to CsA 200 times (Bossard et al., 1991; Liu et al., 1991), while mutations to tryptophan (W) in *E. coli* CyP18 and human CyP40, proteins lacking the corresponding conserved W121, enhanced the susceptibility to CsA (Liu et al., 1991; Hoffmann et al., 1995). All luminal cyclophilins, except AtCYP20-2, lack this tryptophan and are probably not inhibited by CsA. For instance, the activity of spinach TLP20 is abolished in the presence of CsA (Paper I) while the TLP40 activity remains unaffected even at 10 μ M CsA (Fulgosi et al., 1998).

Redox regulation mediated by thioredoxin (Trx) and glutaredoxin has for a long time been known to affect photosynthetic processes in the stroma. During the last years PPIases in the chloroplast have also been reported to be redox controlled (Motohashi et al., 2003; Gopalan et al., 2004; Lima et al., 2006). Therefore, potential cysteines to be involved in formation of disulfide bridges and redox control in the luminal cyclophilins were analyzed.

Human CyPA has four cysteines but no disulfide bridges are formed based on the crystal structures. However, a proteomic study identified CyPA as a target of glutathione under oxidative stress (Fratelli et al., 2002) and very recently the same authors proved CyPA to be *in vivo* redox regulated by glutathionylation of C52 and C62 (Ghezzi et al., 2006).

In a similar manner, AtCYP20-3 was found as a target of stromal Trx (Motohashi et al., 2001). The redox regulation was later confirmed and the

disulfide bridges were mapped between C52-C169 and C127-C174 (Figure 8; Motohashi et al., 2003), where the first bridge was suggested to be the most crucial for isomerase activity (Laxa et al., 2007).

The luminal AtCYP20-2 is similar to AtCYP20-3 and Romano and co-workers (Romano et al., 2005) speculate on redox regulation of AtCYP20-2 since the protein contains three out of four cysteines present in AtCYP20-3. However, AtCYP20-2 cannot form the most crucial bridge since the corresponding C167 (in AtCYP20-3) is missing. This together with the fact that addition of DTT to AtCYP20-2 containing fractions had little effect on the activity (Paper III, Shapiguzov, Edvardsson and Vener, unpublished results) contradicts the theory by Romano et al.

AtCYP26-2 and AtCYP28 have three and five cysteines, respectively, and are potential targets for redox regulation while AtCYP38/TLP40 with one cysteine and AtCYP37 with no cysteines are unlikely to be redox regulated.

Luminal FKBP

The first FKBP structure, solved by NMR in 1991 (Michnick et al., 1991; Van Duynes et al., 1991), confirmed the identity of two distinct PPIase families with unrelated three-dimensional structures. The structure of FKBP12 consists of six antiparallel β -strands and one α -helix crossing above them, which might resemble a half barrel curved around the α -helix. The amino acids creating the hydrophobic pocket are Y26, F36, F46, V55, I56, W59, H87, I90, I91, L97 and F99 (the following numbers referring to human FKBP12 unless otherwise noted). As in the case of cyclophilins, the FKBP residues required for isomerase activity and drug binding are highly conserved. The residues important for activity and inhibitor binding have been extensively studied in mutational analyses performed by scientists from Vertex Pharmaceuticals (Aldape et al., 1992; Park et al., 1992; Futer et al., 1995; DeCenzo et al., 1996). They concluded that D37, R42, W59, H87 and F99 are the most important residues for PPIase activity.

Figure 8 displays an alignment of human FKBP12 and all Arabidopsis FKBP

targeted to the lumen, although the presence of AtFKBP17-1, AtFKBP17-2 and AtFKBP17-3 in the lumen still awaits experimental confirmation. In the top rows, the luminal targeting peptides are aligned around the twin arginine motif pointing out that all, except AtFKBP16-2, probably are transported via the TAT-pathway. The translocation across the thylakoid membrane by this Δ pH-dependent mechanism has, however, been experimentally proven only for AtFKBP13 (Gupta et al., 2002).

AtFKBP13 is the most conserved luminal FKBP and the only one found active when total lumen was fractioned (Paper III). The most important residues for activity are conserved except H87, which is substituted with alanine. H87 is often replaced (Vallon, 2005) and mutational studies of human FKBP12

concluded that the importance of this change is highly dependent of the new residue. A substitution of histidine to alanine, as in the case of AtFKBP13, decreased the activity by 23%. On the other hand, a substitution with phenylalanine had no effect on the activity while 90% of the activity was lost in the construct containing leucine (Futer et al., 1995).

All luminal FKBP, except AtFKBP13, contain two or less of the five important residues (Figure 8) where D37 and F99 seem to be the most conserved. Based on this, one may assume that these proteins are not active isomerases. However, recently the activity of recombinant AtFKBP20-2 was reported (Lima et al., 2006), although 500 times lower than that of AtFKBP13. This very low activity may be the reason why we were not able to identify this enzyme during chromatographic separation (Paper III). Such a low activity would not be detected in the background noise and especially not at the low protein concentrations found in the luminal fractions.

W59, missing in all luminal FKBP except AtFKBP13, was proposed to be the docking site for proline in the substrate (Michnick et al., 1991). We can assume that some of these FKBP might still have very low activity but their primary functions have evolved towards purposes in which a rapid isomerase activity is not essential. The importance of W59 has also been studied in two human isoforms of FKBP12 suggesting improved substrate binding in the presence of W59, while an isoform lacking a couple of residues, including W59, on the other hand showed a greater stability of the protein hydrophobic core (Fulton et al., 2003).

The best studied luminal FKBP is AtFKBP13 which has been shown to be regulated by redox changes (Gopalan et al., 2004; Paper III and IV). Oxidation introduces two disulfide-bridges, C84-C96 and C185-C190 (Figure 8), necessary for isomerase activity (Gopalan et al., 2004). As will be discussed later (see Redox regulation of chloroplast PPIases) this is in contrast to many stromal proteins, such as AtCYP20-3, which are active at reducing conditions. The protein AtFKBP16-2 has four cysteines in the exact corresponding positions as AtFKBP13 (Figure 8) and although not yet reported this enzyme is probably also redox sensitive.

The two cysteines present in the last 16 residues of AtFKBP20-2 (Figure 8) have been shown to form a disulfide bridge when oxidized, but the isomerase activity was not affected by the changes in redox state (Lima et al., 2006). Other potential targets for redox regulation are AtFKBP16-3 and AtFKBP17-3 with two cysteines each while AtFKBP16-1, AtFKBP16-4, AtFKBP17-1, AtFKBP17-2, AtFKBP18, and AtFKBP19 contain one or no cysteines and are not able to form any intramolecular disulfide bridges.

hFKBP12 -----MSSLGSFVGCSPPEKKRCRFLVNNSLAKAEAINRNKQKVSDEPESFAQLSSCRRERAIIGFPFISGLLNDVNSALA----- 79

hFKBP13 -----MAMAMEI SLFPVSGSMALSAGKSRNSVRI SRVQSVFSVAHVPRRMWQLSGFGSVLTLDFPSLAAPV----- 71

hFKBP16-1 -----MASTTLTQSLYTRFRPTFFSSSSSFCSSSCLSSSDECKPLSVKRVFVGWGLFASLSLAPLADADA----- 73

hFKBP16-2 -----MAASPTLLPLGASRNLTKNESSRYIAARVILASRETRQSCCKINLSREEMLVLLGVSGGLSMSSLAAYA----- 76

hFKBP16-3 -----MILTKLVHPLHLSLSSSIFPRRKKQKXPYKCSLSPGCCKVIRIETVDPAPYPCBGRVNLGCLLAPASGLISTGSAEA----- 82

hFKBP16-4 -----MIRCFAMTVLIVGAPLITVHTFPTSPKLRIPAKSSAPSSSSSSAAARSLSLSLIAVTSVSSVSSFCSPSALA----- 78

hFKBP17-1 -----MANLFTAPFLSLKPFKTKASVHQCXASSNPEPESSPPPPQPLASQOKRKNVTTDWAASLITREFGIAGLAWAGLAFVTS----- 94

hFKBP17-2 -----MATLFTA VPSHRRFVSPQHPKSLLOSLSVTFNWPQPAVAVTLEQQOQLTWTIVSPVTRFGLIGAGFWAGFLAVGVVQMKSRDLVFG----- 94

hFKBP17-3 -----MASISSTRHMASNQHSLRPLRITSI SEADQSPINQVVAIVSVPISRKNDAIILLSSILPITSEFVLTIPSSSEA----- 71

hFKBP18 -----MASISSGFCFPFAPALAGTSSTRCRTVAARADQSDFLAPLRSSGNGCQVNNVSGPGLLIGALSVDKSDQDFASA----- 88

hFKBP20-2 -----MVTILSTPLRFLTCLEKLSLRSRNSRVSCCSLSEBPKQCLSRRLSIVYVIVASPCILLFALSSSA----- 67

hFKBP12 -----GVOVETISFGDRITFKRGQTCVHYVTGMLDE-----GKKFDSRRRN-----KPFKFMIGK----- 52

hFKBP13 -----EWTGDFEVSYSGLKQKVVYCGPEAVKGLIKAKHVKLEN-----GVFDSYVNG-----KPLTRRIGV----- 142

hFKBP16-1 -----QMKPEVIRTKLKEQSVRYQETIECE-----REAEGLVLEAVCCRAN-----GVFVHTVDFQFSGES-----SPVKLLIDR----- 141

hFKBP16-2 -----TRIDYATVGDPLCEIYVAKSGLGFCDDLVYFDEAPFCVIVNHLVAPAL-----GVLDSYKKA-----RPLTRRIGV----- 145

hFKBP16-3 -----AGLPEDKPRLECAECKELENVPMVYTESGLQYKDKVGRG-----PSPVPGFVAANVAVPS-----CQIFDSSLKKG-----LPYLFVVG----- 157

hFKBP16-4 -----VSTRRALRASKLPESDFTLPLNGLKYDILKVGNG-----ABA VKSRVAHVVAKWK-----GITFMPSQGLGVGGG-----TPYGFDPVQOSER----- 163

hFKBP17-1 -----EQIKTRIEVSQEVANTRDVEEKEIVLPLNGLRYDQRYVGG-----ATPRAQDLVIDLKGQVGTG-----OVFVDFGTQDKKKW-----KPLALVYVSSKPYSS----- 184

hFKBP17-2 -----EEDNTRGLEKQEEIILPLNGLRYDQRYVGG-----ATESSVYVDFVYKGVHGTG-----OVFVDFGFGKG-----KSLAMVWDSRKYSS----- 169

hFKBP18 -----RERRKRVIPLEEYTGEGELKFYDIEEKG-----PVAETESQAQVHPCRYRITAISTRSEKLIAGNRSTIA-----QTYEFKVSPTPKER----- 155

hFKBP19 -----SQFADMPALGKRDYKTKMYPDYETQSGLOYKDLRVYCG-----PLAKKDKVIVWDSYTIQY-----SRIFEARNKYGGSGFEGDDEKFFKPLGS----- 179

hFKBP20-2 -----KTKSKSPYDERRLLEQNKRIQRENNAPDEFFNFVREGFVKVLASDNYIKADSGLIYRDLRVYCG-----DFFADQGVTFHIGYNES-----GRRIDSYTIQG-----SPARIKGT----- 171

hFKBP12 -----QEVIRQVEGVAQ-----NSVQAKAKTISDYAYGATGHPG-----IIPPHALQFDVLLKLE----- 107

hFKBP13 -----GEVIKWDQIILGSDGIPP-----LITGKRTRIRPEIAYGRDAGKGGG-----LIPASVLPDIEHYTKA----- 208

hFKBP16-1 -----NDVLESLEKVLVYG-----KAGKRALLPEISVYKNEILKPIPEBFGPRR-----SLSHANEPQAEICQLKVL----- 207

hFKBP16-2 -----GKVRGLDQIILGREGVPP-----RVGGRKQIPEKLAYPEPAGCPSGD-----CNPENATLYDINVEIYTPGSMNR----- 217

hFKBP16-3 -----GQVIKGLDGLLS-----KAGKRUVIPCLAPFKFGLASAGPGR-----RVAENSPVEIDSLFETPLDSEEE----- 223

hFKBP16-4 -----GNVKGIDLSEVHG-----RVGGRUVIPPEIAYGKQVQ-----EPPNVAIEDIELSIKQSPFGTFVKIVEG----- 230

hFKBP17-1 -----SKVLPSEIETARS-----KVGRUVIPESQGNMSQEP LFPNFFRQRLFTTFNFRLANGEGSGLGHLFQIELNSTR LHR----- 229

hFKBP17-2 -----KGLCEIIVLRS-----KAGKRUVIPESLGFVDEAE LRSQ-----LQIPNASEIYVIEIDRVSTIA PA----- 247

hFKBP17-3 -----KGLCQSIIEHLRS-----KAGKRUVIPESLGFDRNVE FQG-----LEPESPADYIIRIVTVYCFQTY----- 234

hFKBP18 -----KREFDVFNLSLFAQAPKPPAMVITEGKVGGRKRVIPPEAGYQKMAN-----EIPGGAFFELNI ELRVTVP P PEEK----- 232

hFKBP19 -----NEVIPAFESA VSG-----MALGGIRRLIVPEELGQFDINDYNKSGPRMFTSQOR-----ALDFVLRNQLIDKPLFDVLLKIVPN----- 256

hFKBP20-2 -----NALVPEFEMGIRD-----KPGGRRRILPELGGPVPST-----FFSKQPELFDVLLSIQNGERRITIGFVSDVTS----- 242

In my evaluation and prediction of active PPIases, only residues experimentally proven important for PPIase activity were considered. Hence, residues involved in binding of CsA or FK506, or predicted to interact with peptide substrates, based on structural analyses, were not included. Still, there are many uncertainties in these predictions since the mutational studies are not complete, especially not for cyclophilins, and the change in activity might depend on the properties of the replacing amino acid. However, in an attempt to predict active immunophilins in *C. reinhardtii* and Arabidopsis, including fourteen and four residues for FKBP13 and cyclophilins, respectively, similar results were obtained by Vallon (Vallon, 2005).

Figure 8. Arabidopsis luminal FKBP13 alignment with the human FKBP12. In the top rows luminal targeting peptides were aligned around the double arginine residues with the hydrophobic stretch underlined. Amino acid sequences of mature proteins are aligned in the other two sections. Secondary structure elements of hFKBP12 are indicated above the alignment. Residues crucial for PPIase activity as determined by site-directed mutagenesis of hFKBP12 (Aldape et al., 1992; Futer et al., 1995; DeCenzo et al., 1996), are labelled with asterisks below the alignment and with black background when identical. Other residues, identical or similar to hCyPA are shaded dark and light grey, respectively. Cysteine residues involved in redox control of AtFKBP13 and AtFKBP20-2 are boxed.

POTENTIAL FUNCTIONS OF LUMENAL IMMUNOPHILINS

Although controversial, the chloroplast thylakoid lumen may in some ways resemble the folding environment of the bacterial periplasmic space. Protein folding in the periplasm of *E. coli* is different from folding in the cytosol due to the lack of ATP (Wülfing and Plückthun, 1994). The use of classical chaperones like GroEL:ES, which act in an ATP-dependent manner, is therefore not possible (Young et al., 2004). Instead, the periplasm of *E. coli* contains many protein folding catalysts such as protein disulphide isomerases and prolyl isomerases (reviewed in Duguay and Silhavy, 2004; Mogensen and Otzen, 2005).

When the thylakoid lumen was examined for ATP and ATPase activity, no significant activities were found (Kieselbach et al., 1998) opposite to the evidences for lumenal GTP binding and nucleoside diphosphate kinase activity presented by Spetea and co-workers (Spetea et al., 2004). Therefore, one can at least surmise that the free pool of nucleotides is lower in lumen as compared to other cellular compartments, which might explain the large variety of PPIases in higher plants. 30% (18 of 55) of Arabidopsis PPIases are targeted to the chloroplast where they might assist in folding or perform specific regulatory functions that are absent in non-photosynthetic organisms. The need of all isomerases might however be questioned since yeast mutants lacking all 12 immunophilins were viable and the observed phenotype corresponded to additions of phenotypes from individual deletions (Dolinski et al., 1997). The four PPIases present in the periplasmic space of *E. coli* were previously shown to be involved in the folding of outer membrane proteins (Rizzitello et al., 2001). A combined null mutant of the two parvulins ppiD and SurA was thought to be lethal (Dartigalongue and Raina, 1998) although data regarding a viable quadruple mutant have recently been presented (Justice et al., 2005).

Luminal cyclophilins

The multidomain cyclophilin TLP40, the spinach homologue of AtCYP38, was the first PPIase identified in the thylakoid lumen (Fulgosi et al., 1998). Following the luminal transit peptide is an N-terminal leucine zipper domain, a central acidic region, a putative phosphatase-binding domain and the C-terminal catalytic PPIase domain. TLP40 is known to associate with and regulate the activity of a membrane-bound phosphatase involved in PSII reaction centre protein dephosphorylation and replacement of damaged D1 protein (Vener et al., 1999). Elevated temperature activates the phosphatase, by the release of TLP40 from the thylakoid membrane into the lumen, with subsequent dephosphorylation of PSII reaction centre proteins (Rokka et al., 2000). The rebinding of TLP40 to inside-out thylakoid membrane vesicles (Andersson, 1986) is also decreased at higher temperatures as well as during increase of pH (Edvardsson, Eshaghi, Vener, Andersson, unpublished results). Arabidopsis mutants lacking AtCYP38 show low survival of seedlings and the surviving plants display drastically retarded growth (Khrouchtchova, Edvardsson, Shapiguzov, Fristedt, Paakkarinen, Hansson, Haldrup, Aro, Scheller and Vener, unpublished data) emphasizing the importance of this cyclophilin. Perhaps the crystal structure of AtCYP38, which is on its way (Vasudevan et al., 2005), will contribute with more insights regarding the regulatory mechanism of this complex enzyme.

Purified TLP40 is an active isomerase (Fulgosi et al., 1998) and when spinach lumen was separated, fractions containing TLP40 were active in the isomerase assay with the A-substrate (Paper I). This is, however, not true for Arabidopsis in which AtCYP38 fractions show no activity with either substrate (Paper III). Further investigations of the differences between these two plants surprisingly revealed significant difference in distribution of PPIase activity. When spinach lumen was assayed towards the L-substrate, little activity was found in the TLP20 fractions (Figure 9). The major peak of activity towards the L-substrate was instead found in the fractions eluting around the region of TLP40 (Figure 9). However, the high L-substrate activity found in these fractions cannot unambiguously be assigned to TLP40, as the number of PPIases in spinach is not known due to the lack of a complete genome sequence. It is unlikely that AtFKBP13, the second active PPIase in Arabidopsis, has a homologue in spinach since it does not bind to the anion-exchange column at pH 7.8 (Paper III), while all spinach PPIase activity was bound under these conditions (Paper I). These data demonstrate the species-dependent nature of active PPIases in the chloroplast thylakoid lumen and reinforce the suggestion that the physiological roles of many luminal cyclophilins and FKBP are not related to general PPIase activity.

TLP20 was first identified as the most active PPIase in spinach lumen (Paper I) and AtCYP20-2 is a highly active enzyme, even if AtFKBP13 is the most active PPIase in the lumen of Arabidopsis (Paper III). Further characterization of this

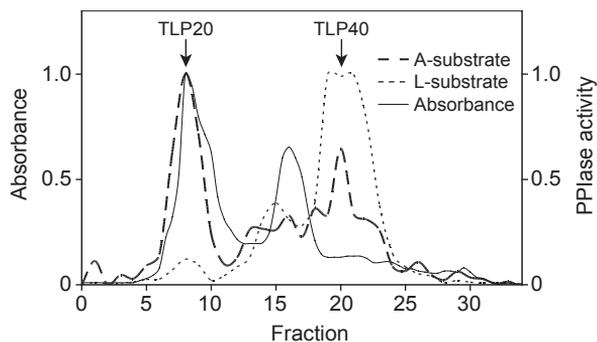


Figure 9. Fractionation of spinach lumen PPIase activities towards two different substrate peptides. Chromatographic separation on a Resource Q (anion-exchange) column identical as in Paper I. Activity in the fractions was examined with both A- and L-substrates (dashed lines) and the protein absorbance at 280 nm is shown with a solid line (as indicated in the figure insert). The elution positions of TLP20 and TLP40, indicated with arrows, were determined by immunoblotting like in Paper I.

protein in Arabidopsis revealed that AtCYP20-2, like TLP40, associates with the thylakoid membrane (Paper II) and that distribution of PPIase activity is very similar between soluble and membrane associated fractions (Paper III). Mutants lacking AtCYP20-2 surprisingly showed no phenotypical differences when compared to wild type and the total PPIase activity was not decreased (Paper IV) indicating that a stable isomerase activity is important in the lumen. A potential explanation could be that other luminal PPIases compensate for the loss of isomerase activity of AtCYP20-2 by redundant functions and pathways, as found in the periplasmic space of *E. coli* (Rizzitello et al., 2001). However, when examined with quantitative two-dimensional gel-electrophoresis no change in the abundance of any other immunophilin, that could explain the activity compensation, was observed (Paper IV). Considering the lack of, or very low activity of most luminal immunophilins (see sequence analyses section) and that the redox regulated AtFKBP13 is the only active PPIase in AtCYP20-2 deficient mutants (Paper III and IV) suggests this protein to be the only enzyme involved in the activity compensation. This is also supported by the fact that the luminal isomerase activity is more redox sensitive in mutants lacking AtCYP20-2 (Paper IV). A special feature of AtCYP20-2 is that the protein is equally expressed in all tissues, including roots, in contrast to other chloroplast PPIases that are only expressed in photosynthetic tissues (Romano et al., 2004). A potential function of AtCYP20-2 could be a general long-term role in adaptation or regulation of light harvesting in PSII. This is supported by its association with PSII supercomplexes and increased abundance during high light (Paper II) and cold acclimation (Goulas et al., 2006). Identification of the five proteins whose abundance did change in the AtCYP20-2 deficient mutants

might also provide clues regarding the function and interacting partners of AtCYP20-2 (Paper IV).

AtCYP26-2 is the only luminal cyclophilin for which the luminal localization is based only on theoretical prediction. AtCYP26-2 together with AtCYP28 and AtCYP37 are highly divergent from the human CyPA with an amino acid identity around 10%. One hypothesis is that these proteins have evolved specific functions and might only play an important role under certain conditions.

Luminal FKBP

No multidomain FKBP is localized in the lumen and very little is known about the functions of the 11 single domain FKBP residing in this compartment, where the predicted localization of AtFKBP17-1, AtFKBP17-2 and AtFKBP17-3 still awaits confirmation. The luminal localization of AtFKBP16-1 was very recently confirmed in a cold acclimation experiment in which the abundance of both AtFKBP16-1 and AtFKBP18 was decreased almost three times (Goulas et al., 2006). Microarray databases such as GENEVESTIGATOR (<https://www.genevestigator.ethz.ch>; Zimmermann et al., 2004) also present data regarding up or down regulation of many luminal immunophilins in response to stress situations. However, the significance of these findings remains unclear since no specific interaction partners or regulatory mechanisms have been ascribed to these proteins.

AtFKBP13 is the smallest and so far, the most studied luminal FKBP. Its translocation to the lumen via the Δ pH-pathway has been confirmed by an *in vitro* import assay (Gupta et al., 2002). Found to interact with the Rieske subunit of the Cyt b_6f complex in the stroma (Gupta et al., 2002), this enzyme is also thought to play an intricate role in redox regulation in the lumen, as will be discussed in the next section. AtFKBP13 is the most active PPIase on both substrates (Paper III) and its activity is regulated by formation of two disulfide bridges (Gopalan et al., 2004). The crystal structure of both the active oxidized form (Figure 10; Gopalan et al., 2004) and of the inactive reduced form (Gopalan et al., 2006) shows that reduction closes the binding pocket and thereby hindering the substrate access to the active site. In the initial redox study with recombinant AtFKBP13 both Trx and DTT (dithiothreitol) were required for inactivation of its PPIase activity (Gopalan et al., 2006). Nevertheless, the activity in both AtFKBP13 fractions and total Arabidopsis lumen was found to be drastically decreased by addition of DTT alone (Paper III and IV). However, it cannot be excluded that some other redox-mediating factor, present in lumen, participated in this reduction.

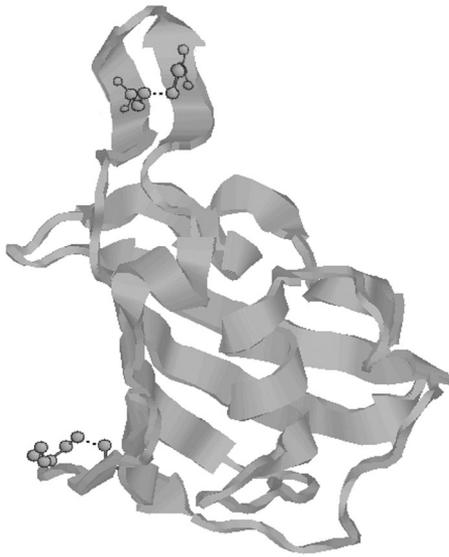


Figure 10. Three-dimensional structure of AtFKBP13. Cysteins involved in redox regulation are indicated as ball and stick models. Reduction of the disulfide bridges (dashed lines) inactivates the PPIase activity and reduces the access to the catalytically important amino acid residues. (PDB 1U79; Gopalan et al., 2004).

AtFKBP16-2 is similar to AtFKBP13 and the presence of all four cysteines suggests a similar regulation in the lumen depending on redox state. Although in this case, the *cis-trans* conversion of its substrate might not be the final target of regulation since this immunophilin lacks three of the five conserved residues (Figure 8) and is probably an inactive isomerase.

Recently another luminal redox sensitive FKBP was described (Lima et al., 2006). AtFKBP20-2 has two cysteines in the last sixteen residues, not present in any other Arabidopsis FKBP, which can be reduced by Trx. However, in contrast to AtFKBP13, the PPIase activity of AtFKBP20-2 was not affected by reduction. In fact, the overall activity of AtFKBP20-2 was found to be very low, only 1/500 of AtFKBP13, which might be explained by the lack of three of the five important residues for PPIase activity (Figure 8). Mutants deficient in this protein showed reduced accumulation of PSII supercomplexes and increase in PSII monomers and dimers leading to lower PSII activity and reduced plant growth (Lima et al., 2006).

Redox regulation of chloroplast PPIases

Many chloroplast stromal enzymes are known to be regulated by changes in redox state ($S-S \leftrightarrow 2SH$) where Trx acts as a sensor turning on and off other enzymes depending on present conditions. During light conditions and photosynthetic electron transfer, a reducing environment is created in the stroma and Trx functions as a link between light and enzyme activation. Many enzymes involved in the Calvin cycle are activated by reduction ($S-S$ to $2SH$)

while carbohydrate degrading enzymes are inactivated (Buchanan and Luan, 2005).

The stromal cyclophilin AtCYP20-3 is one of the targets of Trx (Motohashi et al., 2001; Motohashi et al., 2003) and like many stromal enzymes it is active when reduced. Recently identified interacting partners of AtCYP20-3 are the stromal peroxiredoxins (Laxa et al., 2007), which further strengthens the idea of a role in regulation of/by AtCYP20-3 depending on redox status.

A striking finding was that the precursor form (with at least the luminal transit peptide) of AtFKBP13 was able to interact with the redox active Rieske subunit of the Cyt b_6f complex (Gupta et al., 2002). The later identification of two disulfide bridges, which regulated AtFKBP13 activity (Gopalan et al., 2004), extended the redox regulation function from the stroma into the lumen. However, AtFKBP13 was active when oxidised (2SH to S-S) in contrast to AtCYP20-3, which suggested an opposite mechanism for redox control in the lumen as compared to the stroma (Buchanan and Luan, 2005). An elegant mechanism in which AtFKBP13 functions in three distinct ways depending on its maturation, localization and redox state has been proposed (Gopalan et al., 2004; Buchanan and Luan, 2005; Romano et al., 2005). Briefly, the oxidized precursor of AtFKBP13 acts as a chaperone for the Rieske protein in the stroma. During reducing conditions and increased demand of Cyt b_6f subunits, AtFKBP13 is reduced by Trx and the FKBP-Rieske complex is transported across the thylakoid membrane. Upon entry into the lumen, both AtFKBP13 and Rieske protein are processed into mature forms and dissociate. Free AtFKBP13 may then exert its PPIase activity in different regulatory functions possibly on other targets in the lumen. If this holds true it is the first proposed mechanism in which a luminal protein functions in different compartments of the chloroplast depending on its maturation.

Considering that the Rieske subunit is the only protein in the Cyt b_6f complex that is nuclear encoded, a tight control of both its insertion and further actions by AtFKBP13 provides an excellent way for optimizing the fine-tuning of electron flow in the transport chain. The oxidative activation of PPIase activity of AtFKBP13, first studied on recombinant protein (Gopalan et al., 2004; Gopalan et al., 2006), is also supported by the studies on endogenous protein performed in this thesis work. The PPIase activity in fractions of isolated AtFKBP13 is significantly repressed by addition of the reducing agent DTT (Paper III). Moreover, the constant level of PPIase activity and increased sensitivity to reducing agents in mutants lacking AtCYP20-2 is probably due to oxidative activation of AtFKBP13 (Paper IV). However, the enzymes catalyzing the oxidation and reduction of AtFKBP13 *in vivo* are still unknown. One candidate is cytochrome c_{6A} , a protein identified through its interaction with AtFKBP13 in a yeast two-hybrid assay (Gupta et al., 2002). Cytochrome c_{6A} has been proposed to catalyze the formation of disulphide bridges in luminal proteins by a single-step disulphide exchange reaction with subsequent transfer of the reducing equivalents to plastocyanin (Schlarb-Ridley et al., 2006).

Another candidate is HCF164, which recently was found to mediate reducing equivalents from Trx in the stroma, through the thylakoid membrane and into the lumen (Motohashi and Hisabori, 2006). HCF164 is a luminal thioredoxin-like protein anchored in the thylakoid membrane and originally found to be involved in Cyt *b₆f* biogenesis (Lennartz et al., 2001).

Another PPIase that might be involved in regulation of electron transport is the stromal parvulin AtPIN3. In vertebrates, rhodanese is a mitochondrial enzyme involved in formation of iron-sulfur complexes. The rhodanese domain of AtPIN3 might very well serve the same purpose in assembly of iron-sulfur proteins such as the Rieske subunit and ferredoxin.

If other redox sensitive immunophilins such as AtFKBP20-2 and probably AtFKBP16-2 fit into this evolving picture of redox regulation in the lumen, remains to be discovered.

CONCLUDING REMARKS

Summary

During this thesis work the knowledge regarding luminal PPIases has significantly increased, from 1 single identified cyclophilin (TLP40) to the predicted presence of 5 cyclophilins and 11 FKBP in Arabidopsis. 12 of these 16 immunophilins have now been identified in proteomic studies of the lumen. However, the functional characterization of these immunophilins is still at an early stage and regulatory mechanisms have been addressed to only some of them.

In this thesis work a novel cyclophilin, TLP20, was identified and proved to be the most active PPIase in the spinach lumen. In contrary to TLP40, but like most conserved cyclophilins, the PPIase activity of TLP20 was inhibited by the specific inhibitor CsA. The Arabidopsis homologue of TLP20, AtCYP20-2, together with the stromal AtCYP20-3, are the most conserved and only active chloroplast cyclophilins. The remaining Arabidopsis cyclophilins have lost several of the crucial residues involved in prolyl isomerization and might not be active PPIases. The expression of AtCYP20-2 is increased in high light and further characterization revealed that both AtCYP20-2 and AtCYP38, the spinach homologue of TLP40, are membrane associated. The membrane association of AtCYP20-2 was predominantly found with PSII supercomplexes but not with PSII cores. Fractionation of immunophilins from total lumen also revealed some intra-species differences, as the spinach TLP40 was active while the Arabidopsis homologue AtCYP38 was not. Additionally, AtFKBP13, a protein not identified in spinach, was found to be the most active immunophilin in Arabidopsis. However, since the total number of PPIases in spinach lumen is unknown it is impossible to draw any further conclusions regarding these species differences. A steady state of total isomerase activity is thought to be important since mutant plants lacking AtCYP20-2 displayed no visible phenotypical differences and a constant PPIase activity level was preserved. Comparative analyses revealed no increased abundance of any other

immunophilin that could explain the compensation for AtCYP20-2 absence. Our experimental data suggest the compensation of the isomerase activity due to oxidative activation of AtFKBP13.

Future perspectives

Although PPIases are extensively studied in many different organisms very little evidence regarding the *in vivo* functions are known for most of them. The large number of PPIases present in the chloroplast emphasizes their importance. To maintain a precise regulation in the photosynthetic process and to meet all demands during fluctuations in both cellular and environmental conditions they are probably specific to individual targets. Although possibly functionally redundant with regard to the PPIase activity their specific interacting partners need to be elucidated in order to interpret the significance of preliminary data such as up or down regulation of different immunophilins during stress situations.

The use of the reverse genetic approach in which one or several PPIases have been deleted in combination with old and new biochemical and enzymatic techniques, will provide important information about their functions and regulatory mechanisms.

The genome sequencing projects and methodological development have given us the tools. It is now up to us to find the right experimental conditions and to solve the mystery of “Why are there so many immunophilins in the lumen?” Or to cite the last sentence in the final *Calvin and Hobbes* comic strip.

“It's a magical world, Hobbes, ol' buddy...Let's go exploring!”

TACK!

The accomplishment of this thesis would not have been possible without the help of all my co-workers, friends and family. I would like to express my deepest gratitude to the following people:

* Mina handledare **Alexander Vener & Bertil Andersson**. Alex för att din dörr alltid står öppen och för din förståelse för att kombinera forskning med familj och barn. Bertil för platsen som doktorand när du flyttade ner från Stockholm och för dina stora visioner.

* **Maria**, min rumskamrat och labbgranne i ur och skur. Tack för alla trevligt pratstunder om allt mellan himmel och jord. Det vi inte lyckats reda ut om helgaktiviteter, sportsliga framgångar, fantastiska experiment (i alla fall i teorin) och framför allt organisering av ett labb är inte värt att veta. Vi får se till att hålla tangenterna på datorn varma så att vi inte tappar kontakten innan du återvänder till Sverige.

* **Alexey**, my saviour. Finally someone who shared my interest for PPIases. I have really enjoyed these last years with nice chats and good company during endless days in the cold room.

* All **co-authors** for fruitful collaborations, especially Ulrika & Patrick for nice visits and fun in the lab.

* **Cornelia**, den enda i växtgruppen som hängt med under hela min tid. Tack för alla frågor, konstruktiva kommentarer och trevligt sällskap.

* **Sophie**, för sällskap och behövliga pratstunder. Synd att vi inte umgicks mer, tidigare.

* **Björn**, mångfaldens mästare. Du har alltid en hjälpande hand och är allas expert på datorer, orkidéer, inredning mm.

* **Masha**, för smarta kommentarer och åsikter om allt. Synd att vi inte tillbringade mer tid som mammalediga samtidigt.

* **Rickard**, vår nyaste gruppmedlem och en frisk fläkt i labbet. "Keep up the good work and order in the lab"

* **Lorena**, for your helpful way and your wonderful laughter, which always brightens my days.

* Alla tidigare medlemmar i växtgruppen. **Torill & Said** som var mina stöttepelare när jag började. **Julia, Georgios, Markus & Arti**, for nice jokes and company in the lab. Ni har alla bidragit till den person jag är idag.

* Alla människor på **Plan 13**, för att ni gör det så trevligt att gå till jobbet. Tack för att ni alltid svarat på alla mina frågor och försökte tillämpa era kunskaper på mina "gröna" prover. Jag tror nog att era cellinjer egentligen skulle må bra av en rejäl tillsats av klorofyll i mediet. Jag kommer att sakna alla aktiviteter på och utanför avdelningen och inte minst av allt, de smaskiga tillbehören på fikastunderna.

* All **administrativ personal** för att ni sköter den dagliga ruljansen på ett utmärkt sätt och har fått min tillvaro att flyta på.

* **Övriga doktorander och personal** som jag mött under min tid som doktorand. Tack för trevligt småprat beträffande undervisning och tips om olika metoder.

* Alla **övriga vänner** utanför Cellbiologen. Vad vore livet utan er? I alla fall mindre volleyboll, fika, fester och träffar på lekplatsen. Tack för att ni finns och ger mig perspektiv på tillvaron. Ett särskilt tack till mina gymnasievänner **Sanna, Cecilia & Ludwig**. Sanna för att du alltid bara är ett telefonsamtal bort. Det är få förunnat att ha en vän som du. Cecilia & Ludwig för alla långa kvällar, ändlösa diskussioner och barnpassning. Ni ska inte tro att ni slipper oss bara för att ni flytt Linköping. Nu följer vi efter (i alla fall en liten bit).

* **Den Edvardssonska familjen** med släkt och vänner. Ni tog emot mig med öppna armar och har gjort en plats för mig i er familj som jag alltid känner mig välkommen till. Nu ser jag fram emot mer tid tillsammans i "stugan".

* **Mamma & Pappa**, för att ni alltid finns där. Ni betyder allt och utan er hade jag aldrig nått ända hit. Tack för att ni alltid talar om att jag är bra, även när det inte känns så. Tack också till världens underbaraste **Farmor**. Vem kan glömma alla roliga saker vi gjort tillsammans.

* **Jon**, för att du helt enkelt är bäst. Tänk att vi faktiskt blev klara båda två (även om det hade varit skönt med ett par månader till emellan). Tack för att du alltid finns vid min sida och får mig att må bra.

* **Algot & Rakel**, mina små älsklingar. Ni får mig att se fram emot varje ny dag (även om jag inte sovit på natten). Tänk bara att få vakna upp av orden "Mamma, jag älskar dig".

*Hoppas att vi ses snart igen,
Anna*

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