

Linköping Studies in Health Sciences

Thesis 79

# NUCLEOTIDE-BINDING PROTEINS IN THE PLANT THYLAKOID MEMBRANE

Sophie Heurtel Thuswaldner



**Linköping University**

Division of Cell Biology  
Department of Biomedicine and Surgery  
Faculty of Health Sciences, Linköping University  
SE-581 85 Linköping, SWEDEN

Linköping 2006

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ISBN: 91-85643-03-3

ISSN: 1100-6013

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Paper I © 2006-2007, *Current Knowledge in Plant Cell Compartments*, Research Signpost Publisher, Kerala, India.

Paper III © 2006, *Biochimica et Biophysica Acta*, Elsevier B.V., San Diego, USA.

Printed by LiU-Tryck, Linköping 2006.





## ABSTRACT

Life on Earth is dependent on the oxygen produced through photosynthesis. The thylakoid membrane is the site for the light-driven reactions of photosynthesis, which oxidize water and supply energy in the form of ATP, mainly for carbon fixation. The utilization of ATP in the luminal space of the thylakoid has not been considered in the past. In the latest years, increasing evidence for nucleotide metabolism in the thylakoid lumen of plant chloroplasts has been presented; ATP transport across the thylakoid membrane, and GTP binding to the PsbO extrinsic subunit of the water-oxidizing photosystem II (PSII) complex.

In this thesis, various methods for prediction, identification, and characterization of novel plant proteins, are described. Nucleotide-binding motifs and nucleotide-dependent processes are reviewed, and the experimental data is discussed. 1) A thylakoid ATP/ADP carrier (TAAC) in *Arabidopsis thaliana* was identified and functionally characterized, and 2) the spinach PsbO protein was characterized as a GTPase. The *Arabidopsis* At5g01500 gene product is predicted as a chloroplast protein and to be homologous to the well-studied mitochondrial ADP/ATP carrier. The putative chloroplast localization was confirmed by transient expression of a TAAC-green fluorescent protein fusion construct. Immuno detection with peptide-targeted antibodies and immunogold electron microscopy showed the thylakoid as the main localization of TAAC, with a minor fraction in the chloroplast envelope. TAAC is readily expressed in etiolated seedlings, and its level remains stable throughout the greening process. Its expression is highest in developing green tissues and in leaves undergoing senescence or abiotic stress. It is proposed that the TAAC protein supplies ATP for energy-dependent reactions during thylakoid biogenesis and turnover. Recombinant TAAC protein was functionally integrated in the cytoplasmic membrane of *Escherichia coli*, and was shown to specifically transport ATP/ADP in a protonophore-sensitive manner, as also reported for mitochondrial AACs.

The PsbO protein stabilizes the oxygen-evolving complex of PSII and is ubiquitous in all oxygenic photosynthetic organisms, including cyanobacteria, green algae, and plants. So far only the 3D-structure of the cyanobacterial PsbO is available. Four GTP-binding motifs in the primary structure of spinach PsbO were predicted from comparison with classic GTP-binding proteins. These motifs were only found in the plant PsbOs, in the  $\beta$ -barrel domain of the

homologous 3D-structure. Using circular dichroism and intrinsic fluorescence spectroscopy, it was shown that MgGTP induces specific structural changes in the PsbO protein. Spinach PsbO has a low intrinsic GTPase activity, which is considerably stimulated when associated with a dimeric PSII complex. GTP stimulates the dissociation of PsbO from PSII under both inhibitory and non-inhibitory light conditions. A role for PsbO as a GTPase in the function of the oxygen-evolving complex and PSII repair is proposed.

## ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their roman numerals (I-III):

- I. Spetea, C., and Thuswaldner, S. (2006) *Update in nucleotide-dependent processes in plant chloroplasts*. Book chapter accepted in Current Knowledge in Plant Cell Compartments. (B. Schoefs, ed.) Research Signpost Publisher, Kerala, India.
- II. Thuswaldner, S., Rojas-Stütz, M., Lagerstedt, J. O., Bouhidel, K., Der, C., Leborgne-Castel, N., Mishra, A., Marty, F., Schoefs, B., Adamska, I., Persson, B. L., and Spetea, C. (2006) *Identification of an ATP/ADP carrier in the Arabidopsis chloroplast thylakoid membrane. Heterologous expression and functional characterization*. Under revision in J. Biol. Chem.
- III. Lundin, B\*, Thuswaldner, S\*, Shutova, T., Eshaghi, S., Samuelsson, G., Barber, J., Andersson, B., and Spetea, C. (2006) *Subsequent events to GTP binding by the plant PsbO protein: structural changes, GTP hydrolysis and dissociation from the photosystem II complex*. Biochim. Biophys. Acta, in press, doi:10.1016/j.bbabi.2006.10.009.

\* These authors equally contributed to this work.



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## ABBREVIATIONS

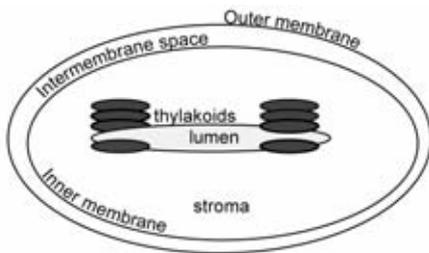
Listed below are selected abbreviations used in the text.

AAC	ADP/ATP carrier
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AMPL	Arabidopsis Membrane Protein Library
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
MCF	Mitochondrial carrier family
NDPK	Nucleoside diphosphate kinase
NTT	Nucleotide translocator
OEC	Oxygen-evolving complex
PS	Photosystem
PsbO	33-kDa extrinsic subunit of PSII
TAAC	Thylakoid ATP/ADP carrier
TM	Transmembrane



## Photosynthesis in plant chloroplasts

Oxygenic photosynthesis in cyanobacteria, green algae, and plants is essential for life on our planet. In plants, this process takes place in the chloroplast. This organelle consists of six distinct compartments: the chloroplast envelope, with the outer and inner membrane enclosing the soluble intermembrane space, the soluble stroma, the thylakoid membrane, and its luminal space (*Figure 1*).



*Figure 1: The chloroplast and its compartments: the envelope membrane, The soluble stroma, and the thylakoids.*

The electron transport chain in the thylakoid membrane oxidizes water and drives the production of ATP and NADPH, used for carbon fixation in the stroma. Four protein complexes in the thylakoid membrane are involved in the light-driven reactions: photosystems (PS) I and II, cytochrome  $b_6/f$ , and the ATP synthase. Since the water-oxidizing PSII complex is the initiation point of the photosynthetic process, this complex has been the focus of intense study ever since the concept of the two photosystems was established through the experiments of Emerson (Emerson *et al.*, 1957) and Duysens (Duysens *et al.*, 1961). PSII is also the primary target for light-induced inactivation (photoinhibition), leading to damage of the reaction center D1 subunit (*Figure 2*). To overcome the photoinhibition, damaged D1 protein is rapidly degraded and replaced by a newly synthesized protein (Mattoo *et al.*, 1984; Ohad *et al.*, 1984). D1 protein degradation proceeds in at least two steps with different nucleotide requirements (Spetea *et al.*, 1999). The primary step is GTP-dependent, while the secondary step depends on ATP and zinc. Several studies have shown that the FtsH and DegP<sub>2</sub> proteases, both active on the stromal side of the thylakoid

membrane, are involved in the D1 protein degradation (Lindahl *et al.*, 2000; Haußühl *et al.*, 2001).

PSII is composed of over 30 proteins encoded by both the nuclear and the chloroplast genome (Barber, 1998), (Figure 2), and functions normally as a dimer (Bianchetti *et al.*, 1998). The structure of the PSII complex was determined in cyanobacteria by X-ray crystallography (Zouni *et al.*, 2001; Kamiya and Shen, 2003; Ferreira *et al.*, 2004; Kern *et al.*, 2005), and a model structure of the oxygen-evolving complex (OEC) was proposed (Ferreira *et al.*, 2004). Three extrinsic OEC proteins (Yamamoto *et al.*, 1981; Åkerlund and Jansson, 1981) located on the luminal side of the thylakoid membrane (Andersson and Åkerlund, 1978), stabilize the complex, and support the PSII activity, namely PsbO, PsbP, and PsbQ [reviewed in (Seidler, 1996)]. The photosynthetic proteins are highly abundant, as opposed to proteins involved in the biogenesis and turnover of the photosynthetic apparatus, as well as in adjustments to different environmental conditions. Those may be present in 10<sup>6</sup>-fold lower amounts, which make them hard to identify and localize by classic proteomic approaches.

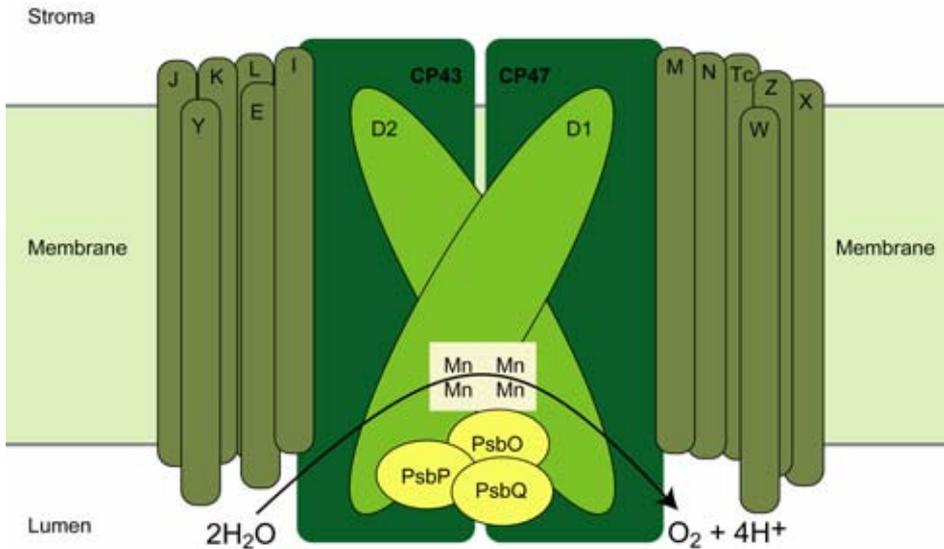


Figure 2: The plant PSII complex with intrinsic and extrinsic subunits. The oxygen-evolving complex (Mn<sub>4</sub>) produces oxygen and protons from water. For clarity, LHCII is not shown.

The thylakoid lumen has been considered to be mainly a proton sink and containing only a few proteins, directly involved in the photosynthetic process. On the contrary, biochemical and proteomic studies of the last decades have shown that it contains about 80 different proteins (Kieselbach *et al.*, 1998; Peltier *et al.*, 2002; Schubert *et al.*, 2002) among which members of the immunophilin (Fulgosi *et al.*, 1998), chaperon (Schlicher and Soll, 1996), carbonic anhydrase (Park *et al.*, 1999), violaxanthin deepoxidase (Hieber *et al.*, 2000), peroxidase (Kieselbach *et al.*, 2000), and protease (Adam, 2001) families have been identified. The luminal space has also been shown to have an active nucleotide metabolism (Spetea *et al.*, 2004), although luminal preparations have previously been tested for ATP and ATPase activity with no conclusive results (Kieselbach *et al.*, 1998). Two nucleotide-binding proteins were identified in the spinach thylakoid membrane: the PsbO protein and a nucleotide carrier of 36.5 kDa (Spetea *et al.*, 2004). Furthermore, a nucleoside diphosphate kinase (NDPK), NDPK3, was localized and characterized in the lumen. This protein serves as a link between the ATP transport by the nucleotide carrier and GTP binding to PsbO (*Figure 3*), by catalyzing the transfer of  $\gamma$ -phosphate from ATP to GDP. Three isoforms are known in plants, each with distinct sub-cellular location (PAPER I). In pea, mitochondrial NDPK has been shown to interact with the mitochondrial ADP/ATP carrier (AAC) with indications for a possible function in activation of a G-protein (Knorpp *et al.*, 2003).

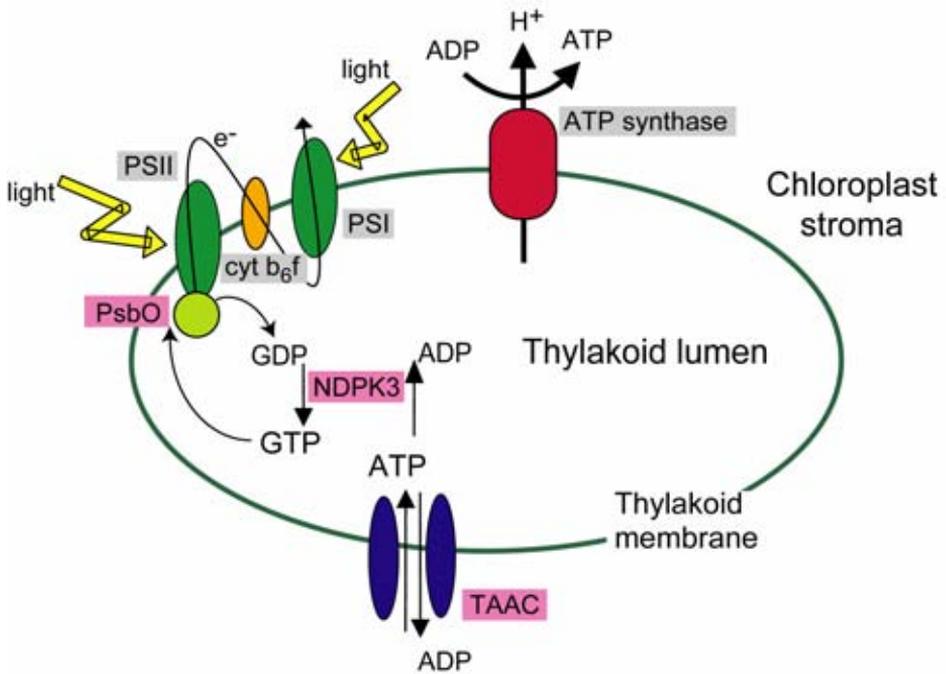


Figure 3: Electron transport, ATP synthesis, transport, and metabolism in the thylakoid membrane. Shown are the complexes involved in electron transport: PSII, cytochrome  $b_6f$  and PSI. ATP is synthesized by the  $H^+$ -translocating ATP synthase, and translocated into the lumen by the thylakoid ATP/ADP carrier. The luminal NDPK3 converts ATP to GTP, which is bound and hydrolyzed by the PsbO subunit of PSII.

The experiments included in this work were carried out using *Spinacia oleracea* (spinach) and *Arabidopsis thaliana* (Arabidopsis). Spinach is a model plant with a long tradition in photosynthesis research; it is reasonably fast growing, and gives a large amount of material for biochemical experiments. It is also commercially available year around. An important drawback is that the spinach genome is large and not sequenced. Arabidopsis is a weed used as a model plant and appreciated by researchers for its small genome (5 chromosomes), short generation time, ease of transformation, and large number of offspring. Its genome was fully sequenced in 2000 (A. G. I., 2000) which makes it easy to search for putative proteins in publicly available databases. Drawbacks in comparison with spinach are that its organelles are fragile and sensitive to inactivation during preparation, and that its small size makes it difficult to obtain enough quantities of sample material for experiments.

The focus of this work was to identify, and characterize the activity of, nucleotide-binding proteins in the thylakoid membrane. This was accomplished by using an appropriate set of methods available.

## **Methods for prediction, identification, and characterization of plant proteins**

The Arabidopsis genome encodes approximately 25,000 predicted protein sequences (A. G. I., 2000). Of these proteins, 15 % are predicted to be targeted to chloroplast [using TargetP, (Peltier *et al.*, 2004)], 18 % are predicted as membrane proteins (Ward, 2001), and at least 2 % are predicted as chloroplast membrane proteins (Sun *et al.*, 2004). Many of these have been assigned putative functions based on homology with known family members, but the task to identify and localize the proteins, and experimentally confirm or resolve plant specific functions, remains to a large extent. Below, a selection of methods available for this task is described. Several of these require a considerable amount of plant material, and optimization of culturing is therefore of importance. For growing of large plants of Arabidopsis, hydroponic systems have been developed (Norén *et al.*, 2004).

### **Bioinformatic tools and databases**

*In silico* analyses and database searches are valuable tools for initial studies of putative proteins in the post-genomic era. There are several extensive resources openly accessible on the web that collect and structure information from different sources and predictive programs. Here, a few that have specialized on plants are mentioned.

TargetP [<http://www.cbs.dtu.dk/services/TargetP>, (Emanuelsson *et al.*, 2000)] is a neural network-based tool widely used for prediction of intracellular targeting and cleavage site of transit peptides. It uses N-terminal sequence information to predict a mitochondrial, chloroplast, ER (for subsequent transport through the secretory pathway), or “other” localization. As most eukaryotic proteins are synthesized in the nucleus and transported to their final localization, this is a valuable tool for prediction of targeting. The N-terminal targeting sequence usually contains enough information to distinguish between the mitochondrion, the chloroplast and the ER. The predictions are made based on known patterns of the primary sequence of signal peptides (with destination ER), mitochondrial targeting peptides, and chloroplast transit peptides.

TMHMM 2.0 [<http://www.cbs.dtu.dk/services/TMHMM-2.0>, (Krogh *et al.*, 2001)] and HMMTOP 2.0 [<http://www.enzim.hu/hmmtop>, (Tusnady and Simon, 2001)] are two of the most popular programs available for prediction of transmembrane domains [more tools evaluated in (Lao *et al.*, 2002)]. The number of predicted membrane-spanning regions is valuable information when designing experiments to elucidate the topology of a protein. Unfortunately, different software often gives different results for the same protein. The discrepancy between the results is likely due to the variability of the TM span recognition in eukaryotic proteins, as many of the programs are trained with a set of known proteins of bacterial origin (Ikeda *et al.*, 2002). Although the tools available deliver more and more accurate predictions, an alignment is usually a more reliable way to get information about the topology if the structure of a homologous protein is available.

The Plant Membrane Protein database, ARAMEMNON [<http://aramemnon.botanik.uni-koeln.de>, (Schwacke *et al.*, 2003)], is searchable for both identified and putative proteins and contains a lot of information about prediction of transmembrane domains, homologous proteins in the plant, orthologues in other species, predicted subcellular location and bibliographic references. Its comprehensive interface makes it easy to use and to get an overview of the collected information.

The NSF Plant Genome Program provides the PlantsT database [<http://plantst.genomics.purdue.edu>, (Tchieu *et al.*, 2003)], which contains information on proteins with emphasis on mineral uptake and translocation. For each listed protein/gene, classification, alignments, and phylogenetic trees are given.

Complementary to the enzyme classification system according to the directives of the Joint Commission on Biochemical Nomenclature, of the International union of pure and applied chemistry (IUPAC), and the International Union of Biochemistry and Molecular Biology (IUBMB), where each enzyme is assigned a four-digit EC number, a classification system for membrane transport proteins has been developed and is recommended by the IUBMB (Saier, 2000). This system (including information about each classified protein family/subgroup) is found in the Transporter Classification Database (TC-DB, <http://www.tcdb.org>). See PAPER I for classification of the proteins in this thesis.

The Arabidopsis Membrane Protein Library [AMPL, <http://www.cbs.umn.edu/arabidopsis>, (Ward, 2001)] sorts the proteins in

families based on the TIGR gene models. Furthermore, it gives graphical representations of microarray data: relative expression levels of each gene in different plant tissues, developmental stages or during stress, derived from the AtGenExpress project using data from the TAIR website.

Genevestigator [<https://www.genevestigator.ethz.ch/at>, (Zimmermann *et al.*, 2004)] is a database containing experimental microarray data from Arabidopsis annotated by the Grüssens Laboratory and the Functional Genomics Center Zurich. The results are presented in comprehensive graphics showing gene expression in different tissues and up- and down regulation during different developmental stages, and during stress conditions, similar to the AMPL.

The Plastid Proteome Database [PPDB, <http://ppdb.tc.cornell.edu>, (Friso *et al.*, 2004)] is dedicated to plant (presently mostly Arabidopsis) plastids and aims at being a central, curated experimental and predictional data deposit for plastid proteins and their properties. The database is searchable based on gene name, annotations, proteome experiments, plastid subproteomes, function etc.

## **Localization studies**

### Chloroplast import

Chloroplast import is a classic method for studying localization, where radiolabeled proteins are used. The gene of interest is cloned into a vector and *in vitro* transcribed and translated. The gene product is imported into chloroplasts, which thereafter are fractionated and the proteins analyzed by gel electrophoresis. Pea chloroplasts have been the model of interest for this assay, although after the sequencing of the Arabidopsis genome, this plant has gained interest (Aronsson and Jarvis, 2002).

### Green fluorescent protein

Since the discovery of the green fluorescent protein (GFP) (Shimomura *et al.*, 1962), and more importantly that the expression of the protein in other organisms creates fluorescence (Chalfie *et al.*, 1994; Inouye and Tsuji, 1994), the GFP has greatly contributed to our knowledge about protein localization and cellular pathways. The original GFP was isolated from the jellyfish *Aequorea victoria* and (together with its derivatives and orthologs) is unique in that the fluorophore is composed of amino acid residues contained in the polypeptide chain (Tsien, 1998). The technique to engineer a GFP tag onto a protein of interest

is relatively easy, and makes it possible to non-invasively monitor the spatial and temporal dynamics of fusion proteins in plants. The fluorophore is located within a barrel and is very resistant to denaturation. The most stable fusions are made using the N- or C-terminus, allowing the protein to fold correctly (Dixit *et al.*, 2006). The fusion DNA is transferred into plant cells by electroporation or into intact leaves by biolistic genetic transformation. Most reliable results are obtained through stable transformation of the plants. The localization of the GFP-construct is studied using a confocal laser-scanning microscope.

### Immuno detection with peptide-specific antibodies

Low abundant hydrophobic proteins, a description that matches transport proteins in the thylakoid membrane, are often difficult to isolate on a gel for further identification by, e.g., mass spectrometry (Rais *et al.*, 2004). Instead, antibodies are produced towards a synthetic peptide, corresponding to a chosen amino acid sequence of the protein. This peptide should be specific enough to identify only the protein of interest in the annotated genome.

### Immunogold electron microscopy

Peptide-specific antibodies are also used to pinpoint the location of the protein within a tissue with electron microscopy resolution. It is essential that the antibody is specific, and that it binds to an accessible part of the protein, e.g., the N- or C-terminus. The tissue (leaf) sample is incubated with the antibody followed by a corresponding secondary antibody coupled to gold particles, large enough to be visualized by transmission electron microscopy.

### Mass spectrometry-based proteomics

The low abundance of many proteins and the complexity of samples have created a need for highly sensitive analytical methods. After the sequencing of genomes and the availability of this information through databases, in combination with technical advances that allowed identification of proteins, mass spectrometry [reviewed in (Aebersold and Mann, 2003)], has become the method of choice for analyses of complex samples. Through this technique, amino acid sequence, post-translational modifications, and protein-protein interactions can be solved and studied. A mass spectrometer consists of an ion source, a mass analyzer, and a detector. The sample is ionized, the peptides separated according to mass and charge, and the number of ions at each mass-to-charge ratio is registered. Ionization is commonly achieved by matrix-assisted

laser desorption, or by electrospray ionization, where the latter allows for coupling of liquid chromatography separation in-line with the mass spectrometer, and thus analysis of complex samples.

The best result is obtained when used with small sets of proteins in a known context, but rapid advances are made also in large-scale proteomics. Sample preparation is crucial for a clear result, and is often done by 1-, or 2-dimensional gel electrophoresis, alternatively by in-line liquid chromatography. A critical amount of protein is needed for analyses, which often excludes very low abundant proteins. Because of the difficulty to analyze the obtained data, there is a need for enrichment of the proteins of interest in the sample to be analyzed.

### ***Functional characterization***

#### Circular dichroism

Circular dichroism (CD) spectroscopy gives atomic resolution data of proteins in solution. It provides useful information on protein secondary structure, as well as conformational changes that take place upon substrate binding (Sharom *et al.*, 1999). As measurements are done with the protein in solution, difficulties may be experienced with membrane proteins. It also gives less specific structural information than comparable methods as X-ray crystallography or protein NMR spectroscopy. Despite this, it is an appreciated method as it is quick and does not require large amounts of protein and extensive data processing.

#### Tryptophan fluorescence

The three aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine) may contribute to intrinsic fluorescence of proteins. Fluorescence from tryptophan is stronger than from the other two amino acids, and has long been known to be sensitive to the polarity of its local environment. It can be measured to gain information about, e.g., the folding and substrate binding of a specific protein (Vivian and Callis, 2001), since the spectra differs depending on whether the tryptophan is buried in the core of the protein or accessible to the solvent at the surface of the protein. A useful feature is that tryptophan often can be substituted for other amino acids by site-directed mutagenesis, with minimal effect on structure and activity.

## Heterologous expression

To study the biochemical properties of novel proteins, cloning of the gene and expression of the protein in *E. coli* can be performed. This strategy has been used successfully to characterize plant membrane proteins, including plastidic nucleotide transporters (NTTs) (Tjaden *et al.*, 1998), and AACs (Haferkamp *et al.*, 2002). If successfully targeted to the membrane, the system can be further used for, e.g., transport activity or topology studies. Methods have also been developed in other bacteria like *Lactococcus lactis* (Monné *et al.*, 2005).

Other expression systems, like *Saccharomyces cerevisiae*, are also widely used. This unicellular yeast is appreciated as a genetic tool (Barbier-Brygoo *et al.*, 2001) due to the many knockout strains lacking specific transport systems available. These have made it possible to clone the corresponding genes from plants and complement the missing transport function (Dreyer *et al.*, 1999).

The use of unicellular algae, like the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschyzon melolae*, also seem promising as expression systems (Shimogawara *et al.*, 1998; Minoda *et al.*, 2004).

*Xenopus* oocytes is another expression system used for identification and characterization of transporters from higher eukaryotes (Romero *et al.*, 1998; Nour-Eldin *et al.*, 2006). Isolation of the desired mRNA from a tissue enriched in the target transporter is followed by injection into oocytes. *Xenopus* oocytes are suitable for this approach because of their low endogenous transport activity and efficient translation apparatus (Nour-Eldin *et al.*, 2006). Their large size also make them excellent for biophysical studies like patch-clamp measurements of currents in ion channels (Liu *et al.*, 2006).

## Activity

Even though a putative function can be assigned to a protein based on sequence homology with a family of proteins, the final proof for the activity has to be obtained experimentally. A radioactive uptake assay is a method to measure transport activity and kinetic properties of recombinant transport proteins inserted into a bacterial membrane or incorporated into liposomes. With *E. coli* or another suitable expression system, uptake of radioactive labeled molecules (e.g. nucleotides) can be measured directly (Tjaden *et al.*, 1998), while in liposomes, the recombinant protein first has to be purified and reconstituted into the artificial membranes (Palmieri *et al.*, 2006). While expression in *E. coli* is a fast method, reconstitution into liposomes includes more experimental steps, but

with the advantage of being a “cleaner” system without additional *E. coli* proteins interfering with the activity measurements. This problem can also be encountered when using *in vivo* systems, e.g., intact mitochondria [used in (Haferkamp *et al.*, 2002)]. In addition to the endogenous protein, other processes are active and may lead to hydrolysis or degradation of the added radioactive molecule. The obvious benefit working with the endogenous protein *in vivo* is closer agreement with the function of the protein in the living organism.

## Phenotypic analyses of knockout mutants

Phenotypic study of mutant plants lacking a certain protein is a way to investigate the effects of protein deficiency on the whole organism. Experiments can be performed on intact plants, detached leaves, or in isolated preparations under normal or stress conditions. Stress can be, e.g., high light exposure during shorter or longer periods, darkness, drought, temperature variation etc.

Insertional mutagenesis is a valuable tool in plant functional genomics. T-DNA (Krysan *et al.*, 1999) or transposons are effective to disrupt plant genes, and several mutagenized populations have been created for Arabidopsis and made available through public databases [*T-DNA*: SIGnAL, <http://signal.salk.edu/tabout.html>, (Alonso *et al.*, 2003); FLAGdb/FST, <http://genoplante-info.infobiogen.fr>, (Samson *et al.*, 2002); GABI-Kat SimpleSearch, <http://www.mpiz-koeln.mpg.de/GABI-Kat>, (Li *et al.*, 2003; Li *et al.*, 2006); *Transposon*: Arabidopsis Genetrap, <http://genetrap.cshl.org>, (Wisman *et al.*, 1998)]. Since the sequence of the T-DNA is known, one advantage with this method for generation of mutants is that the T-DNA easily is localized by PCR amplification of the DNA regions flanking the T-DNA insertion site of an individual plant. This allows mapping to a specific gene in the sequenced genome. A large enough collection of these mapped genes makes it possible to find almost any gene knocked out in a corresponding mutant plant. The same gene may be represented in the databases by multiple mutant lines, where the gene are knocked out by T-DNA insertions in different locations, and some mutant lines may have more than one T-DNA insertion, thus having more than one gene knocked out. To ensure that experimental results originate from the gene of specific interest, it is recommended to analyze more than one mutant line, alternatively to secure that the used mutant line only contains one T-DNA insertion or that other insertions does not interfere with measurements.

## **Methods for identification of nucleotide-binding proteins**

### Nucleotide affinity labeling

This method allows for identification of the nucleotide-binding proteins during denaturing conditions. The sample is incubated with a radioactive nucleotide derivative (e.g. N<sub>3</sub>-ATP), which, upon a brief exposure to UV-light, becomes covalently bound to polypeptides with nucleotide-binding sites. The resulting radioactive products are analyzed by gel electrophoresis and visualized by phosphorimaging. Using this method, two nucleotide-binding thylakoid proteins were identified, namely the nucleotide carrier and GTP-binding to the PsbO protein (Spetea *et al.*, 2004).

### Nucleotide affinity chromatography

Affinity chromatography is performed by coupling a molecule of interest (e.g. a nucleotide) to a solid matrix by a reactive group, and using this to extract proteins reacting to the coupled molecule, from a sample mixture (Lolli *et al.*, 2003). Bound proteins are eluted under denaturing conditions and analyzed by gel electrophoresis and mass spectrometry. The method is useful for isolation of nucleotide-binding proteins, e.g., NDPK (Sommer and Song, 1994) from a known context.

## AIM

An NDPK has been shown to exist in the thylakoid lumen, and nucleotides are needed, e.g., for the degradation of the D1 protein of the PSII complex. It was also shown that nucleotide-binding proteins are present in the thylakoid membrane. In light of this knowledge, this thesis aimed to characterize and study nucleotide-binding proteins, and to provide an overview of the nucleotide-dependent processes, in plant chloroplasts. More specifically:

- 1) To identify and characterize a thylakoid ATP/ADP carrier from Arabidopsis,
- 2) To study the events following GTP binding to the spinach PsbO, namely structural changes, GTP hydrolysis and dissociation from the PSII complex.



# THYLAKOID NUCLEOTIDE-BINDING PROTEINS

There are different kinds of nucleotide-binding proteins including nucleotide transporters, proteins that use nucleotides for energizing various processes [e.g. ATP-binding cassette (ABC) proteins, using the energy from ATP hydrolysis for transport or other reactions], signaling proteins (e.g. GTPases), and NDPKs (reviewed in PAPER I). In this thesis, the focus is on two nucleotide-binding proteins in the thylakoid membrane: a thylakoid ATP/ADP carrier (TAAC) and PsbO as a GTPase. The 36.5 kDa spinach nucleotide carrier reported previously (Spetea *et al.*, 2004), was found to have a homolog in Arabidopsis that is encoded by the At5g01500 gene, which is further characterized in PAPER II.

## ***Nucleotide-binding motifs***

The nucleotide-binding motifs characterize proteins that may have very different biological functions. There are both motifs that are common for different nucleotides and those that are specific for, e.g., ATP or GTP. A common motif found in GTP- and ATP-binding proteins is the phosphate-binding loop (P-loop) (Saraste *et al.*, 1990). This specific sequence has also been called motif A, has the consensus pattern [AG] – X(4) – G – K – [ST] (PROSITE accession number PS00017), and is involved in binding the  $\beta$ -phosphate of a bound nucleotide.

Members of the GTPase superfamily share four conserved sequence motifs: G1-G4, binding different regions of the GTP molecule (Bourne *et al.*, 1991). G1 is the P-loop motif mentioned above, G2 (D – X(n) – T) binds the  $\gamma$ -phosphate group of the nucleotide, G3 (D – X(2) – G) is involved in  $Mg^{2+}$ -binding, and the G4 motif (N – K – X – D/E) determines the specificity for guanine over other nucleotides. The domains flanking the G2 and G3 motifs change conformation depending on binding of GTP or GDP to the protein. These four GTP-binding motifs are partly conserved in PsbO, and are all found in the  $\beta$ -barrel domain of the protein, as judged from comparison with the 3D-structure of the cyanobacterial homologue (PAPER III). These domains are plant specific, and are thus not found in the cyanobacterial or green algae PsbO proteins.

The AACs of the mitochondrial carrier family (MCF) have a unique signature: RRRMMM. The first two arginines are involved in interaction with a specific inhibitor, carboxyattractyloside (Nury *et al.*, 2006). These two residues are

conserved in the TAAC, although the AAC signature in total is only partially conserved (RRqMql, where the lower case letters indicate non-conserved residues) (PAPER II), as in the case of two other AACs (Nury *et al.*, 2006). The relevance of this signature for binding and/or transport of adenine nucleotides is not clearly understood. Another consensus sequence, namely the MCF motif, is highly conserved in the TAAC protein (PAPER II). Part of this motif is directly related to the basket shape of the carrier (Nury *et al.*, 2006). Several adenine nucleotide analogues can bind to the carrier with a high affinity without being transported, showing that binding requires a lower specificity than transport. A selectivity filter consisting of a ring of four well conserved positively charged residues (K22, R79, Y186 and R279 in the bovine AAC) is responsible for the uniqueness of the transport of adenosine nucleotides (Nury *et al.*, 2006). These residues are all conserved in the primary sequence of TAAC (PAPER II).

The ATP synthase alpha and beta subunits signature (PS00152) contains binding sites for ATP and ADP. This pattern is present in the ATP synthase localized in the cytoplasmic membrane of eubacteria, the mitochondrial inner membrane, and the thylakoid membrane of chloroplasts (Futai *et al.*, 1989), as well as in vacuolar ATPases in eukaryotic cells (Nelson, 1989).

The ATP-binding cassette (ABC) transporter-type domain profile (PS50893) is characteristic for the ABC transporter superfamily. The ABC module binds and hydrolyzes ATP and energizes a variety of biological processes, e.g., transport [reviewed in (Higgins, 2001)].

Other known nucleotide-binding motifs are, e.g.,: the biotin carboxylation (BC) domain profile (PS50979, ATP-binding), the GoLoco/GPR motif profile (PS50877, GTP-hydrolyzing), the HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domain (PS50910, suggested to be nucleotide-binding based on structural similarity), the HIT (histidine triad) domain profile (PS51084), the tubulin subunits alpha, beta, and gamma signature (PS00227, GTP-hydrolyzing), the phosphatidylethanolamine-binding protein family signature (PS01220), and the histidine kinase domain profile (PS50109, ATP/ADP-binding).

## **Nucleotide transporters**

The compartmentalization makes it possible to separate different functions in the cell, but it also emphasizes the need for efficient transport pathways between different locations. The cellular membranes act as barriers between the in- and

outside of the organelles, and transport proteins inserted in the membranes function as door-openers for molecules involved in transmembrane trafficking.

### ***Nucleotide transporters in plant chloroplasts***

Two structurally and phylogenetically different types of nucleotide transporters have been identified in plant chloroplasts, namely the plastidic nucleotide translocator (NTT) (Kampfenkel *et al.*, 1995; Möhlmann *et al.*, 1998), and the TAAC (PAPER II). For a review on the ATP-binding transporters in chloroplast, see PAPER I. The NTT belongs to the ATP/ADP antiporter (AAA) family, and resides in the chloroplast inner envelope. The AAA proteins possess twelve putative TM domains. The functional unit is a monomer as compared to the mitochondrial AAC, which is organized as a homodimer. Two isoforms, NTT1 and 2, exist, and their major physiological role is to provide the plastid with ATP (Reiser *et al.*, 2004). The TAAC belongs to the MCF, represented in animals, plants, and yeast and consisting of nuclear-encoded proteins in the range of 30 kDa. It has no orthologues in prokaryotes, suggesting that this family of proteins has emerged as a consequence of a primitive eukaryotic cell incorporating an ancient aerobic prokaryotic cell. The structure of most members displays six TM domains in three homologous repeats of about 100 amino acids each. The mitochondrial AAC is the best studied member of this family, and it is found in the mitochondrial inner membrane importing ADP to the matrix and exporting ATP to the cytosol through the intermembrane space. In addition to the mitochondrion, these carriers have also been found in the peroxisome, hydrogenosome and amyloplast (Laloi, 1999). The structure of the bovine mitochondrial AAC has been solved to the resolution of 2.2 Å in the conformation stabilized with carboxyatractyloside (Pebay-Peyroula *et al.*, 2003).

### ***A Thylakoid ATP/ADP carrier***

The Thylakoid ATP/ADP carrier (TAAC) is the first AAC found in the chloroplast, and the first ATP transporter found in the thylakoid membrane. The concept of a nucleotide transporter in the thylakoid had not been considered in the past, but with the finding of nucleotide metabolism in the thylakoid lumen (Spetea *et al.*, 2004) the need for such a carrier was realized. The encoded *Arabidopsis* TAAC protein (TrEMBL Q9M024) contains 415 amino acids and an N-terminal plastid transit peptide. The TAAC protein was confirmed to correspond to the ATP transport activity of a MCF member described previously in the thylakoid membrane by Spetea *et al.* (2004). By aligning the protein

sequence of the TAAC with that of the bovine AAC, six transmembrane helices were predicted, and a model of the secondary and tertiary structure was constructed (PAPER II). Visualization of fluorescence by transiently expressed GFP-tagged TAAC demonstrated a chloroplast location for TAAC, and immuno detection and immunogold-labeling with peptide-specific antibodies, localized the TAAC protein to the thylakoid membrane, with a minor fraction in the chloroplast envelope (PAPER II).

The TAAC gene, At5g01500, was successfully expressed in *E. coli* and shown to be inserted into the cytosolic membrane (PAPER II). The TAAC-mediated transport of nucleotides across the bacterial membrane was measured by uptake of radiolabeled ATP. The results show that ATP and ADP are specifically transported following Michaelis-Menten kinetics, as opposed to other nucleotides. In thylakoids, we made use of the NDPK activity in the thylakoid lumen [described in (Spetea et al., 2004)], and the amount of GTP produced in the luminal space was measured. Analysis of knockout mutant plants deficient of the TAAC protein (Institute National de la Recherche Agronomique, FLAGdb) show a decrease to 60-70 % of wild type levels, in the GTP production following ATP import (Figure 4, unpublished results). These results confirm the impact of TAAC on the nucleotide metabolism in the lumen, with further implications for, e.g., D1 protein turnover. The NDPK activity may depend on other sources of ATP than import by TAAC, why GTP production in the lumen of knockout plants could not be completely abolished. As an alternative, other, yet unidentified, transporters may partially compensate for TAAC absence.

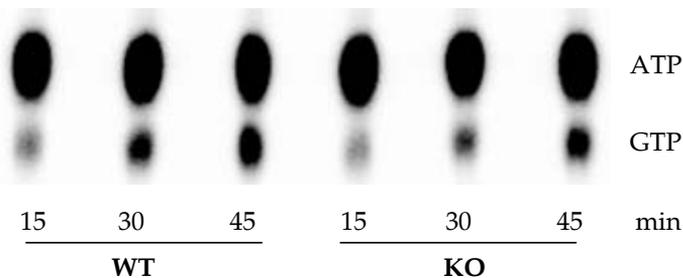


Figure 4: Analysis of the GTP production following ATP import in wild type and knockout mutant plants lacking the TAAC protein. The amount of GTP was measured after 15, 30, and 45 minutes.

TAAC transcript levels in the plant cell was measured by Northern blot, and results show a different pattern than those retrieved from collections of microarray data (PAPER II). Expression was highest in developing green tissues (leaves, flower buds, and siliques) and in senescent leaves, following the pattern of the TAAC protein. Expression was low in mature leaves, where the predominant location of TAAC was in the thylakoid. This implies that TAAC may have multiple functions and its location depend on the developmental stage of the plastids, with an initial location to the envelope and participation in the development of the thylakoid, and thylakoid localization in mature chloroplasts (PAPER II).

## **GTP-binding proteins**

GTP-binding proteins, or GTPases, are proteins that bind and hydrolyze GTP. The GTPase superclass is the largest protein family with signaling function in eukaryotic cells, 20 distinct families divided into 57 subfamilies have been identified.

### ***GTP-binding proteins in plant chloroplasts***

GTP-binding proteins identified in the plant chloroplast use the energy from hydrolysis of GTP to perform various functions. Translocon receptors at the outer membrane of chloroplast Toc34 and Toc159 and their homologues, and two signal recognition particle GTPases (SRP54 and SRP43) participate in translocation of proteins across the envelope and thylakoid membrane, respectively; translation factor EF-Tu is active in translation, and dynamins (ADL1 and ADL2) function in chloroplast division. In addition to these, the well-known 33-kDa extrinsic subunit of PSII (PsbO) in spinach has been shown to bind GTP (Spetea *et al.*, 2004). For a review on GTP-binding proteins in chloroplasts, see PAPER I.

### ***The PsbO protein as a GTPase***

The PsbO protein is known to function in stabilizing the OEC, where the water-splitting reaction that generates O<sub>2</sub> as well as electrons to the transport chain, takes place. PsbO is present in all known oxygenic photosynthetic organisms, showing a high degree of conservation (De Las Rivas and Barber, 2004), though its functional role may differ. Mutant studies in cyanobacteria have shown that the PsbO protein is not essential for the water oxidation process (Burnap and

Sherman, 1991), whereas in *Chlamydomonas reinhardtii* and Arabidopsis, it dictates the PSII stability (Yi *et al.*, 2005). The structure of the cyanobacterial PsbO from *Thermosynechococcus elongatus* at 3.5 Å resolution bound to PSII was solved (Ferreira *et al.*, 2004) and made it possible to model the plant PsbO (De Las Rivas and Barber, 2004).

As opposed to spinach, in Arabidopsis, PsbO isoforms are encoded by two genes: At5g66570 and At3g50820, expressed as PsbO1 and PsbO2 protein, respectively. The two isoforms have been shown to have different functions, where PsbO1 supports PSII activity while interaction between PsbO2 and PSII regulates the turnover of the D1 protein (Lundin *et al.*, 2006).

Spectroscopic measurements show that MgGTP binding to PsbO induces specific changes in the secondary and tertiary structure of the protein in solution (PAPER III). These changes in the PsbO structure are attributed mostly to modifications in the protein domain containing, or located close to, the predicted G domains, and were not detected in the absence of Mg<sup>2+</sup>. This is in line with the requirement of Mg<sup>2+</sup> for high affinity binding of GTP by small GTPases (Bourne *et al.*, 1991). For measurement of the GTPase activity of PsbO (PAPER III), <sup>32</sup>P-GTP was added to the purified PsbO or isolated PSII complexes, and the production of GDP was measured. The results show that purified PsbO has a low intrinsic activity, which is strongly enhanced by association of the protein to the PSII complex in its dimeric form (PAPER III). The PsbO recombinant protein was obtained by cloning of the spinach *psbO* gene and subsequent purification [also done earlier by (Betts *et al.*, 1994)]. Analyses show similar activity for both the recombinant and the purified endogenous protein (PAPER III). The effect of GTP on the release of PsbO and D1 protein was also studied. The mechanisms of GDP-, and GTP-binding of small GTPases are well known, and following GTP hydrolysis, the inactive GDP-form of the protein may be released from the membrane. Photoinactivation of PSII electron transport has been known to induce dissociation of the PsbO protein from its docking site (Hundal *et al.*, 1990). GTP stimulates the dissociation of PsbO from PSII under both inhibitory and non-inhibitory light conditions (PAPER III). D1 protein degradation was stimulated by GTP as a consequence of photoinactivation of oxygen evolution.

It is proposed that higher plant PsbO is not only able to bind GTP, but also to carry out hydrolysis (PAPER III). This conclusion is based on bioinformatic, as well as experimental evidence from spectroscopic measurements, and activity measurements with radioactively labeled GTP. As there are no reports of

interaction between PsbO and the proteases involved in D1 protein degradation, located in the stroma-exposed regions of the thylakoid membrane, it is proposed that the GTPase activity of PsbO is involved in repair events occurring in the grana regions (PAPER III), such as monomerization of damaged PSII complexes. This monomerization is followed by migration to the stroma lamellae, D1 protein degradation and *de novo* synthesis (Aro *et al.*, 2005).



## CONCLUSIONS

- There is a thylakoid ATP/ADP carrier (TAAC) shown by prediction and experimental evidence to be localized to the thylakoid membrane with a minor fraction in the chloroplast envelope. The TAAC transports ATP and ADP in a counter exchange mode, as shown in *E. coli*.
- The expression of At5g01500 (the TAAC gene) is highest in developing green tissues and in leaves undergoing senescence or abiotic stress. We propose that the TAAC protein supplies ATP for energy-dependent reactions during thylakoid biogenesis and turnover.
- PsbO was characterized as a GTPase. Four GTP-binding motifs in the structure of spinach PsbO were predicted and MgGTP shown to induce specific structural changes in the PsbO protein.
- The GTPase activity of the spinach PsbO is optimal when associated with a dimeric PS II. A role for PsbO as a GTPase in the function of the OEC and D1 protein turnover is proposed.



## FUTURE PERSPECTIVES

There are several directions this project could take in the future, some ideas for further study of TAAC and PsbO and their functions in the thylakoid membrane are listed below.

- Integration of purified TAAC into liposomes and further studies of the kinetic properties of the protein would give valuable information about transport activity from a clean, isolated environment.
- Mutational study of heterologously expressed TAAC to elucidate topology of the protein, as well as the function of the N-terminus.
- Phenotypic analyses of Arabidopsis knock-out mutants lacking TAAC, including photosynthetic measurements, high light tolerance etc.
- Study of the role of GTP in light-induced monomerization of PSII.
- Crystallization of PsbO and analysis of structural dynamics induced by GTP.
- Predictional and functional characterization of other thylakoid transporters essential for the nucleotide metabolism, e.g., a thylakoid phosphate transporter.



## ACKNOWLEDGEMENTS

This work was supported by the Graduate Research School in Genomics and Bioinformatics (FGB) in which Sophie Heurtel Thuswaldner was enrolled during the course of this thesis.

I would like to express my appreciation to everyone around me that has supported me during my time as a graduate student, in particular to:

My supervisor **Cornelia Spetea Wiklund**, for your presence, your encouraging holiday gifts, your experimental skills, and always valuable comments on my work.

**Bengt L. Persson**, my other supervisor. Though stationed in Växjö, or later, Kalmar, and often traveling, I have a standing offer of help whenever needed. Thank you for your understanding and encouraging attitude and your engagement in my work.

**Björn Lundin**, my desk neighbor and closest co-worker. Always there, and ready to air your opinions, and to listen to mine, for work and play. My first tango partner, not to forget! ☺

**Anna Edvardsson**, “senior scientist” in the lab, confident in every task! Your humility and honesty make a difference. Thank you for support and for being a friend “when the going gets tough”.

**Maria Hansson**, excellent organizational skills and always ready with a helping hand. Thank you for your companionship in the lab and “on the road”.

**Maria (Masha) Turkina**, Thank you for bringing tricky questions to our group meetings. ☺

**Lorena Ruiz Pavon**, always with a joyful laugh! I’ve really enjoyed having you as a co-worker and desk neighbor, both for work discussions and small talk.

**Alexey Shapiguzov**, easy-going Russian having a solution to every problem. Thank you for being flexible and helpful, always with a smile.

**Rikard Fristedt**, bringing a fresh breeze into the group with your interest, ideas, and air of youth. ☺

**Georgios Merkouropoulos**, your every-day “hello”, and practical questions made me feel valuable in the lab even when experiments weren’t going so great. “Down to earth”-guy when we all needed it.

**Marcus Evertsson**, starting as graduate students at the same time, we helped each other solving practical issues. I hope you got to do what you dream of, when you left us.

**Arti Mishra**, my first master thesis student as a co-supervisor. Your interest and ambition has been an inspiration to me, thank you for bringing a taste of India.

**Julia Vainonen**, short but pleasant acquaintance in the plant group.

**Elisavet Koutzamani**, thank you for being friendly, generous, and including me in your sphere of friends.

**Anna Gréen**, optimistic and smiling in all situations. Thank you for caring.

**Eva Hellqvist**, Thank you for nice company during coffee breaks and fun at parties!

**Anna Lanemo Myhrinder**, talkative and full of stories! Thanks for turning the dullest Monday morning into a gay occasion!

**Emelie Djerf**, Part of the group on floor 13, although not always as a graduate student. Thank you for always giving a nice word and an interesting comment.

**Nabila Aboulaich**, thank you for being my best friend during FGB workshops!

**Peter Strålfors**, for encouragement and interesting discussions in the corridor, when traveling with FGB and elsewhere.

**Maria Dahlström**, Thank you for being a nice, sharing, experienced discussion partner about problem-based learning and groups.

**Administration: Monika, Viveca, Anette and more...** Thank you for being patient with my questions and solving my problems in a nice way.

**Everyone on floor 13**, Thank you for helping out, bringing a smile, and making our floor a good place to work in. Not to forget all our tasty “fika”! ☺

**Jens O. Lagerstedt**, collaborator in Växjö/Kalmar and my “mentor”. You taught me how it is to be a graduate student, and how it could be in another group and another university. I truly appreciate all your help, guidance, and company during dinners and late nights at the lab.

**Fredrik Lundh**, a friendly face in Kalmar and always engaged in projects and activities. It's always nice to see you.

**Dijon Group:** Karim Bouhidel, Christophe Der, Nathalie Leborgne-Castel, Francis Marty, Benoît Schoefs. Thank you for taking care of me in France, helping me improve my french, and showing me how things are done in your lab.

**Jonas Frid**, Thank you for being there, listening, and often with some good advice. Short encounters, spontaneous meetings, late night discussion over several cups of tea – I'm privileged to be your friend. I hope my presence have helped you during your hard times, too.

**Albert Thuswaldner**, Thank you for listening to all my frustration and being by my side. Thank you for keeping up when I didn't cope.

*I hope this wasn't our last encounter!*

*Take care,*

*Sophie*



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