Xanthophyll Cycle in the Light of Thylakoid Membrane Lipids - Membrane Packing, Curvature Elastic Stress and Enzyme Binding

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2007

Xanthophyll cycle in the light of thylakoid membrane lipids

Membrane packing, curvature elastic stress and enzyme binding

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Doctoral dissertation

By permission of the Faculty of Science of Lund University, Sweden,
to be defended in lecture hall K:D at the Center for Chemistry and
Chemical Engineering, Getingevägen 60, Lund, on May 15th, 2007
at 10.30 am for the degree of Doctor of Philosophy

PhD thesis
Department of Biochemistry
Lund University
P.O. Box 124
SE-221 00 Lund
Sweden

ISBN 978-91-7422-157-2

Printed by Media-tryck, Lund, Sweden

Organization	Document name
LUND UNIVERSITY P.O. Box 124 SE-221 00 Lund Sweden	DOCTORAL DISSERTATION
	Date of issue April 2007
	Sponsoring organization
Author(s) Anna Szilágyi	

Title and subtitle

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Abstract

The xanthophyll cycle involves the light-dependent and reversible conversion of violaxanthin to zeaxanthin. The enzyme, violaxanthin de-epoxidase (VDE) catalyses the conversion of violaxanthin to zeaxanthin, via the intermediate antheraxanthin. VDE is a membrane-hosted enzyme during its activity. Low lumenal pH, violaxanthin, ascorbate and monogalactosyldiacylglycerol are required for activity. The formed zeaxanthin is involved in the protection of the photosynthetic apparatus from overexcitation. Apart from the energy-dissipating role, the xanthophyll cycle has been associated with a modulation of the physical properties of the thylakoid membrane.

During photosynthetic activity, the lumenal pH drops and VDE undergoes a conformational change causing the active enzyme to bind to the lumenal side of the thylakoid membrane. Binding studies of VDE to the thylakoid membranes showed that the conserved histidine residues in the lipocalin region are involved in the conformational change of the enzyme.
By altering the ratio between the lamellar and non-lamellar structures in the thylakoid membrane, the activity of VDE was influenced. When the thylakoids were treated with linolenic acid which increases non-lamellar prone structures in the membrane, enhanced zeaxanthin formation was seen. A model is proposed involving membrane curvature stress, thylakoid membrane packing and the xanthophyll cycle. According to this model the different xanthophyll pigments have different preferences for different membrane structures and can themselves induce differentially curved membrane regions. Zeaxanthin with its increased molecular hydrophobic length affects lipid packing, and brings about a release of curvature stress leading to a less favoured lipid environment for VDE.
Xanthophylls, based on their rigid molecular structure, have been suggested to modify membrane structure, more specifically to decrease membrane fluidity. We have shown that accumulation of zeaxanthin in the isolated thylakoid membrane resulted in an increased rigidity of the membrane measured as a red-shifted fluorescence emission spectrum using laurdan, as a probe.
These results further support the hypothesis that xanthophyll cycle pigments are located in the lipid phase of the thylakoid membrane and affect membrane physical properties.

Key words: xanthophyll cycle, zeaxanthin, VDE, histidine, enzyme binding, thylakoid membrane, membrane fluidity, lipid packing, curvature elastic stress, HPLC, laurdan, fluorescence spectroscopy

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language English
ISSN and key title:		ISBN 978-91-7422-157-2
Recipient's notes	Number of pages 117	Price
	Security classification	

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Publication list

This thesis is based on the following publications, which are referred to in the text by their roman numerals.

I **Role of histidines in the binding of violaxanthin de-epoxidase to the thylakoid membrane as studied by site-directed mutagenesis**
Gisselsson A, Szilágyi A, Åkerlund H-E
Physiol Plant (2004) 122: 337-343

II **Membrane curvature elastic stress controls the maximal conversion of violaxanthin to zeaxanthin in the violaxanthin cycle – influence of α-tocopherol, cetylethers, linolenic acid, and temperature**
Szilágyi A, Sommarin M, Åkerlund H-E
(2007) Submitted

III **Laurdan fluorescence spectroscopy in the thylakoid bilayer: the effect of violaxanthin to zeaxanthin conversion on the galactolipid dominated lipid environment**
Szilágyi A, Åkerlund H-E
Manuscript

Abbreviations

ABA	Abscisic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Proton translocating ATP synthase
CAB	Chlorophyll a/b binding proteins
CP	Chlorophyll-binding proteins
DGDG	Digalactosyldiacylglycerol
DPPC	Dipalmitoyl-phosphatidylcholine
DTT	Dithiothreitol
ELIP	Early light induced proteins
Fd	Ferredoxin
GP	General polarisation
H_{II}	Inverted hexagonal phase
IPP	Isopentenyl diphosphate
L_{α}	Fluid phase
Laurdan	6-Lauroyl-2-dimethylaminonaphthalene
LHCI	Light-harvesting complex I
LHCII	Light-harvesting complex II
MGDG	Monogalactosyldiacylglycerol
$NADP^{+}$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NPQ	Non-photochemical quenching
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PQ	Plastoquinone
PSAT	Phospholipid:sterol acyltransferase
PSI	Photosystem I
PSII	Photosystem II
qE	pH- or energy-dependent component of NPQ
qI	Photoinhibition
qT	State transition
RP-HPLC	Reversed phase - high performance liquid chromatography
SE	Sterol ester
VDE	Violaxanthin de-epoxidase
ZE	Zeaxanthin epoxidase

1 Preface

Photosynthesis, is the driving force of all living organisms on Earth. In the photosynthetic process energy from sunlight is utilised to convert water, carbon dioxide and minerals to sugars, proteins and all other components required for a living organism. The molecular oxygen we breathe is also a product of the process. Photosynthesis is carried out by higher plants, algae and some bacteria, on the land and in the sea. It takes place in the chloroplast, a specialised organelle, using photosynthetic pigments that act as light receptors capturing the energy of sunlight. The main pigments are called chlorophylls that absorb light in the visible spectrum, blue and red light most effectively. Green light is reflected, which is why leaves appear green. Photosynthetic organisms have evolved to take advantage of a broader range of the light spectrum than the absorption by chlorophylls offer. They have employed carotenoids another group of pigments for this reason. These yellow-orange pigments make the autumn leaves of the forest so colourful. However, a particular group of carotenoids, namely xanthophylls, are also involved in minimizing light damage when too much light is absorbed. To carry out photosynthesis more efficiently, chlorophylls are organised into pigment-protein complexes.

The protection of the photosynthetic machinery from serious damage is of special importance. Perhaps the most prominent photoprotective mechanism is the formation of zeaxanthin from violaxanthin, termed the *xanthophyll cycle*. With increasing light intensities, zeaxanthin and antheraxanthin are formed from violaxanthin by the enzyme violaxanthin de-epoxidase (VDE). In the presence of zeaxanthin too much absorbed light can be safely dissipated as heat, thus reducing the risk of photodamage. In the dark, zeaxanthin is transformed back to violaxanthin by zeaxanthin epoxidase, the other enzyme of the cycle.

All protein complexes and pigments required by the photosynthetic process are embedded in a special membrane, the *thylakoid membrane* of the chloroplast. Lipids are the basic building blocks of biological membranes. The thylakoids are mainly composed of specific lipids containing one or two sugar residues. The interesting question is what special roles these sugar-containing lipids have in the photosynthetic membrane and how adequate membrane fluidity is maintained for different physiological processes, e.g. during the process of photosynthesis and the function of xanthophyll cycle.

During my doctoral studies I have centred my interest around the interplay between the xanthophyll cycle and the lipid phase of the thylakoids using spinach as a model plant. This thesis is a summary of our investigations and findings, and it is based on three papers (I-III). The summary below starts with an introduction to photosynthesis and continues with discussions related to the xanthophyll cycle and its enzymes. I also present results on the effect of sterol esters in cold acclimation. Changes in the pigments of the xanthophyll cycle indicated the extent of stress. In the last chapter, I propose a new model in which the conversion from violaxanthin to zeaxanthin, and membrane curvature stress is discussed.

Paper I discusses the role of conserved histidine residues in binding of VDE to the thylakoid membrane. In Paper II the conversion of violaxanthin to zeaxanthin in thylakoids was followed at different temperatures and in the presence of lipid packing modifiers. The physical status of the thylakoid membrane upon conversion was studied in Paper III with fluorescence spectroscopy employing laurdan, a fluorescent probe.

2 Introduction to photosynthesis

In eukaryotes, photosynthesis takes place in specialized organelles, the chloroplasts (Fig. 1A). Evolutionary, the chloroplasts of eukaryotes originate from endosymbiotic cyanobacteria and have their own circular DNA. However, during evolution, most of the ancestral genes appear to have been either completely lost or transferred to the nucleus. Chloroplasts are surrounded by a double-layered envelope (outer and inner membranes) and contain an inner membrane network, called the thylakoids. The thylakoid membranes, where photosynthesis takes place, are further organised into stacked grana and unstacked stroma lamellae regions, surrounded by the fluid stroma. The thylakoid lumen is the compartment enclosed by the thylakoid membrane. Photosynthesis is a sequence of light-dependent and light-independent reactions. The light-dependent reaction is the first stage of the photosynthetic system, which converts solar energy into chemical energy. In this process molecular oxygen, ATP and NADPH are produced. To conduct the light-independent reactions (carbon fixation and the Calvin-Benson cycle), light is not directly required but the products of the light reaction are required to convert carbon dioxide into glucose. These reactions take place in the stroma, and the products will be further used in metabolic processes.

2.1 The thylakoid membrane

The thylakoid membrane is the most abundant biological membrane on Earth. It is the membrane milieu where the photosynthetic machinery is embedded (Fig. 1B), housing all light-harvesting and energy-transducing functions. The mature thylakoid membranes can differentiate into grana and stroma lamellae, also called stacked and unstacked regions, respectively. The grana are stabilized by stacking of thylakoids. It is generally assumed that light-harvesting complex II (LHCII) is responsible for this stacking. The stacked regions consist of a

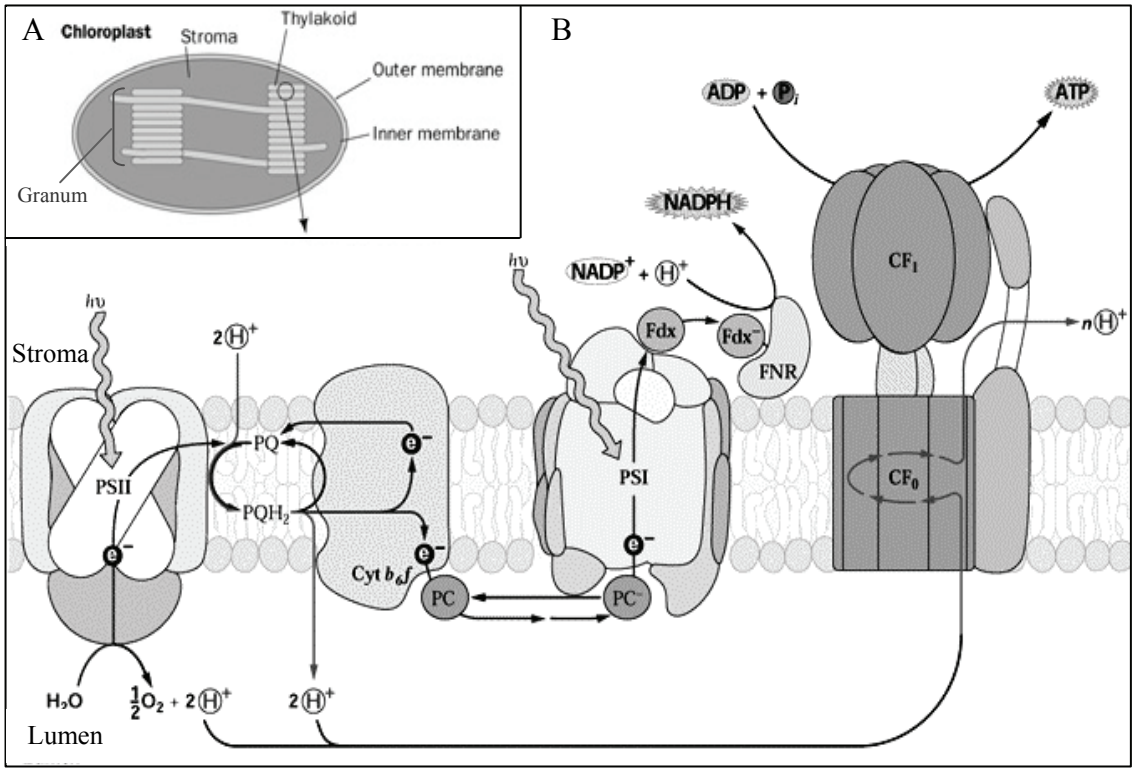

Figure 1. A) Chloroplast with the thylakoid membrane. B) A thylakoid membrane section depicting the scheme of photosynthetic electron transport. Photosystem II (PSII), cytochrome b_6/f complex, photosystem I (PSI) and the proton translocating ATP synthase (CF_o, CF_I) are illustrated in the membrane. Modified from Buchanan et al. (2000).

central grana core, the margins and the two end membranes (Albertsson, 2001) (Fig. 2). The stroma lamellae are single pairs of membranes that interconnect the grana stacks. The continuous thylakoid membrane system encloses an aqueous interior, the thylakoid lumen. A three-dimensional thylakoid-model suggests a helical arrangement of stroma lamellae around the cylindrical granum of stacked membranes (Mustárdy and Garab, 2003).

The thylakoid membrane is also called the photosynthetic membrane. It contains protein complexes of photosynthesis such as the photosystems (PS) I and II, cytochrome b_6/f, and the ATPase (Fig. 1B). The two PSs are segregated in the thylakoid membrane: PSI with light-harvesting complex I (LHCI) located to the stroma-exposed regions and PSII with LHCII

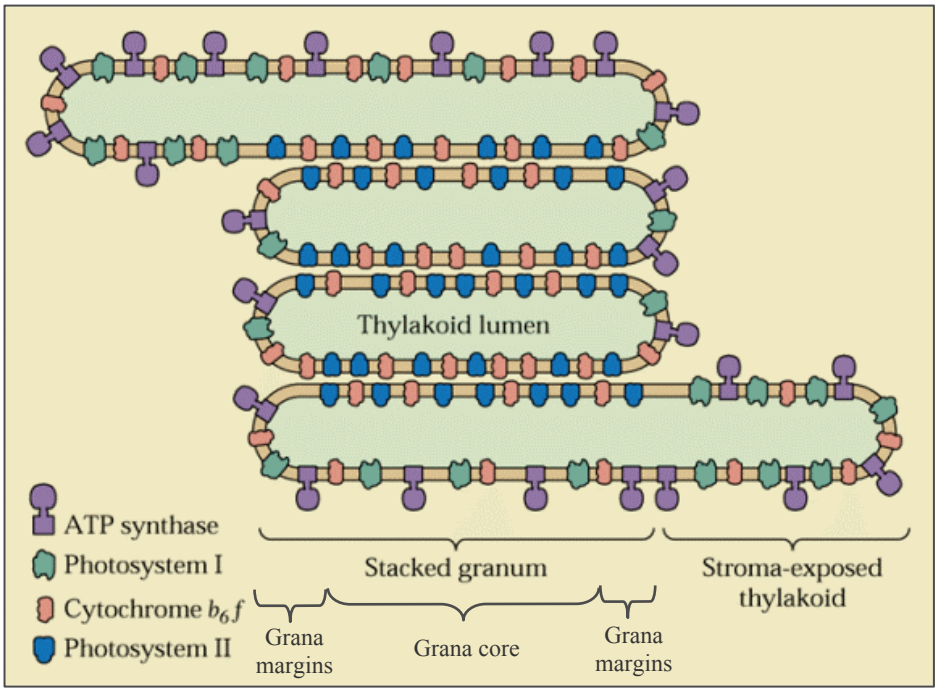

Figure 2. A model of domain organisation and distribution of protein complexes in the thylakoid membrane, modified from Buchanan et al. (2000).

located to the stacked grana core (Albertsson, 2001). The ATP synthase is enriched in the non-appressed regions, whereas the cytochrome b_6/f-complex is evenly distributed (Fig. 2).

Light is captured by antenna proteins together with bound chlorophylls and channelled to the reaction centre. The core of the reaction centre of PSII is a special pair of chlorophylls, P680. When P680 becomes excited, an electron will move away to Q_B via pheophytin and Q_A. From the Q_B site, electrons are transported via plastoquinone (PQ), the cytochrome b_6/f complex and plastocyanin to PSI. Here, another photon is absorbed by the antenna complex (LHCI) and excite the special pair of chlorophylls (P700) in the reaction centre. Electrons will leave P700 and after passing a few redox carriers reduce ferredoxin (Fd), which, in turn, reduces $NADP^+$ to NADPH, using two electrons and one proton. To complete the electron transport, the electron hole in the PSII reaction centre is filled from the manganese cluster, which oxidises water on the lumenal side

and molecular oxygen is produced. The production of protons in the water-splitting complex and the proton pumping across the membrane, facilitated by the PQ pool, drives the generation of ATP from ADP and inorganic phosphate, a reaction catalysed by the ATP synthase (Fig. 1B).

2.2 Photosynthetic pigments

Photosynthetic antenna systems play a significant role in absorbing light energy for photosynthesis. Most photosynthetic eukaryotes are known to possess light-harvesting complexes (LHC). LHC proteins are highly conserved and bind various kinds of pigments capable of light absorption. The three major classes of pigments found in plants and algae are the chlorophylls, the carotenoids and the phycobilins. Carotenoids and phycobilins are called accessory pigments since they transfer the absorbed energy to chlorophylls.

Chlorophylls *a* and *b*, are the main photosynthetic pigments of higher plants. A chlorophyll molecule consists of a porphyrin ring and a phytol tail. Chlorophyll *a* and *b* differ by a single functional group in the porphyrin ring. In the central cavity of porphyrin ring a magnesium ion is coordinated.

Carotenoids are characterized by a large (35-40 carbon atoms) conjugated polyene chain, often terminated by rings. They are split into two major classes, carotenes (non-oxygen-containing carotenoids, such as β-carotene) and xanthophylls (oxygen-containing carotenoids, such as lutein and zeaxanthin). Carotenoids are abundantly synthesized by plants and some microorganisms. In higher plants the major carotenoids are β-carotene and the xanthophylls such as neoxanthin, violaxanthin, antheraxanthin, lutein and zeaxanthin whose separation from chlorophylls is shown in Figure 3. Violaxanthin, antheraxanthin and zeaxanthin are interconverted in the xanthophyll cycle (Fig. 4).

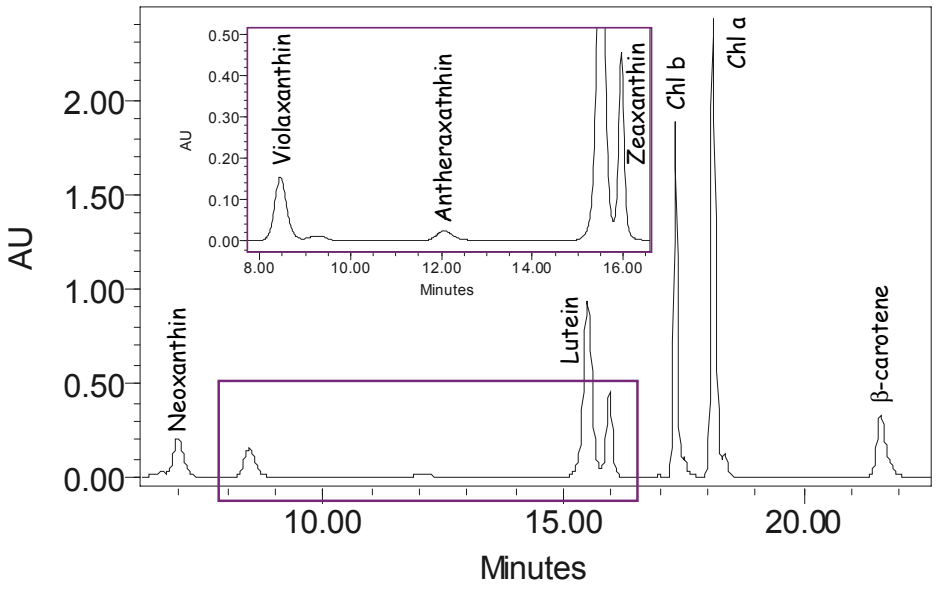

Figure 3. Typical RP-HPLC separation profile showing carotenoids and chlorophylls in a spinach thylakoid extract.

Pigments are not evenly distributed in the thylakoid membrane basically because of their binding to proteins. The stroma lamellae are enriched in chlorophyll *a* and β-carotene correlating well with the high content of PSI (Arvidsson et al., 1997). On the other hand, the granal-region has a high content of PSII and LHCII and shows enrichment in chlorophyll *b*, lutein and neoxanthin. Unlike the pigments mentioned above, the xanthophyll cycle pigments do not correlate with the distribution of protein complexes along the thylakoid membrane, they are almost uniformly distributed as revealed by fractionation of mechanically fragmented thylakoids (Arvidsson et al., 1997).

Pigment analysis constitutes an analytical challenge because pigments are often part of complex structures and/or mixtures. To assess changes of the xanthophyll cycle pigments we have used a chromatographic separation method (Felti et al., 2005), RP-HPLC (reversed phase - high performance liquid chromatography) on octadecylsilane (C18) columns of acetone-extracted pigments. However, pigments with very similar structure might be difficult to separate using classical RP-C18 columns. This is the case for zeaxanthin (Fig. 4) and lutein. Lutein is a structural isomer of

zeaxanthin and the only difference between the two is the position of one double bound. To achieve good separation, I have in my work used a special HPLC bounded phase parameter; a so-called non-endcapped (residual silanol sites present) C18 column with a mobile phase of methanol/acetonitrile/ethyl acetate, essentially according to Thayer and Björkman (1990).

2.3 Photosystem (PSII)

The two photosystems, PSII and PSI, acting in series, catalyse the first step of energy conversion. In this part I will focus on PS II, since the PSII-LHCII supercomplex has been discussed extensively in relation to the xanthophyll cycle.

The heart of PSII is the reaction centre, which consists of the D1 and D2 proteins. The reaction centre is surrounded by an antenna system that is a multiprotein complex with chlorophyll-binding proteins (CP), the two innermost are CP47 and CP43. Three minor chlorophyll *a/b* binding complexes (CP24, CP26 and CP29) encompass CP43 and 47. Most peripherally located are the major chlorophyll *a/b* binding complexes, trimers are the dominant form (Åkerlund, 1993). All these antenna proteins take part in the excitation energy transfer to the reaction centre.

Apart from the CP proteins, there are a number of smaller proteins located in between the above-mentioned subunits with mostly unknown functions. One of them (PsbS) is, however, particularly interesting in connection with the xanthophyll cycle. First, this 22 kDa protein, PsbS, was suggested to be closely associated with the PSII reaction centre core based on co-immunoprecipitation (Ljungberg et al., 1984). In the original isolation and characterisation of the protein, Ljungberg et al. (1986) could not find any pigments. Later, a more peripheral location to the PSII reaction centre was proposed for PsbS based on its presence in etiolated plants (Funk et al., 1995a). Sequence homology was found between PsbS,

the CAB (chlorophyll a/b binding proteins) family and ELIPs (early light induced proteins) by Kim et al. (1992). The CAB gene family encodes for the major chlorophyll *a/b* binding complexes of LHCII. However, four transmembrane helices in the folding pattern were predicted in PsbS instead of the three predicted for CAB proteins and ELIPs (Kim et al., 1992). Within the structure the first and third helices are homologous, while the second is related to the fourth (Kim et al., 1994). Similarities between *psbS* and *cab* genes suggested that PsbS could be pigment-binding (Funk et al., 1994). The isolated PsbS was, in fact, found to contain pigments (Funk et al., 1994; 1995b). Derived from the pigment-binding properties of PsbS and the early occurence during development, the function of transient binding of pigments during chloroplast biogenesis seemed reasonable (Funk et al., 1995a). More recently, an additional function was proposed by Li et al. (2000) that PsbS is the site of photoprotective heat dissipation in plants (see section 2.4 and Niyogi et al., 2005).

2.4 Light stress

Plants in Nature experience huge variations of light intensity on a daily basis and therefore the amount of light captured has to be regulated. Light can cause serious damage to the photosystems. If the absorbed light energy exceeds the limit of photosynthetic capacity a subsequent decrease in the photosynthetic efficiency can occur. To assess photochemical performance, measurements of chlorophyll fluorescence was found to be a powerful method (Horton et al., 1994). The basis of the method is that each photon absorbed by a chlorophyll *a* molecule lifts an electron from the ground state to an excited state, forming singlet chlorophyll ($^1Chl^*$). The absorbed light can be used to drive photosynthesis, can be dissipated as heat or re-emitted as light (chlorophyll fluorescence). These three processes occur in competition, and by measuring the yield of fluorescence; information about the other two can be gained. Generally, fluorescence yield is highest when photochemistry and heat dissipation

are lowest. For a recent review regarding chlorophyll fluorescence see Maxwell and Johnson, (2000). Moreover, $^{1}Chl^{*}$ can also be converted into triplet Chl ($^{3}Chl^{*}$), which can interact with ground state molecular O_2, converting it into singlet oxygen ($^{1}O_2^{*}$), a highly reactive oxygen species. Quenching of both the triplet state chlorophylls and singlet oxygen can be accomplished by carotenoids (Niyogi, 2000). Carotenoids also act as antioxidants, scavenging the very damaging reactive oxygen species and free radicals, such as singlet oxygen, superoxide anion and lipid-peroxide radicals.

Non-photochemical quenching (NPQ) is recognised as a central regulatory mechanism for protecting plants from photodamage. Three processes contribute to the NPQ (Müller et al., 2001). The major process, termed the pH- or energy-dependent component (qE), responds rapidly (in seconds), requires the presence of low pH in the thylakoid lumen, and involves the light-induced formation of zeaxanthin from violaxanthin in the xanthophyll cycle (Demmig et al., 1987). The xanthophyll cycle will be discussed in detail in chapter 3. The second component of NPQ is the phenomenon of state transition (qT) (Walters et al., 1991). State transition involves the reversible phosphorylation and movement of LHCII. It is a way of adjusting the incoming light to the different needs of the photosystems. When PSII gets more light energy than PSI, LHCII becomes phosphorylated and moves towards PSI. The reverse occurs when PSI is more highly excitated than PSII. The contribution of qT to the overall quenching is small. Photoinhibition (qI) is the third NPQ component and it relaxes within hours. A drastic decline in the maximum photosynthetic yield can be observed when photoinhibition takes place.

Since the formation of zeaxanthin is strongly involved in the quenching process, the mechanism of qE is of special interest. When the conversion by violaxanthin de-epoxidase (VDE) from violaxanthin to zeaxanthin was inhibited by dithiothreitol (DTT), pE was also inhibited (Horton et al., 1994). Preventing the formation of zeaxanthin by genetic mutation of

VDE in *A. thaliana* (*npq1*) resulted in reduced qE. This demonstrated that violaxanthin de-epoxidation is required for the rapidly responding element of NPQ. In a mutant (*npq2*) accumulating zeaxanthin constitutively, qE still required low pH. This indicated that the presence of both zeaxanthin and low pH are necessary for the efficient dissipation of excess energy (Niyogi et al., 1998). More recently, yet another component was proposed to be required for qE. Li et al., (2000) isolated a qE-deficient mutant. The *A. thaliana* mutant (*npq4*, deleted *psbS* gene) with a fully functional xanthophyll cycle (Peterson and Havir, 2000) lacked qE, most probably due to the missing PsbS protein in the LHCII complex (Li et al., 2000). The conclusion is, that the presence of low pH, zeaxanthin and PsbS is essential for the energy-dependent component of NPQ, although further studies are needed for the precise characterisation of qE as proposed by Niyogi et al. (2005).

3 The xanthophyll cycle

The xanthophyll cycle (Fig. 4) is a mechanism by which oxygenated carotenoids interconvert, on a timescale of minutes. The pH-dependent conversion of violaxanthin, a xanthophyll with two epoxide groups, first to antheraxanthin (one epoxide group) and then to zeaxanthin (no epoxide group) occurs in high light and is catalysed by the enzyme violaxanthin de-epoxidase (VDE). Under low light, zeaxanthin epoxidase (ZE) becomes active and forms violaxanthin from zeaxanthin. The light-dependent conversion of xanthophylls was first discovered by Sapozhnikov et al. (1957) and the field of xanthophyll cycle research was expanded during the 1960s and 1970s with major contribution by the groups of Yamamoto and Hager. The main features of the cycle were first described in 1962 by the Yamamoto group using spinach leaves. Identification of several other biochemical parameters of the cycle followed, such as the requirement of ascorbate and lipids by VDE (Yamamoto and Higashi, 1978), the characterisation of ZE (Siefermann

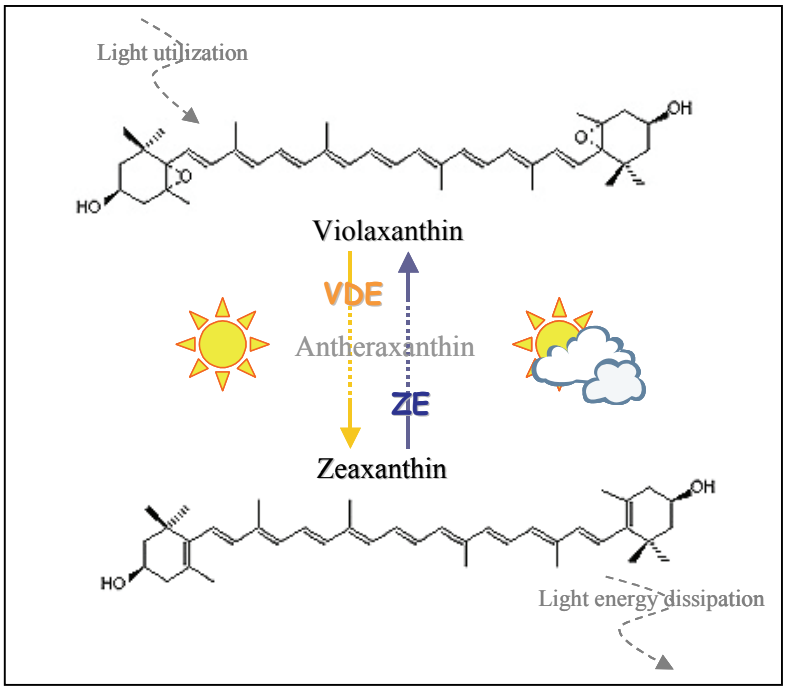

Figure 4. The xanthophyll cycle

and Yamamoto, 1975b) and the effect of DTT, inhibitor of VDE (Yamamoto et al., 1972). The breakthrough came in 1987 when Demmig et al. (1987) coupled the formation of zeaxanthin from violaxanthin by VDE to the process of NPQ. As discussed in the section 2.4, the low lumen pH, zeaxanthin formation, and an LHCII subunit (PsbS) were found to be required for the qE component of NPQ (Li et al., 2000).

3.1 Different types of xanthophyll cycles

The xanthophyll cycle is widespread in Nature and so far three different types have been found (Müller et al., 2001). The violaxanthin cycle (referred to as xanthophyll cycle throughout this thesis) in higher plants, green and brown algae consists of the interconversion of violaxanthin and zeaxanthin via the intermediate antheraxanthin, catalyzed by two enzymes VDE and ZE (Fig. 4). In high light diatoms and most eukaryotic algae convert diadinoxanthin to diatoxanthin in a one-step de-epoxidation reaction by diadinoxanthin de-epoxidase. This type of xanthophyll cycle is called the diadinoxanthin cycle. The

mechanism of diadinoxanthin conversion and comparisons to the violaxanthin cycle have been investigated (Latowski et al., 2002; Goss et al., 2006). Under prolonged high-light stress some algae can use both the violaxanthin cycle and the diadinoxanthin cycle (Lohr and Wilhelm, 1999). The parasitic plant *Cuscuta reflexa* performs a unique type of xanthophyll cycle in which lutein-5,6-epoxide is converted directly to lutein presumably by VDE in the lutein-5,6-epoxide cycle (Bungard et al., 1999). The striking feature about *C. reflexa* is that it lacks neoxanthin, and instead accumulates lutein-5,6-epoxide. The biosynthetic pathway of lutein-5,6-epoxide is unknown, however, as lutein is a carotenoid of the α-carotene branch, it seems possible that the accumulation of lutein-5,6-epoxide in *C. reflexa* reflects the compensatory synthesis of α-carotene-derived xanthophylls when production of the β-carotene-derived neoxanthin is lacking (Bungard et al., 1999). See 3.2 for explanation of carotenoids biosynthesis.

3.2 Functions of the xanthophyll cycle

The main function of the cycle, via the production of zeaxanthin, is to allow harmless energy dissipation by the NPQ process. NPQ has been discussed in detail in section 2.4. Here, additional functions of the cycle and the role of zeaxanthin will be discussed.

Carotenoids are tetraterpenoids derived from C5 isoprene units (isopentenyl diphosphate, IPP) and are therefore biosynthetically connected to the synthesis of other isoprenoid compounds such as tocopherols, chlorophyll phytol and plastoquinones. IPP originates from the plastid-localized mevalonic acid-independent pathway. IPP is converted to the phytoene chain in a series of reactions followed by isomerisation into the non-cyclic lycopene. Lycopene is the precursor for cyclisation yielding the two branch substances, α-carotene and β-carotene. β-Carotene can be further hydroxylated, via α-cryptoxanthin and β-cryptoxanthin, to lutein and zeaxanthin, respectively. The two

branches appear to be tightly regulated and compensatory with respect to each other (Taylor et al., 2005). Violaxanthin is formed from zeaxanthin through the introduction of 5,6-epoxy groups into the 3-hydroxy-β-rings. This process proceeds via the mono-epoxidated intermediate antheraxanthin.

The phytohormone, abscisic acid (ABA) is important in the control of plant responses to abiotic stress, especially water deficit. Mutants deficit in ABA are unable to control stomata opening and closure in response to water stress, leading to a wilty phenotype. The β-carotene-derived violaxanthin serves as a precursor in the synthesis of ABA and therefore the concentration of violaxanthin may influence the formation of ABA. To protect plants from additional water-loss during drought, ABA concentration in the xylem increases and the stomata close. In addition, moderate water deficit, at constant light intensity, leads to increased zeaxanthin formation. As the water deficit becomes sustained more violaxanthin will be converted to zeaxanthin. Thus, in drought-stressed plants violaxanthin must be converted into both ABA and zeaxanthin. It has a question whether there are two separate pools of violaxanthin, one, in the root, for ABA synthesis and the other one in the leaf, for the xanthophyll cycle (reviewed by Seo and Koshiba, 2002). Gene expression studies in tobacco revealed that ZE mRNA levels in leaves are under circadian control, peaking during the day, followed by an increased level of ABA production (Audran et al., 1998). ZE is encoded by the nuclear *aba* gene (Martin et al., 1996). ABA biosynthesis mutants (*aba1*, *aba2*, and *aba3*) have been isolated to elucidate the link between zeaxanthin epoxidation and ABA production. The carotenoid biosynthetic pathway was reviewed by Fraser and Bramley, (2004).

Carotenoids, including xanthophylls, have a broad range of functions also in non-photosynthetic organisms, especially in relation to human health and nutrition (Demmig-Adams and Adams, 2002). Carotenoids exert protective function as biological antioxidants. They fulfil an essential role

as scavengers of reactive oxygen species (Niyogi, 2000), among them the lipid-peroxide radical. Increased levels of lipid peroxidation when exposed to high light during growth were observed in the *Arabidopsis* mutant (*npq1*) lacking functional VDE (Havaux et al., 2000) pointing to the role of zeaxanthin in protection of thylakoid lipids against photodamage.

Of the many carotenoids circulating in human sera, only lutein and zeaxanthin are accumulated in the macula of the eye. It is thought that these two pigments protect the macula from light-induced damage and scavenge free radicals formed in the photoreceptors, while at the same time they improve visual acuity by absorbing light of short-wavelength. Several epidemiological studies have supported the observation that high intake of carotenoids decrease the risk of cataract formation (opacification of the eye's natural lens) as well as the age-related macular degeneration. Evidence for the protective effect of carotenoids in the eye was reviewed by Chiu and Taylor, (2007). Another substance important in vision with a carotenoid origin is vitamin A, derived from β-carotene, also called a provitamin A carotenoid. In photoreceptor cells of the retina retinal, the most active form of vitamin A, is bound to a protein (rhodopsin). Further links between rhodopsin and zeaxanthin will be discussed in section 4.5.

In vitro cell culture experiments have shown that carotenoids inhibit cell proliferation, transformation and micronucleus formation as well as modulating expression of certain genes. These properties are all consistent with a protective effect against carcinogenesis. It has been shown that the higher intake of lycopene from tomato ameliorates the risk for prostate cancer about 35 % (Giovannucci, 2002).

The carotenoids are responsible for colours of many fruits (citrus fruits, tomatoes, paprika) and flowers (daffodil), as well as the colours of many

birds (flamingo, canary), insects (lady bird), and marine animals (salmon).

Xanthophylls, based on their rigid molecular structure, have been suggested to modify membrane structure, more specifically to decrease the membrane fluidity, especially in the fluid phase (Gruszecki and Strzalka, 1991; Subczynski and Wisniewska, 2000; Socaciu et al., 2002). We have shown that accumulation of zeaxanthin in the isolated thylakoid membranes resulted in an increased rigidity of the membrane measured as a red-shifted fluorescence emission spectrum using laurdan (Paper III). In model membranes made of the main thylakoid lipids, both viola- and zeaxanthin exert rigidifying effect, although violaxanthin was less efficient in that respect. My overall conclusion drawn is that the operation of the xanthophyll cycle producing zeaxanthin has an additional membrane-stabilising role. This ensures the physiological function of the membrane even at elevated temperatures, in addition to protecting against overexcitation.

3.3 Violaxanthin de-epoxidase (VDE)

Two enzymes work in the xanthophyll cycle of higher plants (Eskling et al., 2001), VDE and ZE. The former is a de-epoxidase and the latter is an epoxidase enzyme (Gilmore, 1997). Both have been found to belong to the group of lipocalin proteins (Gryzb el al., 2006). VDE requires low pH, ascorbic acid (Bratt et al., 1995), all trans xanthophylls (Yamamoto and Higashi, 1978), and lipids forming inverted hexagonal phase (H_{II}) (Latowski et al., 2004) for its activity.

VDE is a 43 kDa enzyme, located in the thylakoid lumen and it is responsible for the conversion of violaxanthin to zeaxanthin. It is a nuclear-encoded protein, which is targeted to the chloroplast and then to the lumen by a transit peptide. The transit peptide is cleaved from the

mature protein. The mature lumenal protein is 341- 349 amino acids long, depending on plant species.

The activity of the enzyme is strongly pH-dependent. At high lumenal pH (at low light intensities) the enzyme is water-soluble. Upon illumination the electron transport chain develops a proton gradient across the thylakoid membrane and the lumen becomes acidic. This low pH triggers a conformational change of VDE (Arvidsson et al., 1997) allowing it to bind to the membrane. Emanuelsson et al. (2003) suggested, using site-directed mutagenesis and chemical modification by diethylpyrocarbonate, that this conformational change is triggered by the protonation of the four histidine residues rather than that of carboxyl groups in the C terminus. The negatively charged C-terminal of VDE was suggested to be involved in the membrane binding process (Bugos and Yamamoto, 1996). Hieber et al. (2002) studying deletion mutants of *A. thaliana* VDE, with 72%, 85% and 94% of the C terminus truncated, respectively, showed that the C terminus is not involved in the membrane binding. However, it is essential for activity.

The four histidines are located in pairs and close to each other (Fig. 5). Mutant forms of VDE were created by substitution of 1, 2 or 4 histidines by either alanine or arginine (Emanuelsson et al., 2003). Replacing all four histidines resulted in complete inactivation of the enzyme. Mutants with one histidine substituted for alanine or arginine were active, as well as some mutants with a replacement of a pair of histidine. These results led to the conclusion that histidines are important for VDE activity.

Figure 5. Schematic illustration of the three regions in spinach VDE. Starting with the cystein-rich N terminus, then the lipocalin region, and the highly negatively charged C terminus. The four histidines and the 11 cysteins are also marked. Cystein and histidine residues are conserved between different species.

In Paper I we studied the pH-dependent membrane binding of native and mutant VDE to thylakoids. Thylakoids from spinach and wheat were sonicated on a pH-scale from 4.7 to 7.1 to release unbound VDE. The inflection point, the pH value where half of the enzyme is bound, was found to be 6.6 for spinach VDE in agreement with earlier results (Bratt et al., 1995) and 6.0 for wheat VDE (Fig. 6). The cooperativity (Hill constant), with respect to protons, for binding the enzyme to the thylakoid membrane was found to be 3.8 and 2.9 for spinach and wheat VDE, respectively. The value of 3.8 in the spinach VDE case is close to the previously published value of 4.0 (Bratt et al., 1995) and proportional to the four histidine residues found in the structure. The lower cooperativity found for wheat fits with the three histidines in wheat VDE. We propose that the cooperativity in binding the enzyme to the membrane is a result of protonation of the histidine residues of VDE.

When the native and wildtype recombinant spinach VDE was compared in terms of cooperativity, the value of 3.7 was found for both. Mutants with one or two histidine substitutions showed a lower cooperativity than that of the wildtype. The inflection points were also shifted to lower values (6.0-6.1) than that of the binding to the inside of the thylakoids (pH 6.6). However, it is important to emphasise the differences between the two types of experiments, namely the release of unbound VDE using sonication and the binding experiments. In the former experiments VDE was released from the inside, and in the latter VDE was externally added. Possible reasons for the shift from 6.6 to 6.0 could be variations (i) in the thylakoid membrane composition, (ii) in the surface charge density and (iii) in the concentration of the enzyme in the two methods. Taken together, we propose that protonation of the conserved histidine residues (four in spinach and three in wheat) at low pH induces a conformational change of the enzyme. This change makes the enzyme surface more hydrophobic and promotes binding to the thylakoid membrane.

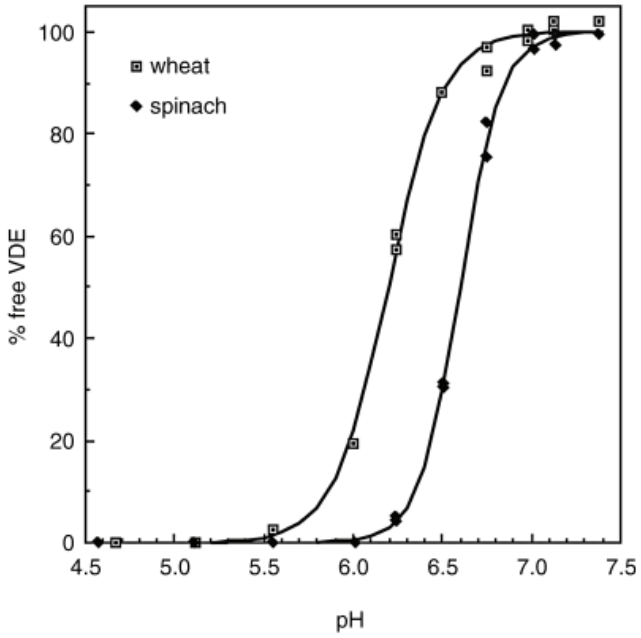

Figure 6. Release of spinach and wheat VDE from the inside of the thylakoid membrane, as a function of pH. The release of free VDE from the thylakoid lumen was made by sonication. The released enzymes were assayed in a spectrophotometric enzyme assay at pH 5.2.

Additional membrane properties that may be involved in binding and releasing of VDE are proposed in Paper II (see section 4.5).

VDE was one of the first proteins from plants identified as a member of the lipocalin family (Bugos et al., 1998). The main structure of the lipocalin region in lipocalin proteins consists of eight β-strands forming an antiparallell β-sheet. Another family feature is the binding of small, hydrophobic molecules, such as xanthophylls with a size of about 30 Å or lipids. Lipocalin fall into two main subfamilies, the kernel and the outlier lipocalins. Retinol binding protein is an example of the kernel lipocalins. VDE and ZE are plant outlier lipocalins with enzymatic activity. For more detailed information about lipocalins see a recent review by Gryzb el al. (2006). Secondary structural prediction made by Emanuelsson et al. (2003) showed the location of histidines in the lipocalin region and also predicted that the typical β-barrel structure including a number of loops

could serve as the active site of the enzyme binding MGDG and/or violaxanthin.

Monogalactosyldiacylglycerol (MGDG), the characteristic lipid of the thylakoid membrane, was found to be required by VDE *in vitro* (Yamamoto and Higashi, 1978). Recently the effect of two phospholipids and two galactolipids on VDE activity has been compared (Latowski et al., 2004). One lipid prone to forming bilayer and one non-bilayer lipid were selected in both groups. It was found that only phosphatidylethanolamine (PE) supported VDE activity and not the chemically very similar phosphatidylcholine (PC). The same was found with the two galactolipids; conversion of violaxanthin by VDE was supported only in the presence of MGDG, and not digalactosyldiacylglycerol (DGDG). From these results it was concluded that the type of structure formed by the lipids determines VDE activity, rather than the chemical nature of the individual lipids. This is because only PE and MGDG are non-bilayer prone lipids forming an inverted hexagonal phase (H_{II}) in aqueous media. In line with these results, Goss et al. (2005) found that bilayer-forming lipids (PC and DGDG) were not able to induce efficient xanthophyll de-epoxidation. Another important result of this study was that higher concentrations of PC and DGDG are required to solubilise violaxanthin than PE and MGDG. Even in the presence of high concentrations of DGDG or PC, where violaxanthin is completely solubilised, de-epoxidation by VDE was completely inactive (Goss et al., 2005). However, contradictory to these results Yamamoto (2006) reported that both MGDG and DGDG could support VDE activity, although DGDG at a lower rate. The reason for this discrepancy is not known, but source, purity and handling of lipids and pigments are critical parameters. In addition to its role in pigment solubilisation, MGDG has been proposed to actually form H_{II}-structure in the thylakoid membrane (Latowski et al., 2002). This feature of MGDG will be discussed in chapter 4.

Ascorbate, the electron donor to VDE, as well as viola- and antheraxanthin are the substrates of VDE. Ascorbic acid becomes oxidised to dehydroascorbate upon zeaxanthin formation. Bratt et al. (1995) studied the activity of VDE as a function of both pH and ascorbate concentration. They found that VDE has a pH-dependent K_m for ascorbate and suggested that the acid form of ascorbate is the true substrate for VDE. A transport system of ascorbate from the high pH stroma into the lumen was proposed.

3.4 Location and availability of violaxanthin

It is difficult to obtain a clear picture of where violaxanthin is located under normal photosynthetic activity, what controls its availability for conversion and where zeaxanthin is located under conditions of overexcitation when NPQ takes place. These issues have not yet been experimentally settled. Since carotenoids are lipophilic compounds, neither the lipid phase nor membrane proteins can be ruled out as potential locations.

Despite the correlation between the level of zeaxanthin (+antheraxanthin) and NPQ, the conversion of violaxanthin is limited even if the stress level is increased and prolonged. The amount of violaxanthin remaining unconverted in the thylakoid membrane normally ranges between 20 to 50% and has often been referred to as inaccessible (Siefermann and Yamamoto, 1974; 1975a). To answer the question of inaccessible violaxanthin, investigations should begin with the location of the pigment.

As a starting point for the discussion, many studies suggest that pigments of the xanthophyll cycle are protein-bound (e.g. Ruban et al., 1994) as they can be found in pigment-protein complexes after fractionation or purification (Bassi et al., 1993; Verhoeven et al., 1999; Jahns et al., 2001; Ruban et al., 2002). They have been found in the crystal structure of the

major LHC (Liu et al., 2004). It has also been shown that xanthophyll cycle pigments can occupy the binding site for lutein and neoxanthin in recombinant LHCs (Croce et al., 1999). The problem is, however, that the binding properties of xanthophylls to proteins is much weaker than that of other carotenoids and also xanthophyll cycle pigments are often found in sub-stoichiometric quantities. Another weak point of the protein-binding model for violaxanthin, is that it would require binding sites for violaxanthin on all pigment proteins (see below).

An opposing theory, however, locates xanthophylls in the lipid phase of the thylakoid membrane. This view is supported by several findings. Arvidsson et al. (1997) using two-phase partitioning to fragment the thylakoid membrane showed that xanthophyll cycle pigments have no preference for any particular regions of the thylakoid membrane. This is in contrast to other pigments and proteins in the membrane (see section 2.2). In the same study, adding VDE and ascorbate to either right-side-out or inside-out thylakoid vesicles resulted in conversion of the same amount of violaxanthin. An extended high light treatment of spinach plants revealed that even if the amount of xanthophyll cycle pigments increase in high light, the amount of pigment-protein complexes remained constant or even reduced (Eskling and Åkerlund, 1998). In model membrane systems resembling the thylakoid membrane Latowski et al. (2002) found a restricted conversion of violaxanthin by VDE. Note that these systems did not contain pigment-protein complexes and the conversion was still found to be limited as with the natural thylakoid membrane (Thayer and Björkman, 1990; Arvidsson et al., 1997; Paper II). Even a flip-flop mechanism suggested to convert antheraxanthin into zeaxanthin would more reasonably take place in the lipid phase than in pigment proteins. All these findings point to the lipid phase of the thylakoid membrane as a place where violaxanthin is located and most probably where the conversion takes place.

Other possible explanations to the restricted conversion of violaxanthin have been product feed-back inhibition by zeaxanthin (Latowski et al., 2002; Havir et al., 1997) and that some of the violaxanthin is in the *cis*-form (Latowski et al., 2002) rather than the all-*trans* form required by VDE for active de-epoxidation reaction.

A compromise to the two opposing main theories would be if a small part of the xanthophyll cycle pigments were bound to proteins (inaccessible violaxanthin fraction) while the rest is free in the lipid matrix (accessible for de-epoxidation). Interesting questions, which will be addressed later in this thesis, are how the xanthophyll cycle pigments in the lipid part of the membrane affect its packing, and how the packing affects the xanthophyll cycle.

3.5 Zeaxanthin epoxidase (ZE)

The enzyme carries out the reaction of epoxidation of zeaxanthin to violaxanthin upon low light or in darkness (Fig. 4). It is a stromally located enzyme (Siefermann and Yamamoto, 1975b) with a pH optimum of 7.0-7.5. ZE belongs to the group of lipocalin proteins (Bugos et al., 1998; reviewed by Gryzb el al., 2006). Oxygen and NADPH are co-substrates for the epoxidation reaction by ZE (Siefermann and Yamamoto, 1975b). Flavin adenine dinucleotide is needed as a cofactor. During the characterisation of *Nicotiana plumbaginifolia* mutants deficient in ABA biosynthesis Martin et al. (1996) found zeaxanthin accumulation. The gene (*aba*) responsible for the mutation was found to encode ZE. The mature enzyme is 67 kDa. The product of ZE activity, violaxanthin is a precursor of the synthesis of ABA.

3.6 The xanthophyll cycle as a stress indicator

Chilling temperatures increase the sensitivity of plants to photoinhibition due to restricted photosynthetic energy utilisation (Krause, 1994). The degree of violaxanthin to zeaxanthin conversion varies in response to the stress level, and the level of zeaxanthin (+antheraxanthin) directly correlates to the degree of NPQ under restricted conditions (Gilmore and Yamamoto, 1993). The maximal photochemical efficiency (F_v/F_m) and Yield ($F`_m - F_t$) / $F`_m$ are often used to reflect the extent of stress and the tolerance of the plant to different stress situations.

Sterols are important plant membrane components, enriched in the plasma membrane (Hellgren and Sandelius, 2001). Free sterols are one of the major regulators of membrane fluidity (Demel and de Kruyff, 1976; Bloch, 1983). One important part of the regulation of free sterols in the membrane involves acylation (reviewed by Sturley, 1997). This generates sterol esters (SE) that cannot participate in the bilayer formation, but they are generally thought to provide a storage pool of sterols. Genes responsible for acylation of sterols have been well characterized both in animals and in yeast (Chang et al., 1997). Although plants have been found to accumulate SE under different conditions (Dyas and Goad, 1993), a plant gene was just recently characterized in *Arabidopsis* (Banas et al., 2005). The *At1g0410* gene encodes a phospholipid:sterol acyltransferase (PSAT) (Banas et al., 2005). PSAT is an intracellular enzyme catalysing the synthesis of SEs by an acyl CoA-independent reaction in which fatty acids are transacylated from phospholipids to sterols. Results from *in vivo* studies suggested that AtPSAT (*Arabidopsis thaliana* PSAT) was involved in regulation of the free sterol content of the plant cells (Banas et al., 2005).

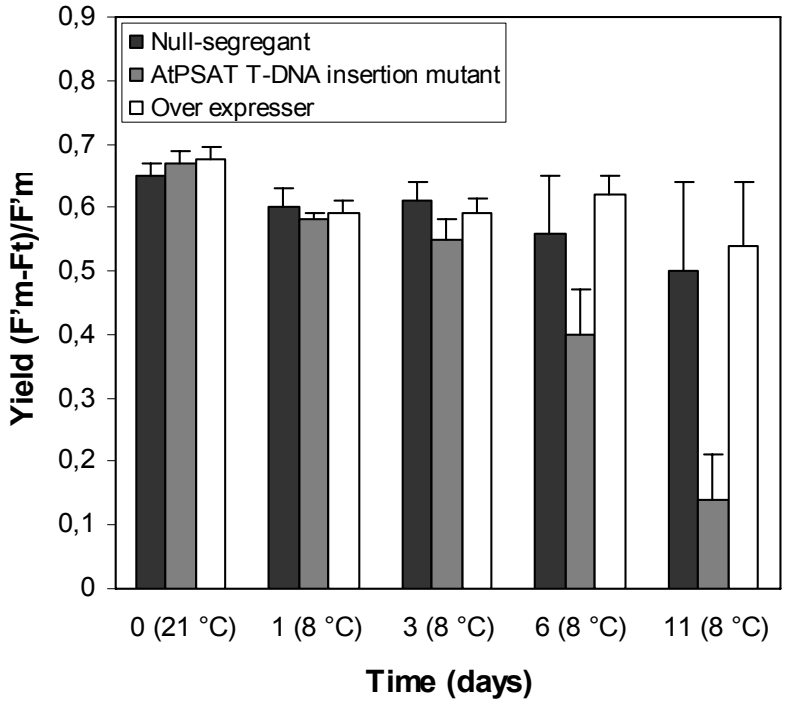

Figure 7. PSII efficiency during chilling stress. The photochemical quantum yield (F`m - Ft) / F`m of PSII in leaves of null-segregant (black bars), AtPSAT T-DNA insertion mutants (grey bars) and overexpresser (white bars). Plants were grown at 21 °C and then transferred to 8 °C. Standard deviations of three independent experiments.

A proper regulation of free sterols is expected to be particularly important in situations involving adjustment to chilling temperatures. We have therefore, together with Anders Carlsson (SLU, Alnarp) and co-workers, studied how changes in expression of AtPSAT could affect plants when shifted to low temperature (unpublished). A knockout mutant and an overexpresser was grown under ambient temperature and then exposed to sustained low temperature. Knocking out PSAT in *Arabidopsis* resulted in senescence of older rosette leaves after 6 weeks at 2 °C and after 2 weeks at 8 °C and constant light. After 4 months at 2 °C all rosette leaves were gone in the mutant plants. Control plants (overexpressers and null-segregates) grew well under the same treatments.

The photosynthetic efficiency of *Arabidopsis* plants, exposed to chilling stress, was assessed. A reduction in chlorophyll content, reduction in the fluorescence parameter reflecting photochemical activity (Fig. 7) and a

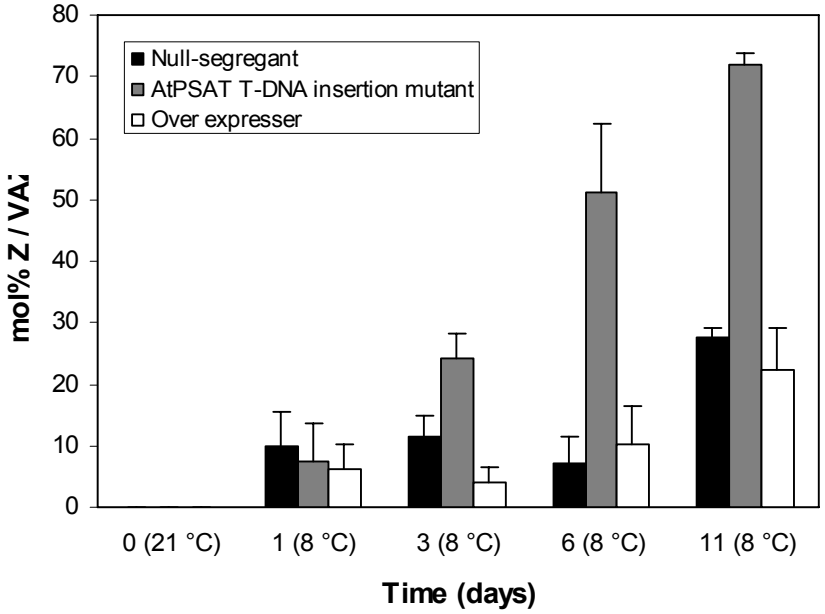

Figure 8. Effect of low temperature on the amount of zeaxanthin in *Arabidopsis* leaves. Xanthophylls were analysed by RP-HPLC (see section 2.2). Null-segregant (black bars), AtPSAT T-DNA insertion mutants (grey bars) and overexpresser (white bars). The amount of zeaxanthin (Z) was measured as mol% of the total pigments of the xanthophyll cycle pool (VAZ).

drastic change in the xanthophyll cycle (Fig. 8) was seen. Thus photosynthesis became restricted, although the thylakoids are non-sterol-containing membranes. The link between SE-synthesis and photosynthesis is not obvious. However, the indirect effect of SEs on photosynthesis points to a central role in cellular homeostasis.

4 Thylakoid membrane lipids

The photosynthetic membranes of higher plant chloroplasts have an unusual lipid composition, as they consist mainly of polar, but uncharged, galactolipids. This is in contrast to most other eukaryotic membranes, e.g. the plant plasma membrane, which contain large amounts of phospholipids (Sprague, 1987).

The thylakoid membrane is a very complex membrane with respect to both structure and function because it has to carry out many biochemical reactions. Furthermore, it has to cope with the destructive effect of light and oxygen stress and repair the damage they cause. Hence, it is no surprise that the photosynthetic membrane has an ingenious structure to fulfil all its functions.

4.1 Plant galactolipids and their structures

The major thylakoid lipids are MGDG and DGDG (Fig. 9) amounting to about 50 mol% and 30 mol% of the total polar lipids, respectively. The remainder is made up of approximately equal proportions of mainly anionic lipids such as sulfoquinovosyl diacylglycerol and phosphatidylglycerol together with a small amount of PC (Selstam and Wigge, 1993).

Despite similarities in structure of MGDG and DGDG, the physical properties of these two lipids are very different due to the different size of the mono- and digalactosyl groups. MGDG with a small head group has a cone-like geometry. Therefore, when dispersed in aqueous media, MGDG tends to form an inverted hexagonal structure (H_{II}), with the polar head group facing towards the centre of micellar structures rather than forming a conventional bilayer. In contrast, DGDG with two galactose moieties in the head group have a more cylindrical shape, so they form lamellar phases and hence bilayers (Sen et al., 1981a) (Fig. 10). The most noteworthy common feature of these two thylakoid lipids is the high degree of unsaturated fatty acyl residues amounting to 90% (mol) linolenic acid as Brentel et al. (1985) found in wheat leaves. Binary mixtures of MGDG and DGDG result in the formation of overall lamellar structures with intrinsic curvature elastic stress. In the natural thylakoid membrane, the dominance of non-lamellar lipids is compensated for the presence of lamellar-phase-prone lipids and proteins leading to a lamellar

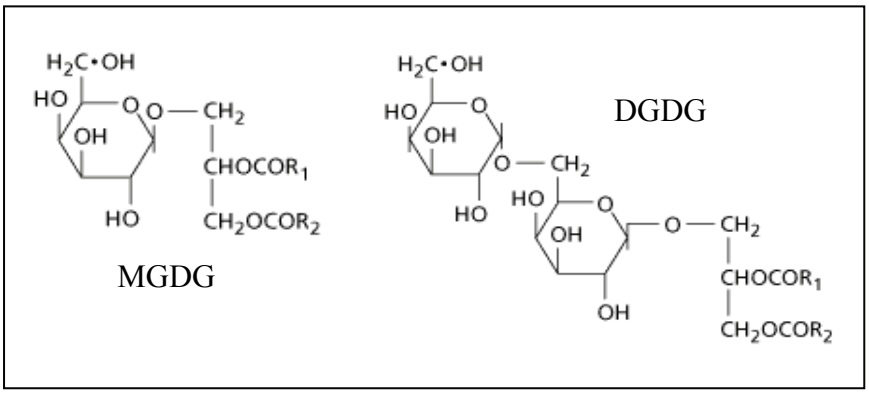

Figure 9. Molecular structures of the main thylakoid lipids, MGDG and DGDG.

structure even at such a high MGDG content. The ratio of MGDG and DGDG must be under tight control for adequate membrane function.

The existence of H_{II} phases for MGDG in the thylakoid membrane has been indicated, however the exact arrangement is still unclear. Electron microscopic view of freeze-fractured membranes revealed the presence of inverted hexagonal structures sandwiched within the bilayer (Sen et al., 1981b). However, Garab et al. (2000) proposed that H_{II} lipids can be sequestered from the membrane and form H_{II}-phases attached to the surface of the membrane. Goss et al. (2006) suggested that the presence of H_{II} lipids is crucial for both pigment solubilisation and VDE activity. They also hypothesised that lateral segregation of MGDG into proper H_{II} structures within the plane of the membrane occurs upon high light providing preferred docking site for the pH-activated VDE. In line with this lateral segregation model (Goss et al., 2006) we propose a model in which MGDG-rich regions can be formed and are incorporated in the bilayer as highly curved regions (Paper II and section 4.5 concerning curvature stress in the membrane).

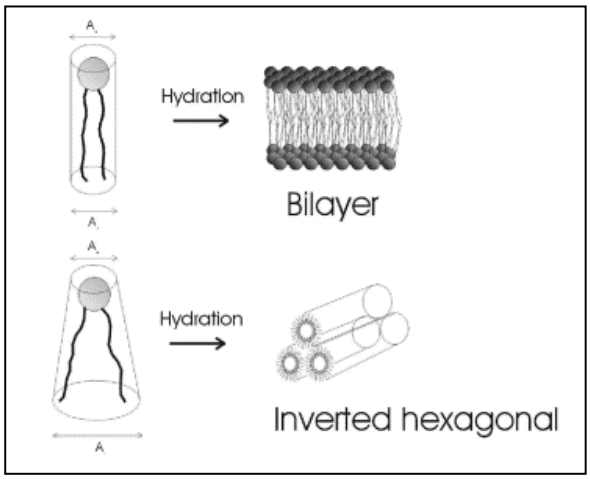

Figure 10. Molecular geometry of bilayer (top, DGDG, PC) and inverted hexagonal phase (bottom, MGDG, PE) forming lipids. The different structural arrangements of bilayer- and non-bilayer-forming lipids take up in aqueous media is also shown. Modified from Hafez et al. (2001).

4.2 Effect of temperature

Several lipid bilayer structures exist depending on the type of lipids, chemical, and physical conditions such as temperature. The simple way to distinguish between these phases is to determine the order of hydrocarbon chains. In this respect, bilayer arrangements fall into two major categories, the highly ordered, crystallised, gel phase (L_β) and the disordered, ′melted′, fluid phase (L_α). Changes in temperature leads to transition of the phase character. Cellular membranes must be in a fluid state for normal cell function. The lipid bilayer structure provides the membranes with their fluid characteristics. Note that at low temperatures, the bilayer is in a gel state and tightly packed. Consequently, the effect of temperature, lipid packing and membrane fluidity are closely connected. At higher temperatures, the bilayer ′melts′ and the hydrophobic interior resembles a fluid, allowing the lipid molecules to move around, rotate and exchange places. This also allows movement of other membrane components, i.e. membrane located xanthophylls, and allows the flip-flop mechanism of antheraxanthin to make the other epoxide group available for conversion by VDE.

The thylakoid membrane is thus susceptible to temperature fluctuation and yet must successfully cope with e.g. the incidence of too much light

combined with radiation-induced high temperature. It has been shown that the maximal conversion of violaxanthin to zeaxanthin is temperature-dependent and zeaxanthin production is suppressed at low temperatures (Arvidsson et al., 1997). The authors suggested that the tight packing of lipids at low temperature gave rise to the decreased zeaxanthin formation, influencing the availability of violaxanthin for VDE. We carried out the same type of experiments and confirmed the findings that violaxanthin de-epoxidation is suppressed at low temperatures. In addition, we also carried out temperature shift experiments to exclude the possibility that VDE had become irreversibly inactivated during the long (3 h) incubation time. Thylakoids were incubated with ascorbate at 4 °C for 50 min until the VDE reaction had almost stopped. Then the temperature was raised to either 25 °C or 37 °C (Fig. 1 in Paper II). Active conversion was rapidly resumed and the final level of violaxanthin conversion was the same as for the samples only exposed to the higher temperatures. It was concluded that by increasing the temperature gel phase ′melts′ to fluid phase, which in turn could promote the formation of curved regions, by non-lamellar lipids. These curved regions in the membrane could serve as preferred binding sites for VDE, consequently more violaxanthin was converted by VDE.

Even though de-epoxidation of violaxanthin can be modified by temperature, the question remains open why violaxanthin conversion becomes restricted at a specific level determined by the temperature. In Paper II we investigated the role of temperature on violaxanthin availability in a temperature-jump experiment (Fig. 7 in Paper II). Violaxanthin de-epoxidation was first run at 4 °C for 120 min, to reach essentially full conversion at that temperature. The enzyme reaction was reversibly stopped by increasing the pH in the reaction medium. The temperature was then raised to 37 °C where more violaxanthin should be available. Here violaxanthin and zeaxanthin should have the possibility to exchange with inaccessible violaxanthin whether it was present in the fluid regions in the gel of the membrane or bound to proteins. As the

conversion reached its maximum level at 4 °C no further zeaxanthin production was observed even though more violaxanthin was made available by transiently increasing the temperature to 37 °C. Finally, the temperature was again lowered and the conditions were changed back to low pH to resume VDE activity. Remarkably, no further conversion was observed. If it were just a question of availability a significant increase in the amount of zeaxanthin would be expected. Thus, it seems as if no more violaxanthin could be converted to zeaxanthin at this temperature although it had become available. One possibility is that zeaxanthin with its increased hydrophobic length might cause a stretched lipid structure with tighter packing and this in turn could inhibit further violaxanthin de-epoxidation.

4.3 Lipid packing and its effect on VDE activity

Lipid packing modifiers were employed to study the effect of either enhanced lamellar or non-lamellar structure formation on the violaxanthin conversion in the thylakoid membrane (Paper II). Unsaturated C-18 fatty acids, cetylethers and α-tocopherol were tested. Free fatty acids have been shown to modulate membrane lipid composition as they facilitate the formation of H_{II} structures in model membranes (Prades et al., 2003), as shown by X-ray diffraction and ^{31}P-NMR spectroscopy. We found that the higher the degree of unsaturation in the fatty acid tail was, the more violaxanthin was converted by VDE. In the case of linolenic acid the maximal level of violaxanthin de-epoxidation obtained was close to 100%. Cetylethers (poly(oxyethylene) alkyl ethers) are also known to affect lipid packing. In bacterial membranes supplemented with different cetylethers during cell growth the phase equilibrium was shifted towards non-lamellar structures (Wieslander et al., 1986). Addition of 8-cetylether to thylakoids caused a severe inhibition of violaxanthin conversion. The inhibition could be tuned by altering the size of the polyoxyethylene head group (Fig. 5 in Paper II). α-Tocopherol, a naturally occurring lipid-soluble antioxidant in the thylakoid membrane, increased the rigidity of

the membrane (Munne-Bosch and Alegre, 2002) and promoted lamellar phases. The *A. thaliana* mutant, *npq1*, increased the levels of α- and γ-tocopherol compensating for the lack of zeaxanthin (Havaux et al., 2000). Addition of extra α-tocopherol to the thylakoids caused a time- and concentration-dependent reduction of available violaxanthin for conversion (Paper II). These findings indicate that altering the equilibrium between the lamellar and non-lamellar phase by addition of lipid-packing modifiers to the membrane has a strong influence on the maximal level of violaxanthin de-epoxidation.

4.4 Laurdan-labelled thylakoid membranes and liposomes

Our hypothesis is that the xanthophyll cycle pigments are located in the lipid phase and that the conversion takes place in the lipid matrix of the thylakoids. It is then reasonable to assume that xanthophylls have a role to fulfil in the membrane. Xanthophylls have been suggested to decrease the membrane fluidity especially in the fluid phase (Gruszecki and Strzalka, 1991). However, the effect of violaxanthin conversion in native membranes has not been demonstrated. This is in part because the methods available to date are difficult to apply to the thylakoid membrane. To elucidate particular changes in membranous systems, including coexistence of different lipid phases and alteration in the fluidity, several techniques are available. These techniques (differential scanning calorimetry (DSC), electron paramagnetic resonance (EPR), fluorescence recovery after photobleaching (FRAP), nuclear magnetic resonance (NMR) and fluorescence spectroscopy) provide structural and dynamical information on the membrane system (reviewed by Bagatolli, 2006). We utilised fluorescence spectroscopy using a fluorescent probe, laurdan, to assess modulations in the membrane fluidity in response to xanthophyll cycle activity (Paper III). The advantage of using fluorescence spectroscopy is that model membranes and thylakoids can both be studied. In the case of laurdan labelled membranes, lateral

packing information can be obtained directly from the fluorescence images.

There are a number of fluorescent probes available to study the physical properties of lipid structures. Laurdan (Fig. 11) is one of them. Laurdan (6-lauroyl-2-dimethylaminonaphthalene) was first designed and synthesised by Gregorio Weber for the study of the phenomenon of dipolar relaxation of fluorophores in solvents, bound to proteins and associated with lipids (Weber and Farris, 1979). Since then, laurdan has been widely used to assess membrane physical properties, although mainly in phospholipid membrane studies. A great advantage of laurdan among other fluorescent probes is that it partitions to the lipid phase and its solubility in water is negligible (Davenport, 1997). Laurdan is incorporated into the membrane with the fluorescent naphthalene moiety situated at the level of the interface (glycerol backbone) region, and the lauric acid tail anchored in the hydrophobic core (Fig. 11).

Laurdan is a polarity-sensitive fluorescent probe, which means that both the emission and excitation spectra are sensitive to the polarity of water molecules in the vicinity of the probe and their dynamics in the hydrophobic/hydrophilic membrane interface (Parasassi et al., 1986).

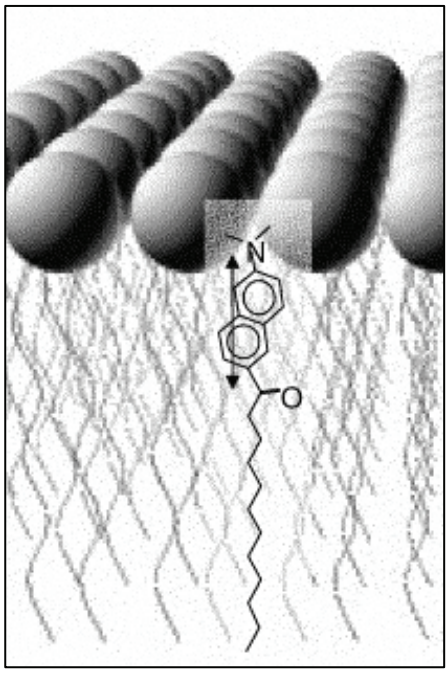

Figure 11. Sketch of laurdan's orientation and its dipolar moment with respect to the surface of the bilayer. The chemical structure of laurdan (6-lauroyl-2-dimethylaminonaphthalene) is also shown. Modified from Bagatolli (2006).

Upon excitation, the dipole moment of laurdan increases and water molecules surrounding laurdan will adopt to this new dipole (Parasassi et al., 1998). In tightly packed gel phase membranes with reduced molecular mobility in the hydrophobic region, the dipolar relaxation of these remaining water molecules is too slow to change the properties of fluorescent emission, thus the emission spectra have only one peak at 440 nm (Fig. 12). However, in loosely packed fluid phase membranes, where the interior is fluid allowing the lipid molecules to move around, dipolar relaxation of water molecules occurs, and it is apparent as a red-shift about 50 nm, increased fluorescence at 490 nm, in the emission spectra of laurdan (Parasassi et al., 1986; 1990). Hence, this phase dependent red-shift in the steady-state emission spectrum of laurdan reflects the excitation energy spent for relaxation of water molecules in the surroundings of the probe, and at the same time reports on the molecular dynamics in the membrane interface region (Parasassi et al., 1990).

For interpreting the average fluorescence signals from fluid- and gel-embedded laurdan, Parasassi et al. (1990) have developed the concept of general polarisation (GP) of laurdan fluorescence. The definition of general polarisation is $GP = (I_g - I_f)/(I_g + I_f)$ where I_g and I_f are the fluorescence intensity maxima of laurdan in gel and fluid phase, respectively (Fig. 12). In principle, higher GP values indicate a membrane in the gel (more rigid) phase with a low rate of solvent relaxation.

We characterised the behaviour of laurdan in model systems made of galactolipids and in thylakoid membranes dominated by MGDG and DGDG. Laurdan showed the same characteristics in galactolipid membranes as in phospholipid membranes (Parasassi et al., 1986; 1990; 1998; Hellgren, 1996; Hellgren and Sandelius, 2001). The fluorescence maximum was shifted about 50 nm when increasing the temperature thus inducing transition from gel to fluid phases (Figs. 2 and 4 in Paper III).

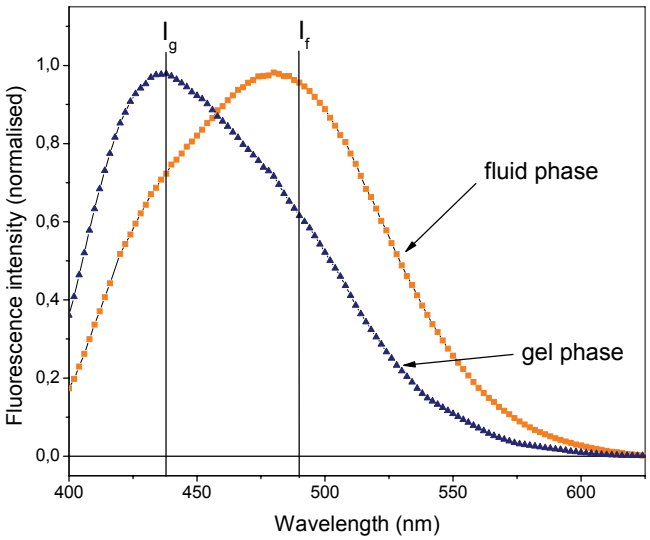

Figure 12. Laurdan emission spectra in gel (10 °C) and fluid (37 °C) phase dipalmitoyl-phosphatidylcholine (DPPC) membranes. The emission spectrum shift is around 50 nm. The generalized polarization parameter that depends on the position of the laurdan emission spectrum contains information about solvent dipolar relaxation processes that occur during the time that laurdan is in the excited state.

Thylakoid membranes with increasing amount of zeaxanthin were produced and laurdan fluorescence was measured. The GP values for laurdan increased in response to zeaxanthin formation indicating a more rigid membrane as the xanthophyll cycle proceeds and more and more zeaxanthin is formed (Table 1 in Paper III). A distinct membrane fluidity decrease has earlier been observed in PC membranes when violaxanthin and zeaxanthin was present (Gruszecki and Strzalka, 2005). The same authors have reported that the activity of the xanthophyll cycle leading to changes in the carotenoid pigments composition in the thylakoid membranes result in distinct modification of the fluidity of these membranes (Gruszecki and Strzalka, 1991). Using laurdan fluorescence spectroscopy with zeaxanthin-incorporated PC bilayers Socaciu et al. (2002) found a more ordered structure than in the absence of zeaxanthin. Our results are in line with the above studies. However, we found that zeaxanthin is more efficient than violaxanthin in making the membrane more rigid. The results in the natural membrane were confirmed by studies on liposomes composed of DGDG-MGDG, where zeaxanthin

addition resulted in a GP value higher than for pure DGDG, and lower than for pure DPPC (Table 2 in Paper III). Zeaxanthin, especially in the fluid phase, decreased the rate of dipolar solvent relaxation, revealing that the molecular mobility of water molecules decreased at the hydrophobic/hydrophilic interface of the laurdan-embedded membrane. The more pronounced effect of zeaxanthin could be explained by its increased hydrophobic length due to the disappearance of epoxi-groups. This could allow the fatty acid chains of lipid molecules to stretch somewhat more leading to tighter packing.

In summary, laurdan fluorescence spectroscopy can be successfully used to report physical properties of model systems made of galactolipids, i.e. thylakoid membrane and liposomes, respectively. In conclusion, our results further support the hypothesis that xanthophyll cycle pigments are located in the thylakoid and that the conversion of violaxanthin to zeaxanthin makes the thylakoid membrane more rigid, because zeaxanthin is more efficient in this respect.

4.5 Membrane curvature stress and violaxanthin conversion

One of the main reasons for lipid diversity in Nature is to maintain fluid bilayers. Adequate fluidity is crucial to membrane integrity and function. Bilayer fluidity and elasticity are collective physical properties resulting from the lipid mixture forming the membrane. Local curvature is another physical parameter that is thought to produce local stress in bilayer membranes. Since proteins and lipids are in active interplay in the membrane, the stress caused by local curvature affects protein-lipid interactions and thereby protein conformation and activity.

Bilayer-prone lipids, such as DGDG and PC, tend to form flat bilayer structures (Fig. 10). By contrast, non-bilayer-prone lipids, such as MGDG and PE, have a tendency to form non-bilayer phases (Fig. 10), i.e. H_{II}, whereby the monolayers bend towards water. Inclusion of MGDG into

DGDG bilayer increases the propensity of each of the DGDG/MGDG monolayers to curve towards water. Such curvature, however, cannot be achieved because the bilayer structure dictates a flat geometry. This leads to the build-up of curvature elastic stress within the DGDG/MGDG or PC/PE bilayers and a redistribution of the intermolecular lipid forces. There is an increase in the lateral pressure near the centre of the bilayer. In the thylakoid membrane the function of MGDG has been an important question. Israelachvili et al. (1980) have suggested that lipids like MGDG may act to pack large protein complexes, i.e. PSII, into biological membranes. Such a function in lipid-protein interactions would prevent MGDG from adopting non-bilayer structures. They also suggested that MGDG lipids could stabilise regions of high membrane curvature. In Paper II we show by increasing membrane fluidity, and inducing structures with non-lamellar properties the propensity for the curved regions and thereby the curvature stress in the thylakoid membrane was increased.

Curvature can be dynamically modulated by changes in lipid composition (McMahon and Gallop, 2005). Unsaturated bonds in lipid chains increase the tendency for curvature. In line with this we observed more violaxanthin conversion when the number of double bound increased in the C-18 fatty acids. The curvature stress is increased by the addition of the corresponding non-bilayer prone lipid. Alternatively, the stress could be decreased by the addition of a bilayer-prone lipid with fully saturated chains or by the addition of substances that induce lamellar arrangements, i.e. cetylethers (Wieslander et al., 1986) and α-tocopherol (Bradford et al., 2003) (Paper II).

Curvature stress was recently shown to be involved in vesicle budding (McMahon and Gallop, 2005), protein folding (Booth, 2005) and on a larger scale in growth, division and movement (McMahon and Gallop, 2005). The yield of folded bacteriorhodopsin protein increased with decreasing bilayer stress. Stressed bilayer prevented bacteriorhodopsin

from entering the bilayer, thus reducing the folding yield (Booth et al., 2001). Also, curvature stress of biological membranes has been shown to affect the activity of a number of enzymes i.e. CTP:phosphocholine cytidyltransferase and phosphoinositide 3-kinase, (Attard et al., 2000; Hubner et al., 1998; Drobnies et al., 2002). VDE may prefer the curved regions most probably enriched in MGDG for proper docking to the membrane. These regions can be promoted by higher temperatures and by additions of polyunsaturated fatty acids. On the other hand, when the incidence of curved regions decrease (e.g. in response to lamellar structure promoting substances), the docking site for VDE also decreases.

Curvature stress affects protein behaviour, but the opposite is also true and the incorporation of a protein or other membrane-located substances into a bilayer will affect the curvature stress. This could be the case for zeaxanthin. We proposed (Paper II) that converting violaxanthin to zeaxanthin increases the hydrophobic length in the molecule (11 double bonds instead of 9 in violaxanthin), and thereby zeaxanthin affects lipid packing, in a way that it decreases membrane fluidity (Paper III). The membrane expands and brings about a release of curvature stress leading to a less favoured lipid environment for VDE. As seen in the temperature-jump experiment, even if violaxanthin was made available, there was no further zeaxanthin formation. This was perhaps because zeaxanthin could indirectly inhibit VDE. A possibility not yet tested is that the changed membrane packing could release VDE from the thylakoid membrane. My current hypothesis emerging from these data is shown in Figure 13.

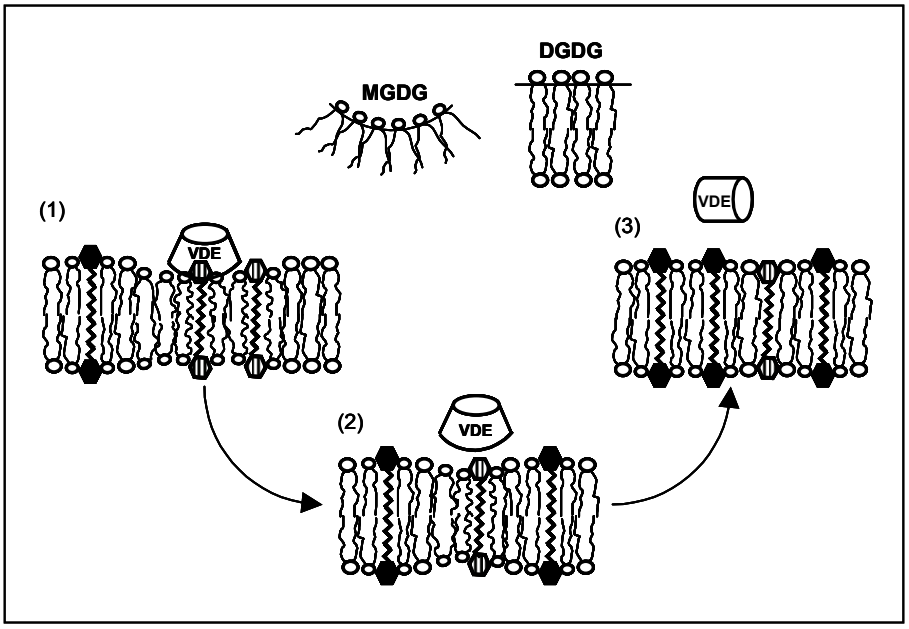

Fig. 13. Schematic model of the effect of curvature stress on xanthophyll cycle.
The thylakoid membrane is composed of 50 mol% MGDG (H_{II} lipid) and 30 mol% DGDG (L_α). MGDG has a high propensity for interfacial curvature. Upon temperature increase, the formation of the MGDG-rich regions gives rise to curvature stress in the bilayer. MGDG serves as an efficient host for violaxanthin (hatched hexagon) and is also required by the enzyme, VDE (truncated cone). (1) The enzyme docks to the membrane and converts violaxanthin to zeaxanthin (black hexagon). (2) As the conversion proceeds, more hydrophobic and stretched zeaxanthin is formed, (3) the membrane expands and brings about a release of curvature stress leading to a less favoured lipid environment for VDE (cylinder) and as a consequence VDE is released from the membrane. Elevated temperature and the presence of linolenic acid facilitate zeaxanthin formation, whereas low temperature, the presence of other membrane modifiers such as 8-cethylether and α-tocopherol during the conversion retard the formation of zeaxanthin.

5 Summary and future perspectives

The xanthophyll cycle involves the light-dependent conversion of violaxanthin to zeaxanthin and it is catalysed by violaxanthin de-epoxidase (VDE). The enzyme is membrane-hosted and its activity is controlled by the pH. Monogalactosyldiacylglycerol (MGDG), an inverted hexagonal phase forming lipid, violaxanthin and ascorbate are required for activity. The zeaxanthin formed is involved in protection of the photosynthetic apparatus from overexcitation. Apart from its energy-

dissipating role, the xanthophyll cycle has been implicated in a modulation of the physical properties of the thylakoid membrane.

VDE is a 43 kDa protein, soluble in the lumen at high pH. During photosynthetic activity, the lumenal pH drops and VDE undergoes a conformational change causing the active enzyme to bind to the lumenal side of the thylakoid membrane. Binding to the membrane is a crucial part of the violaxanthin to zeaxanthin conversion. Four histidine residues in the lipocalin region are highly conserved and in Paper I we show that they are important for the enzymatic activity and membrane binding. More specifically, the cooperativity (Hill constant), with respect to protons, for binding VDE to the thylakoid membrane was decreased upon mutations of these histidines.

The amount of violaxanthin converted, and the zeaxanthin produced was changed by chemically altering the tightness (enhanced lamellar structure formation in the thylakoids) or looseness (more H_{II}-phase prone lipids in the membrane) of the membrane of the isolated spinach (*Spinacia oleracea*) thylakoids (Paper II). Thus, lipid packing of the thylakoid membrane influences the activity of VDE. We propose a model involving membrane curvature stress, thylakoid membrane packing and the xanthophyll cycle. According to this model the different xanthophyll pigments have different preferences for different membrane structures and can themselves induce differentially curved membrane regions. Zeaxanthin with its increased molecular hydrophobic length affects lipid packing, and brings about a release of curvature stress leading to a less favoured lipid environment for VDE. To further test our model, I propose that VDE's preference for the differently curved membranes is studied. Thus, binding of VDE to thylakoid membranes in violaxanthin- and zeaxanthin-form or as a function of temperature should be carried out.

To investigate membrane fluidity of the thylakoids and lipid mixtures a new approach was used. Paper III reports on a special dye, laurdan, which

changes fluorescent properties dependent whether inserted into a gel or a fluid membrane. We found that the conversion of violaxanthin to zeaxanthin made the thylakoid membrane more rigid, zeaxanthin was more efficient in this respect. These results further support the hypothesis that xanthophyll cycle pigments are located in the lipid part of the thylakoid membrane.

Sterols have a special role in maintaining the adequate membrane fluidity in plant membranes, especially during exposure to cold stress. We studied the physiological role of the PSAT enzyme modifying plant sterols in mutants and wildtype of *Arabidopsis thaliana*. By analysing xanthophyll cycle function and chlorophyll fluorescence parameters we could show that a knockout mutant of PSAT had severe problems to adapt to low temperature. This suggests that the activity of PSAT has an important role in cold acclimation.

Populärvetenskaplig sammanfattning på svenska
(Swedish summary)

Fotosyntetiska membraner utsätts för stress vid höga ljusintensiteter och skador kan lätt uppstå. En av de viktigaste skyddande substanserna i växter är karotenoiden zeaxantin. Zeaxantin bildas från violaxantin i xantofyllcykeln och bildningen katalyseras av enzymet violaxantin de-epoxidas (VDE). I aktiv form är enzymet bundet till det fotosyntetiska membranet (tylakoidmembranet). Enzymet kräver lågt pH, askorbinsyra (vitamin C) och en speciell lipid, monogalactosyldiacylglycerol (MGDG). Produkten zeaxantin har olika funktioner. Den viktigaste är att delta i skyddet av fotosyntesmaskineriet mot överskott av ljus. Bildningen av zeaxantin har också föreslagits kunna påverka rörligheten hos komponenter i tylakoidmembranet och är en av de centrala frågorna som jag behandlar i denna avhandling.

VDE har molekylvikten 43 kDa och är löslig i tylakoidernas inre (lumen) vid högt pH. Vid höga ljusintensiteter och därigenom mycket aktiv fotosyntes sjunker pH i lumen, VDE ändrar sin form och binder till membranet. Bindningen till membranet är avgörande för aktiviteten eftersom violaxantin befinner sig i membranet. I arbete I visar vi, med hjälp av riktad mutagenes, att fyra konserverade hisitidiner i VDE är väsentliga för det starka pH beroende enzymet har för sin inbindning till membranet.

I arbete II har vi studerat hur aktiviteten hos VDE påverkas av lipidernas packning i tylakoidmembranet. MGDG är en lipid som krävs för enzymets aktivitet men dess närvaro i membranet leder till inre spänningar i membranet. Genom att tillsätta substanser som ökar eller minskar spänningarna i membranet har vi kraftigt påverkat graden av omvandling av violaxantin. Ju mer spänningar desto mer violaxantin gick att omvandla. Vår slutsats är att VDE kräver spänningar i membranet för

att fungera. Zeaxantin med sin utökade hydrofoba längd föreslogs göra membranet tätare och hjälpa till att minska spänningarna.

I arbete III visar vi för första gången att packning och rörlighet i tylakoidmembran kan studeras med hjälp av en fluorescerande substans, laurdan. Laurdan ändrar sina fluorescensegenskaper när dess egen och närmaste vattenmolekylers rörelse minskar. I detta arbete har vi kunnat visa att violaxantin, zeaxantin och α-tocopherol alla gör membranet mindre rörligt men att zeaxantin är effektivast. Omvandlingen av violaxantin kan nu ses i ett vidare perspektiv. Eftersom växter ofta utsätts för värme samtidigt som de utsätts för hög ljusintesitet ger bildningen av zeaxantin inte bara skydd av fotosyntesmaskineriet utan kan också hjälpa till att stabilisera membranstrukturen.

Summary in Hungarian

(Összefoglaló magyarul)

A zöld növények, a moszatok és bizonyos baktériumok képesek a napfény energiáját átalakítani, és kémiai kötések formájában raktározni. Ezt a kloroplasztokban lejátszódó fizikai-kémiai folyamatot fotoszintézisnek nevezzük. A fotoszintézis során olyan szerves molekulák képződnek, amelyeknek az elégetése (oxidálódása) lehetővé teszi a fotoszintézisre nem képes élőlények életét. A fényenergia begyűjtéséhez a fotoszintetizáló szervezetek speciális pigmenteket használnak, amelyek közül a legfontosabbak a klorofillok és karotenoidok. A karotenoidokat járulékos pigmetnek is nevezik, mivel elsősorban a fényenergia hatékonyabb elnyeléséhez járulnak hozzá. A xantofillok speciális karotenoidok. Közülük kettő (a violaxantin és a zeaxanthin) különös jelentőséggel bír a fotoszintézisben, elsősorban erős napsugárzás esetén.

A tilakoid membrán - más néven a fotoszintetikus membrán - a kloroplasztokban található membránrendszer (12. oldali ábra A része), amely helyet ad az egész fotoszintetikus apparátusnak (12. oldali ábra B része). A tilakoid membrán fokozottan ki van téve az erős napsugárzás okozta károsodásnak. A legfontosabb védekező mechanizmus a violaxantin zeaxantinná alakítása. A zeaxantin violaxantinból való átalakítása enzimatikus módon a xantofill-cikulsban (20. oldali ábra) történik. A közreműködő enzimet violaxantin-deepoxidáznak (VDE) hívják. Egy másik enzim, a zeaxanthin epoxidáz (ZE), zeaxantinból csinál violaxantint sötétben, vagy nagyon kevés fény esetén. A VDE aktív formájában a tilakoid membránhoz kötődik, és a violaxantint zeaxantinná alakítja. Egy speciális, cukor tartalmú tilakoid-lipid (MGDG), aszkorbinsav (C-vitamin) és alacsony pH (savas közeg) szükséges ahhoz, hogy a VDE aktivizálódjon és violaxantinból zeaxantint készítsen.

A zeaxantinnak különböző funkciói vannak. A legfontosabb ezek közül talán a fotoszintetikus apparátus védelme az erős fény káros hatásai ellen.

A kutatások szerint a zeaxanthin ezen kívül befolyásolhatja a tilakoid membrán szerkezetét. Doktori tanulmányomban én is ezzel a tulajdonságával foglalkoztam.

A VDE egy 43 kDa protein, magas pH (lúgos, nincs fotoszintézis) esetén vízoldékony. Maga az enzim a tilakoid membrán által körülzárt vizes közegben, a lumenben található. Amikor beindul a fotoszintetikus folyamat a lumenben lecsökken a pH, a VDE magváltoztatja az alakját és a membránhoz kötődik. Az enzim aktivitása a tilakoid membránban található átalakított violaxantin mennyisége alapján mérhető. Az első cikkben (Paper I) azt vizsgaltuk, hogy a négy megőrzött hisztidin aminosav a fehérjeszerkezetben milyen mértékben befolyásolja a membránhoz való kötődést. Kutatásaink alapján ezek a hisztidinek, meghatározó jelentőségűek a VDE pH-függő viselkedésében, és ezáltal a membránhoz való kötődésében.

A második cikkben (Paper II) azt vizsgáltuk, hogyan változik az enzim aktivitása, ha megváltozatjuk a tilakoid membránban az úgynevezett hajlékonysági stresszt. Az MGDG egy speciális lipid, amelyik a VDE aktivizálásához szükséges, és egyben egyfajta hajlékonysági stresszt is előidéz a membránban. Ha olyan kémiai anyagokat adunk a membánhoz, amelyek az MGDG-hez hasonló szerkezetek képződését stimulálják, több violaxantin alakul át zeaxantinná. Ezzel szemben ha olyan anyagokat juttatunk a membránba, amelyek a hajlékonysági stresszt csökkentik - más szóval csökkentik az MGDG típusú szerkezetek mennyiségét - kevesebb violaxantin alakult át zeaxantinná. Összefoglalva, a VDE a működéséhez igényli ezt a hajlékonysági stresszt a tilakoid membránban. Ezzel szemben a képződött zeaxantin csökkenti ezt a fajta stresszt, meggátolva ezzel az enzim további működését.

Az utolsó cikkben (Paper III) beszámolunk egy kísérletről, amelynek során - egy fluoreszcenciás módszer segítségével- elsőként mutattuk ki, hogy a xantofillok a tilakoid membránban módosítják a membrán

keménységét. Ehhez egy festéket, laurdant használtunk, amelyik megváltoztatja a fluoreszcenciáját, ha a saját, és a környezetében lévő vizmolekuláknak lecsökken a mozgása. A vizsgálatok során kimutattuk, hogy mind a violaxantin, mind pedig a zeaxantin megkeményíti a membránt, de a zeaxantin nagyobb hatást fejt ki. A zeaxantin erős napsugárzás elleni védelme sokkal hatásosabb, mint a violaxantiné. Mivel az erős napsugárzás általában megemelkedett hőmérséklettel is jár, a zeaxantin nemcsak a fotoszintetikus apparátust védi az erős fény okozta károsodásoktól, hanem a tilakoid membránt is stabilizálja, és ezáltal biztosítja a fotoszintézis folyamatosságát.

Acknowledgements

My sincere thanks to:

My supervisor Professor Hans-Erik Åkerlund for introducing me into the world of science, his patience, his never-ending optimism and for those hours when we repaired something, mainly the HPLC. My co-supervisor Professor Marianne Sommarine for her understanding, cheer-ups and endless hours reading-correcting-thinking through my writings. It has been a pleasure to be your student over these years.

Professor Christer Larsson for giving me the chance to make this possible.

Magnus for solving every computer-related problem. Maria for helping me in the gel-room and for encouragement. Lisa for all the nice mama-talks. Sinan for keeping me company in the lab and for baklava. Anna Å and Katja for those hours in Umeå airport. Virginia for the nice chats at lunch. Laura for your friendship. And other past and present colleagues of "Plant Biochemistry" and now Biochemistry.

Adine for all her inevitable help in the lab. Maivi for helping me with all kinds of paperwork. Gert for fixing whatever needed.

Simon P. Gough for proof reading this thesis.

Köszönet a Lundi barátoknak: Szász, Marcian/Szatmári, Rusz és Istók családoknak; az otthoniaknak: Benkő, Simonics és Miholics családoknak, hogy mindenben a segítségemre voltak.

És a családomnak, akik mindig mindenben mellettem álltak-állnak. Köszönet a szeretetért, támogatásért és a belém vetett hitért.

Gáborom, mindez (a thesis és ami mögötte van) és rengeteg más dolog az életemben értéktelen lenne nélküled, a szerelmed, mellettemállásod, és biztatásod nélkül!
Ábel, mostantól jöhet az állatkert, az uszoda és a játéküzlet programok.

To our growing family!

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