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review

The xanthophyll cycle - molecular mechanism and physiological significance

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Abstract

The light-dependent, cyclic changes of xanthophyll pigments: violaxanthin, antheraxanthin and zeaxanthin, called the xanthophyll cycle, have been known for about fifty years. This process was characterised for higher plants, several fern and moss species and in some algal groups. Two enzymes, violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE), belonging to the lipocalin protein family, are engaged in the xanthophyll cycle. VDE requires for its activity ascorbic acid and reversed hexagonal structure formed by monogalactosyldiacylglycerol. ZE, postulated to be a flavoprotein, has not been purified yet and it is known from its gene sequence only. Zeaxanthin epoxidation is dependent on the reducing power of NADPH and presence of additional proteins.

The xanthophyll cycle is postulated to play a role in many important physiological processes. Zeaxanthin, formed from violaxanthin under high light conditions, is thought to be a main photoprotector in autotrophic cells due to its ability to dissipate excess of absorbed light energy that can be measured as a non-photochemical quenching. In addition the zeaxanthin formation is important in protection of the thylakoid membranes against lipid peroxidation. Other postulated functions of the xanthophyll cycle, which include regulation of membrane physical properties, blue light reception and regulation of abscisic acid synthesis, are also discussed.

List of abbreviations:

VDE; violaxanthin de-epoxidase
Vx; violaxanthin
MGDG; monogalactosyldiacylglycerol
DGDG; digalactosyldiacylglycerol
PE; phosphatidylethanolamine
Zx; zeaxanthin
Ax; antheraxanthin
Xc; xanthophyll cycle
ZE; zeaxanthin epoxidase
NPQ; non-photochemical quenching
ABA; abscisic acid
DTT; dithiothreitol
SCR; short conservative motifs
H_{II}; reversed hexagonal structure

Introduction

Light is one of the most important environmental factors influencing photosynthetic activity of green plants. The absorption of light by antenna pigments and the transfer of excitation energy to the reaction centres of Photosystems I and II are the primary steps in this process. To achieve high efficiency of photosynthesis, plants have developed regulatory mechanisms to adapt their photosynthetic apparatus to variable light conditions, which can rapidly

change in both duration and intensity during the day. At low light intensity, the system must be able to convert as much as possible of the available energy into its useful form. However, under high light condition, the available light energy may exceed the plants ability to use the photosynthetic systems at an efficient rate (see, for example, Fryer *et al.* 2002). To avoid damage under such conditions the plants have created several adaptive and protective mechanisms. These mechanisms may operate at various levels of organisational complexity, *e.g.*, as a movement of assimilatory organs, (leaves), translocation of chloroplast within the cell, changes in distribution of pigment-protein complexes in the thylakoid membrane, and others. One of the mechanisms optimising the amount of light necessary for photosynthesis is the xanthophyll cycle, which was discovered by Sapozhnikov and coworkers in 1957. These researchers described a decrease in the content of one of xanthophyll pigments, violaxanthin (Vx), in plants after high light treatment and its subsequent increase in low light or darkness (Sapozhnikov *et al.* 1957). Yamamoto *et al.* (1962) showed, that decrease in the Vx concentration in plants in intensive light was connected to the Vx transformation into the different xanthophyll pigment, zeaxanthin (Zx).

The following studies, mainly by Yamamoto (Yamamoto and Takeguchi 1972, Yamamoto and Kamite 1972, Yamamoto *et al.* 1974, Yamamoto 1979), Siefermann-Harms (1977) and Hager (1980) allowed a more accurate description of the light dependent transformation of xanthophyll pigments in plants. However, the xanthophyll cycle is still the subject of intensive studies concentrated on the explanation of both molecular mechanism of the cycle and its diverse functions in plants.

Forms of the xanthophyll cycle and their distribution

The light dependent changes in the Vx concentration, discovered by Sapozhnikov, are called the xanthophyll cycle (Xc) or the violaxanthin cycle. This process represents a sequence of two reactions, both composed of two steps (Fig. 1). The first reaction is the dependent on the light intensity de-epoxidation of Vx to Zx. The second reaction of

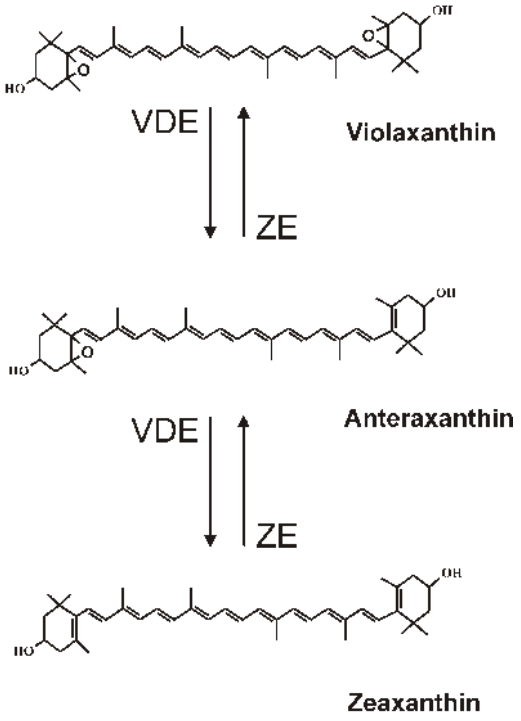

Fig. 1

Fig. 1. Reactions of the xanthophyll cycle typical for higher plants. VDE - violaxanthin de-epoxidase, ZE - zeaxanthin epoxidase.

the cycle is the epoxidation of Zx to Vx. Both these processes have one common intermediate product, xanthophyll monoepoxide called antheraxanthin (Ax). De-epoxidation and epoxidation are catalysed by two different enzymes: the violaxanthin de-epoxidase (VDE) is responsible for the first reaction and the zeaxanthin epoxidase (ZE) is believed to mediate the second reaction.

The Xc, defined as above, was described in the thylakoid membranes of all higher plants, ferns, mosses and some algae (Table) (Stransky and Hager 1970, Adamska 1997). Recently, light dependent and reversible changes of epoxylutein to lutein and of lutein to epoxylutein were found in some higher plants, (*Cuscuta reflexa Roxb*., *Amyema miquelli* and in quercus), (Fig. 2; Bungard *et al.* 1999, Matsubara *et al.* 2001, Garcia-Plazaola *et al.* 2002, 2003).

A modification of the Xc was also described in marine alga, *Mantoniella squamata* (Goss *et al.* 1998).

Table. Occurence of different forms of the xanthophyll cycle in hotoautotrophic organisms

The violaxanthin cycle	The diadinoxanthin cycle	Lack of xanthophyll cyclic changes
Higher plants	*Bacillariophyceae*	*Cyanobacteria*
Ferns	*Chrysophyceae*	Photosynthetic bacteria
Mosses	*Xanthophyceae*	Most of *Rhodophyta*
Phaeophyta	*Rhaphidophyta*	*Cryptophyta*
Chlorophyta	*Dinophyceae*	
Some species of *Rhodophyta*	*Euglenophyta*	

The most intriguing feature of this cycle is that the second step of the de-epoxidation *i.e*. conversion of Ax to Zx is very slow and, as a consequence, and contrary to typical Xc, Ax is accumulated in place of Zx . These observations lead to the conclusion, that VDE from *Mantoniella* has less affinity to Ax than VDEs from other plants. Another kind of xanthophyll transformation was discovered in cells of diatoms. In this systematic group, the violaxanthin cycle does not occur. However, another xanthophyll pigment, diadinoxanthin, is de-epoxidated to diatoxanthin in high light. The reaction reverses after turning light down (Stransky and Hager 1970), (Fig. 3). Recently, it has been shown (Lohr and Wilhelm 1999) that in *Phaeodactylum tricornutum*, in addition to the diadinoxanthin cycle, the Xc also operates. The diadinoxanthin de-epoxidase from the cells of this alga is characterised by higher pH optimum than diadinoxanthin de-epoxidase and violaxanthin de-epoxidases from other organisms (Jakob *et al*. 2001).

The possibility of zeaxanthin synthesis from β-carotene in high light was shown for some representantatives of *Cyanobacteria* (Demmig-Adams 1990), but neither Vx nor Ax was found in these organisms. As it is summarised in Table, except several groups of autotrophs where one or two forms of the Xc have been detected, there are also some other groups, where the Xc has not yet been described. Although, it must be noted that these groups have not been fully explored for the presence of the xanthophyll cycle.

Enzymes of the xanthophyll cycle

Violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE) are two of the six known plant

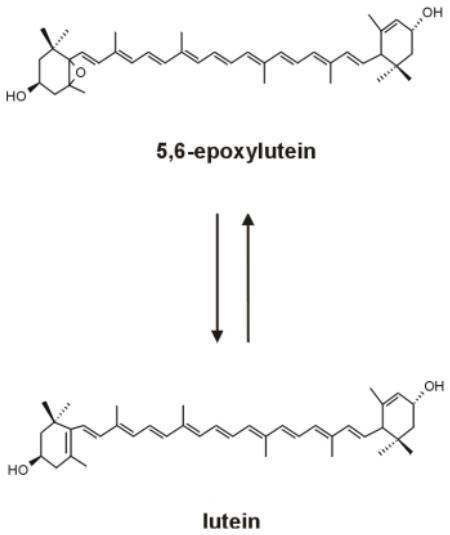

Fig. 2. Light dependent and reversible changes of epoxylutein to lutein (modified from Bungard *et al.* 1999, Matsubara *et al.* 2001).

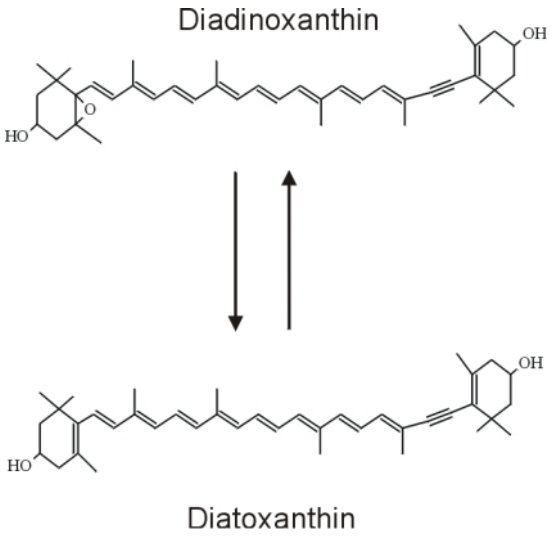

Fig. 3. The diadinoxanthin cycle occuring in diatoms (Lohr and Wilhelm 1999).

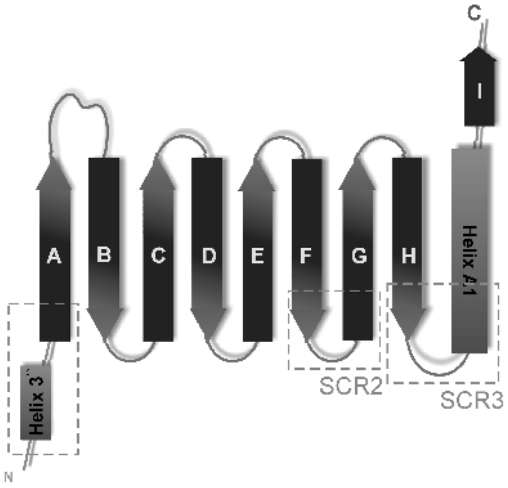

Fig. 4. Scheme of the secondary structure of typical lipocalin domain (modified from Flower *et al.* 2000). SCR - short conservative region, A-J - β-sheets of β-barrel.

Fig. 5. The tertiary structure of model lipocalin, bovine β-lactoglobulin, complexed with retinoic acid (Kontopidis *et al.* 2002). The picture was proceeded in MOLSCRIPT (Kraulis 1991) and Raster3D (Merritt and Bacon 1997).

lipocalin proteins. Biological function of lipocalins was described only for VDE and ZE (Bugos *et al.* 1998, Hieber *et al.* 2000). Lipocalins are widely characterised for animals (Flower 1996) and *Procaryota* (Barker and Manning 1997, Bishop 2000). The main features of these proteins are, firstly, similar tertiary structure, and secondly, their function in organism. The characteristic feature of lipocalins is the presence of eight, antiparallel b-sheets (Fig. 4). Among them, three highly conservative motifs, known as SCR, can be distinguished (Flower *et al.* 2000):

· motif I - first of the eight β-sheets, preceded by a short fragment of α-helix;
· motif II - fragments of the sixth and the seventh β-sheet, together with the loop between these sheets;
· motif III - part of the eighth β-sheet together with the fragment of C-terminal α-helix and the loop between this helix and the eighth β-sheet.

Some of lipocalins, thought previously to contain eight β-sheets (Bugos *et al.* 1998), consist of six β-sheets only as described for VDE and retinol binding protein. The kernel lipocalins (*e.g.* retinol--binding protein, glycodelin or β-lactoglobulin) are conserved in all SCRs and outlier lipocalins (VDE, ZE, lazarillo – a neuronal protein in grasshoppers, and neuthrophil-gelatinase associated lipocalin) are conserved just in two or only one SCR (Åkerstrom *et al.* 2000). Crystalographic research showed that the characteristic structure of β-sheets is responsible for creation of a deep, conical hollow, necessary for substrate binding. The depth of the hollow in examined proteins is about 40 Å (Newcomer *et al.* 1984, Holden *et al.* 1987). The structure like the one shown in Fig. 5 is typical for lipocalins and is associated with their function. All proteins belonging to this class are able to bind and carry small hydrophobic molecules (Pervaiz and Brew 1985). The presence of the hollow in VDE molecules was already predicted (Yamamoto 1979). The depth of this hollow fits the length of Vx molecule and this is why VDE is strictly specific to 3 OH, 5,6-epoxycarotenoids in configuration 3R, 5S, 6R (Yamamoto 1979, Grotz *et al.* 1999). Except for VDE and ZE, there is only one other known lipocalin with enzymatic activity - prostaglandin D synthase (Urade and Hayashi 2000).

VDE is encoded in nuclear DNA. In 1996 cDNA of VDE was cloned for the first time and expressed in *Escherichia coli* (Bugos and Yamamoto 1996). These experiments allowed to determine the number of amino acids (348 residues), and to calculate the molecular mass of VDE as 39.9 kDa, which is close to 43 kDa resulting from its determination us-

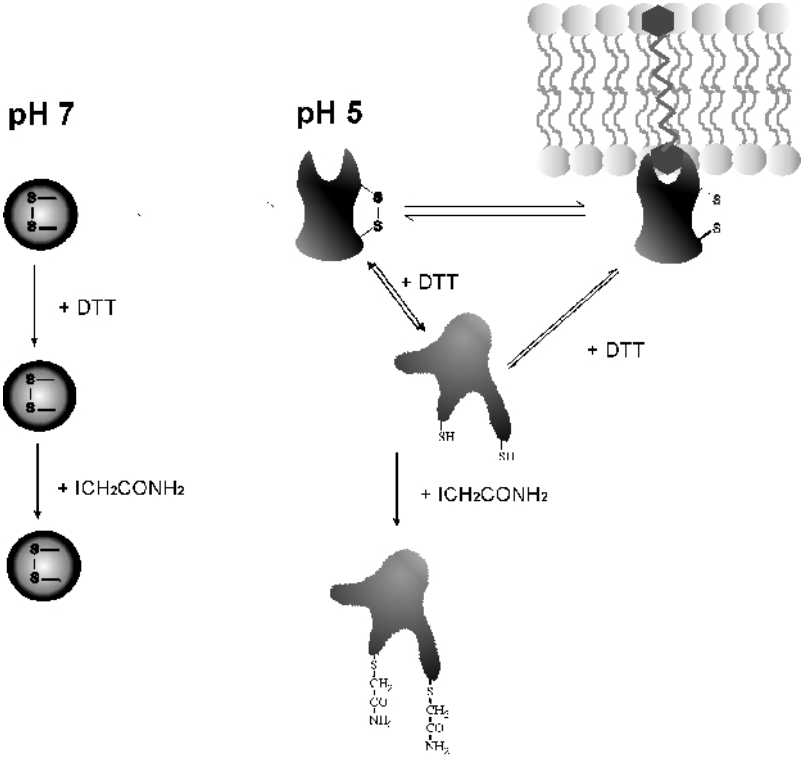

Fig. 6. Conformational changes and pH dependence of VDE (modified from Eskling 1998). In pH 7 or higher, all VDE molecules are in unbound conformation. After pH decrease, conformation of the enzyme changes, (S-bridge is exposed to environment), and VDE molecules bind to the membrane. This process can be detained by reversible inhibition by dithiothreitol (DTT) or irreversible inhibition by iodoacetamide.

ing PAGE (Åkerlund *et al.* 1995, Arvidsson *et al.* 1996, Rockholm and Yamamoto 1996, Havir *et al.* 1997). Calculated isoelectric point amounts 4.57, as compared with the 5.4 found experimentally (Rockholm and Yamamoto 1996).

Besides the lipocalin domain, there are also two other domains in VDE. The first of them is the N-terminal region enriched in cystidyl moieties (11 from all 13 found in VDE). This region most probably contains α-helices. Second, C-terminal domain is charged and enriched in glutamyl residues and it probably contains long α-helices. Domains like those have also been found in VDE of other plants. Comparison of amino acid sequence (deduced from cDNA) indicates a high degree of homology among VDE proteins from different plants. VDEs from *Arabidopsis thaliana*, *Nicotiana tabacum* and *Lactuca sativa* differ just in nine amino acid residues (Hieber *et al.* 2000). Transit peptides of VDE do not show such a high homology. Their structure is similar but the reported amino acid sequences agree in eight residues only (Bugos *et al.* 1998). Knowledge of the amino acid sequence permits to understand some specific properties of VDE. Cysteine enriched domain is responsible for inhibitory effect of dithiothreitol (DTT), which reduces the disulphide bonds in the enzyme molecule (Yamamoto and Kamite 1972, Bugos and Yamamoto 1996). Inhibition (at pH 5.2 and 5.7) is reversible, but it is not reversible after iodoacetamide treatment (Arvidsson *et al.* 1997). After treatment at pH 7.2, the chemicals do not influence enzyme activity which means that in such conditions disulphide bonds are not exposed to the environment. This result shows that the pH-dependent conformational changes in the VDE molecule are necessary for enzymatic activity (Fig. 6).

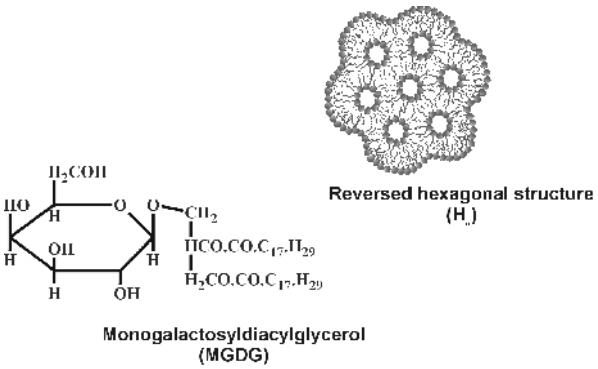

Fig. 7. Monogalactosyldiacylglycerol and inverted hexagonal structure it forms in water.

VDE was identified as a water-soluble and lumen localised protein (Hager 1969). Later, VDE can be either unbound or bound to the thylakoid membrane depending on the lumenal pH (Fig. 6). Connection to the membrane is important for enzymatic activity (Hager and Holocher 1994). At pH lower than 6.0, all VDE molecules are associated with the membrane. If pH increases to 7.0 or more, the VDE exists in an unbound form. In pH 6.6, half of VDE molecules was found to be linked to the membrane

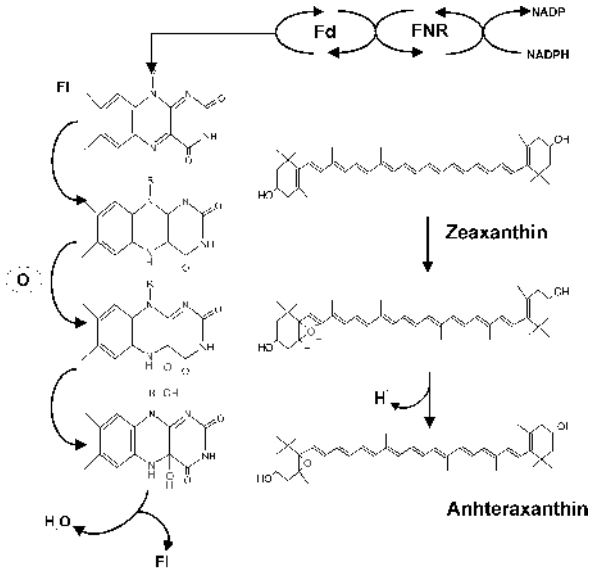

Fig. 8.

Fig. 8. Postulated mechanism of ZE catalysed rection (Bouvier *et al.* 1996). Fl_{ox} -oxidised flavine, Fd - ferredoxin, FNR - ferredoxin:$NADP^+$ oxidoreductase. Flavine (cofactor of zeaxanthin epoxidase) is reduced by Fd. Electrons to Fd reductions came from NADPH, through FNR. Reduced flavin binds oxygen molecule, then the OH^- group is transfered to zeaxanthin, to create the unstable carbocation. In the following reaction H^+ ion is removed and antheraxanthin molecule is formed.

(Bratt *et al.* 1995). These properties of VDE are useful for its purification (Yamamoto and Higashi 1978, Havir *et al*. 1997). Membrane joining occurs by the C-terminal, a charged domain of the enzyme. At low pH, glutamic acid residues are protonated which can facilitate their association with the membrane (Bugos and Yamamoto 1996, Hieber *et al.* 2000).The optimum pH for VDE activity *in vivo* is 4.8 and it increases to 5.2 after isolation (Hager 1969).

Activity of an isolated enzyme can be measured spectrophotometrically. The reaction mixture except Vx has to contain additional components - monogalactosyldiacylglycerol (MGDG, Fig. 7) and ascorbic acid (Hager 1969, Siefermann and Yamamoto 1975b, Yamamoto and Higashi 1978, Latowski *et al.* 2000, Muller-Moule *et al.* 2002). MGDG is the main lipid component of the thylakoid membrane (about 50 % of total thylakoid lipids). According to Yamamoto, MGDG forms *in vitro* micelles containing Vx molecules, which are exposed in this way to VDE (Yamamoto and Higashi 1978). Ascorbic acid (Asc) is thought to be an endogenous electron donor for de-epoxidation and regulator of the VDE activity (Sokolove and Marscho 1976, Yamamoto 1979, Neubauer and Yamamoto 1994, Bratt *et al.* 1995). The mechanism of the transport of Asc through the thylakoid membrane has not been elucidated yet. Different researches showed that Asc transporters can be found in the cell and outer chloroplast membranes but not in the thylakoid membrane. Consequently, it has to be assumed that Asc molecules diffuse across the thylakoid membrane (Foyer and Lelandis 1996). However, concentration of the protonated form of Asc increases as a function of a decrease of the lumenal pH during photosynthesis (Fig. 8). Protonated form of Asc can influence VDE activity simply as a substrate (Bratt *et al.* 1995, Eskling *et al.* 1997). Asc is converted into dehydroascorbate (DHA) during Ax formation. On the other hand, no mechanism engaged in rereduction of DHA into Asc in thylakoid lumen is known. Such process, however, is believed to occur in the stroma. This is why the presence of the DHA transporter in the thylakoid membrane is postulated with the assumption that Asc can freely diffuse across membranes (Bratt *et al.* 1995, Foyer and Lelandis 1996, Eskling *et al.* 1997).

Zeaxanthin epoxidase (ZE) is the second identified plant lipocalin. Its cDNA was first characterized for *Nicotiana plumbaginifolia* (Marin *et al.* 1996), later for *Capsicum annum* (Bovier *et al.* 1996), *Lycopersicon esculentum* (Burbidge *et al.* 1997), *Arabidopsis thaliana* and *Prunus armeniaca* (Hieber *et al.* 2000). ZE also contains tertiary structure characteristic for lipocalin (Fig. 4). Differences between ZE and VDE are in the number of amino acids between motifs I and II. In the case of ZE from *Nicotiana* there are 103 residues, but 65 to 73 residues were found in VDEs from different sources. The increase in amino acid number gives evidence about longer loop, or about the existence of additional β-form in that region of the protein (Bugos *et al.* 1998). The level of homology between motif II and III of ZE and those motifs of other lipocalins seems to be rather low (Flower *et al.* 1993, Flower 1996). The homology to VDE is also low (Hieber *et al.* 2000). However, it should be

noticed that the first motif of different ZEs (except ZE of *Prunus*) aligns well with other lipocalins. In this region, after triptofan and tyrosine residues, follows an unusual cysteine moiety. This position is conserved in every known lipocalin (Hieber *et al.* 2000). Besides the similarity in the tertiary structure, there are no similiarities between ZE and VDE in the amino acid sequence. Contrary to VDE, ZE is proposed to be localised on the stromal side of the thylakoid membrane. ZE is thought to be a monooxygenase, catalysing reaction of epoxidation in positions 5 and 6 of xanthophyll rings (Hieber *et al.* 2000). In plants, this process occurs in the dark or in weak light. Some data indicate, that it can be observed even in high light epoxidation (Siefermann and Yamamoto 1975a, Gilmore *et al.* 1994, Frommolt *et al.* 2001). Optimum pH for ZE activity is 7.5 (Bouvier *et al.* 1996). Until now, in spite of many attempts, an active form of this enzyme has not been isolated, however Marin *et al.* (1996) were able to express this protein in *Escherichia coli*.

Molecular mechanism of the xantophyll cycle and its regulation

Since Sapozhnikov's discovery of light dependent Vx transformation, the mechanism of this reaction is intensively explored. However, after almost 50 years of research on that subject, the molecular mechanism of xanthophylls transformation is still far from being completely understood. In accordance with requirement of the MGDG for the *in vitro* reaction, it has already been proposed that Vx is located in the micelles of that galactolipid (Yamamoto *et al.* 1974). In such structures, believed to consist on average of 28 MGDG molecules, Vx can oscillate and finally come in contact with substrate binding site of the VDE. Then, one of the two epoxy groups of the ionone rings can be de-epoxidated and Vx changed into antheraxanthin. This part of the reaction mechanism is rather easy to explain, but it is not clear how the second step, the transformation of Ax to Zx, occurs. Yamamoto suggested that during oscillation in micelles Ax can make a full turn and the second ionone ring may become accesible to the enzyme (Yamamoto and Higashi 1978). Some researchers suggested that *in vivo* Vx de-epoxidation occurs within thylakoid protein-pigment complexes (Thayer and Björkman 1992) but a great number of experiments demonstrated that the reaction takes place in the lipid part of the membrane. Vx was proven to bind to thylakoid membrane proteins weaker than other carotenoids. Results obtained recently by Ruban *et al.* (2002) suggest, that the Xc pigments are not freely located in thylakoid membranes, but are bound by proteins of light harvesting complexes. However, the same data shows, that these pigments can easily dissociate from their loci. Perpendicular location of Vx in the thylakoid membrane was confirmed by experiments with VDE added from the stromal side of the thylakoids (Åkerlund *et al.* 1995). In addition, Gruszecki (1995) showed, that this pigment locates perpendicularly to the membrane surface in model lipid bilayers.

The main problem of the molecular mechanism of Xc is de-epoxidation of the ionone ring located on the stromal side of the thylakoid membrane, *i.e.* on the opposite side to the enzyme location. An assumption of flip-flop of Ax, created by Vx de-epoxidation has to be made to explain the Ax epoxide group removal. This hypothesis was never fully clarified and Ax flip-flop was hard to imagine in membrane bilayer for thermodynamical reasons. We have proposed a new mechanism for Vx conversion to Ax and Zx, which also takes into account indispensability of MGDG for de-epoxidation (Latowski *et al.* 2002). In accordance with the MGDG properties – hydratation degree of about 5 water molecules per one molecule of MGDG, in comparison to about 50 and 35 in the case of DGDG and PC, respectively (Sen and Hui 1988, Newman and Huang 1975, Finer and Drake 1974, Lis *et al.* 1982) and value of critical packing parameter (Israelachvili and Mitchell 1975) it has been shown that this lipid does not create micelles in water, but it forms reversed hexagonal structures (H_{II}) (Sen *et al.* 1981, Shipley *et al.* 1973) . The new model of Xc functioning is based on the *in vitro* experiments, consisting of PC liposomes enriched in MGDG and Vx, *i.e.* the system which resembles more the thylakoid membrane that the commonly used system composed of MGDG aggregates. The presence of the H_{II} domains inside native thylakoid membranes and the liposomes composed of PC and MGDG has been described by several authors (De

Kruijff *et al.* 1979, Gounaris *et al.* 1983a, b, Harańczyk *et al.* 1995, Sprague and Staehelin 1984, Quinn and Wiliams 1983, van Venetië and Verkleij 1981, Walde *et al.* 1990, Latowski *et al.* 2002). In liposomes composed of PC and MGDG, the de-epoxidation rate of Vx depends on the MGDG/Vx ratio. In order to be de-epoxidated into Ax, Vx has to reach the MGDG domain by lateral diffusion and the rate of de-epoxidation depends on the rate of the lateral diffusion of Vx to these structures (Latowski *et al.* 2002). Due to the presence of H_{II} phase in the MGDG domain, Ax can easily turn in the membrane, performing the flip-flop type of movement. The role of H_{II} structures in Vx and Ax de-epoxidation has been confirmed in experiments with another non-bilayer-forming lipid, phosphatidylethanolamine (PE). Active Vx de-epoxidation was observed when MGDG was replaced by this lipid (Latowski *et al.* 2004).

Until zeaxanthin epoxidase is not isolated and studied *in vitro*, the molecular mechanism of epoxidation of Zx to Vx can not be fully elucidated. However, it is known that for its activity ZE requires NADPH, FAD, ferredoxin and oxygen (Hager 1975, Siefermann and Yamamoto 1975c, Büch *et al.* 1996, Bouvier *et al.* 1996). Other known epoxidases also need additional proteins for their activity. For example, squalen epoxidase needs flavoprotein oxidase and NADPH-cytochrome P450 oxidase (Ono *et al.* 1982, Bouvier *et al.* 1996). Similiar dependence is proposed for ZE. Bouvier *et al.* (1996) suggest that electrons from NADPH are transferred through ferredoxin:$NADP^+$ oxidoreductase to ferredoxin, then to FAD. Reduced FAD is able to bind oxygen molecule and to form hydroperoxyl moiety. Part of this moiety is transfered to Zx, as a hydroxyl radical. Zeaxanthin forms unstable carbocation and then proton from Zx is transferred through FAD to oxygen and finally water molecule is formed. After loss of proton Zx becomes antheraxanthin (Fig. 8). Ax undergoes an analogical reaction of transformation to Vx. In accordance with the data of zeaxanthin preference to membrane protein complexes, it is proposed that transformation Zx - Ax occurs in that place (Gruszecki and Krupa 1993, Jahns and Schweig 1995, Härtel *et al.* 1996, Hager 1966, Arvidsson *et al.* 1993, Bouvier *et al.* 1996, Bassi *et al.* 1993, Ruban *et al.* 1994, Lee and Thornber 1995, Färber and Jahns 1998). Similarly to de-epoxidation reaction, Ax has to perform a flip-flop type movement to be further epoxidated to Vx. However, until now there is no knowledge how second ionone ring can be exposed to ZE. Two mechanisms are possible: (i) Ax molecule turns in precincts of protein complex, (ii) it leaves the complex and translocates to lipid domains, containing reversed hexagonal structures where the flip-flop takes place. It is also possible, that both proteins and lipids are engaged in the Ax flip-flop.

Functions of the xanthophyll cycle

Quenching of excess energy in PSII

Thorough and detailed studies by different research groups showed the dependence between the content of Zx and non-photochemical quenching (NPQ) in chloroplasts (Demmig *et al.* 1987 a, b, Krause 1988, Demmig-Adams *et al.* 1990, Demmig-Adams 1990, Krause and Weis 1991, Demmig-Adams and Adams 1992, Pfundel *et al.* 1994, Demmig-Adams *et al.* 1995, Jin *et al.* 2001, Ma *et al.* 2003). Even better correlation was found between NPQ and the total amount of Zx + Ax (Gilmore and Yamamoto 1993, Adams *et al.* 1995). An increase in NPQ after light treatment and its correlation to Vx de-epoxidation in spinach (*Spinacia oleracea*) leaves, isolated chloroplasts and purified LHC complexes were also observed (Ruban and Horton 1999). In diatoms, the NPQ level is well correlated with the diatoxanthin amount, which was created during de-epoxidation of diadinoxanthin (Lavaud *et al.* 2002 a, b). In other experiments, photoprotection of diatoxanthin during prolonged UV-A and UV-B ilumination of diatoms (*Thalassiosira weissflogii*) has been demonstrated (Zudaire and Roy 2001). These UV-insensitive diatoms have increased activity of the diatoxanthin cycle as an answer to light stress.

It is generally thought that the xanthophyll cycle is one of the main photoprotection mechanisms in autotrophic cells. Induction of energy transfer from chlorophyll to Ax and Zx, in connection with structural transformations make possible energy dissipation by Ax and Zx in LHC (Horton *et al.* 1991,

Frank *et al.* 1994, Gilmore *et al.* 1995, Yamamoto and Bassi 1996, Gilmore and Yamamoto 2001, Li *et al.* 2002, Polivka *et al.* 2002). On the other hand, it has been shown that accumulation of Zx is not the main cause of chlorophyll fluorescence quenching (Leipner *et al.* 2000). Moreover, analysis of *Arabidopsis* mutants and its wild form proved that Zx is not responsible for the dissipation of energy excess in PSII, and that photoprotection mechanisms of the xantophyll cycle are not simply associated with the replacement of Vx by Ax or Zx (Tardy and Havaux 1996, Peterson and Havir 2000). In *Arabidopsis npq1* mutant without active VDE, the extent of photoinhibition was comparable to that in the wild form of the plant after light stress (Havaux and Niyogi 1999). Külheim *et al*. (2002) showed, that in *npq1* mutant the fitness decreased rather in fluctuanting light conditions, than in a response to high light. It has been demonstrated that there is no difference between Vx and Zx in fluorescence quenching from purified LHCII, which was incorporated into liposome, (Gruszecki *et al.* 1997). Experiments with *Arabidopsis* mutant *aba-1* and *aba-2* containing only Zx and lutein showed that the presence of Zx is not sufficient for fluorescence quenching, both *in vivo* and in isolated protein-pigment complexes (Pesaresi *et al.* 1995).

Protection against lipid peroxidation

Degradation of lipids in pea leaves was observed after light stress (Havaux *et al.* 1991). In intensive light, the content of lipids in leaf cells decreased and the saturated/unsaturated lipid ratio increased. The lipid degradation was more significant when Zx formation was inhibited by DTT (Havaux *et al.* 1991). Similiar results came from the experiments in which the lipid contents were measured in response to high illumination in *npq1* mutant (Havaux and Niyogi 1999). In comparison to the wild *Arabidopsis* form, *npq1* mutant had significantly higher level of lipid photooxidation. Interestingly, in tomato leaves, Zx level and lipid degradation (measured as ethylene formation) were also correlated. In 3 °C and high light condition (low level of created Zx), ethylene production was intensive. But in 23 °C and high light condition, ethylene secretion was lower and the Zx content increased (Sarry *et al.* 1994).

It is also possible that Xc and Zx play a role in senescence, as a photoprotectant against lipid photooxidation (Munne-Bosch and Alegre 2002).

Regardless the differences among the authors, the Xc is recognized as one of the main adaptation mechanisms responsible for a fast response to peroxidation and creation of antioxidant substances in thylakoid membranes. This photoprotectant is Zx, which can quench singlet oxygen (Krinsky 1979) and other free radicals (Burton and Ingold 1984, Lim *et al.* 1992).

Blue light reception

Another postulated function of the xanthophyll cycle is blue light reception. Some researchers suggested that Zx is responsible for the blue-light--dependent stomata opening (Srivastava and Zeiger 1995, Quinones *et al.* 1996, Talbott *et al.* 2002), chloroplast movement (Tlałka *et al.* 1999), and phototrophism (Quinones and Zeiger 1994). HPLC measurement of Zx level as a function of chloroplast movement in strong and weak light in *Lemma trisulca* proved a good correlation between these two variables (Tlałka *et al.* 1999). Because of this finding, Zx is supposed to be a photoreceptor in blue-light stimulated chloroplast movement. Moreover, *Zea mais* coleoptiles, grown in darkness, did not show blue-light dependent phototrophism. Interestingly, these coleoptiles did not contain Zx. The degree of increase in Zx level (regulated by red light, darkness periods, and use of DTT) correlated well with the blue-light stimulated phototrophism of maize coleoptiles (Quinones and Zeiger 1994). The DTT-related inhibition of Zx synthesis consequently inhibited blue-light-dependent stomata opening in *Vicia faba* epidermis, what was thought to be a proof of the photoreceptor function of Zx (Srivastava and Zeiger 1995). Similar conclusions came from analysis of blue-light phototrophism or stomata opening activity spectrum and absorption spectrum of Zx (Quinones *et al.* 1996). However, Palmer *et al.* (1996) showed, in experiments correlating the level of protein phosphorylation and blue-light induced phototropism, that there is no connection between Zx, or any other carotenoid, and phototropism.

Regulation of membrane physical properties

Modulatory effects of carotenoids on physical properties of model and natural membranes have been known since the 70-ties. Soon after Xc characterisation, there was a suggestion that this process may regulate physical properties of thylakoid membranes (Siefermann and Yamamoto 1975a, Yamamoto 1979). This hypothesis has been confirmed by other studies (Gruszecki and Strzałka 1991, Tardy and Havaux 1997).

In natural and model membranes, zeaxanthin appears to have the strongest influence on such membrane properties as temperature of phase transition, molecular dynamics, permeability and polarity gradient (Lazrak *et al.* 1987, Gruszecki and Strzałka 1991, Subczyński *et al.* 1991, Subczyński *et al.* 1992, Havaux and Gruszecki 1993, Strzałka and Gruszecki 1997, Tardy *et al.* 1997, Jagannadham *et al.* 2000, Socaciu *et al.* 2000, Kostecka-Gugała *et al.* 2003). It was shown for two antarctic bacterial species, *Sphingobacterium antarcticus* and *Sphingobacterium multivorum*, that membrane fluidity is regulated mainly by Zx (Jagannadham *et al.* 2000). Zx is able to stiffen the membrane in liquid-crystalline phase and this process is connected to orientation of this xanthophyll in the membrane. Long axis of Zx molecule is oriented perpendicularly to the membrane surface and ionone rings are anchored in peripheral, polar zones of the membrane (Lazrak *et al.* 1987, Gruszecki and Sielewiesiuk 1991, Subczyński *et al.* 1992). In addition, Zx decreased molecular dynamics of lipids, limiting heat-induced lipid mobility and preventing membrane desorganisation (Gruszecki and Strzałka 1991, Havaux and Gruszecki 1993, Tardy and Havaux 1997).

Regulation of abscisic acid synthesis

One of the postulated Xc function is its involvement in abscisic acid (ABA) synthesis. It has been shown (Marin *et al.* 1996, Pogson *et al.* 1996, Audran *et al.* 1998) that Vx is one of the intermediate products in that process. One could suppose that conditions causing an increase in VDE activity, would cause decrease in ABA production. Exogenously added ABA, which inhibits its synthesis, resulted in higher concentration of Zx and greater photoprotection of PSII (Ivanov *et al.* 1995).

The existence of a relationship between Xc pigments and ABA synthesis was proven for *Nicotiana tabacum*. After ozone treatment, Vx level in leaves strongly decreased, but Ax and Zx contents changed insignificantly. This changed was accompanied by a pronounced increase in ABA (Pasqualini *et al.* 1999).

Other postulated functions of the xantophyll cycle

Krinsky (1966) suggested that formation of Vx through the incorporation of oxygen to Ax and Zx plays a role in photoprotection by lowering the oxygen level in chloroplasts. Oxygen, together with high light conditions, lead to the destruction of the photosynthetic apparatus. However, according to recent studies, it is known that photoprotection is associated with the reverse process, *i.e.*, conversion of Vx to Ax and Zx (Demmig-Adams 1990). On the other hand, Sapozhnikov and Calvin postulated that Xc takes part in the oxygen evolution during photosynthesis (Sapozhnikov *et al.* 1957, Sapozhnikov 1967 1973), however this proposal turned out not to be correct (Vrettos *et al*. 2001)

The xanthophyll cycle – still a lot of questions

The xanthophyll cycle has been investigated for about 50 years but functions and molecular mechanism of this process are still not completely known. Some hypotheses were disproved, some other were created. Recently, some new aspects of molecular mechanism of Xc action have been elucidated. A new model system to study VDE activity *in vitro* has been worked out. The regulatory role of the molecular dynamics and structure of the membrane in de-epoxidation reaction were demonstrated in model and natural systems. Also the importance of MGDG and H_{II} in the molecular mechanism of the xanthophyll cycle has been described.

Presently, the xanthophyll cycle is thought to play a role in quenching of excess energy in PSII, protection against lipid peroxidation, blue light reception, regulation of physical membrane properties, and regulation of abscisic acid synthesis. There are, however, remaining important questions, *e.g*. Xc regulation in different plant species, Xc regulation during plant growth and senescence, lipid depend-

ence of VDE activity, and an almost totally unknown molecular mechanism of zeaxanthin epoxidation to antheraxanthin and zeaxanthin.

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