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Light Protection in Plants

Characterisation of Violaxanthin de-epoxidase

ERIK HALLIN | BIOCHEMISTRY AND STRUCTURAL BIOLOGY | LUND UNIVERSITY



Light Protection in Plants

Characterisation of Violaxanthin de-epoxidase

Erik Hallin



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DOCTORAL DISSERTATION

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Light Protection in Plants

Characterisation of Violaxanthin de-epoxidase

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Abstract

Plants and algae need light to drive the photosynthetic machinery. An excess of light will, however, result in damage to the photosynthetic machinery and to the rest of the organism. The surplus of energy, when exposed to light stress is converted into less harmful heat energy through a process called non-photochemical quenching. This quenching is partly dependent on the xanthophyll pool located inside the thylakoid membrane. During light stress the xanthophyll violaxanthin is converted to zeaxanthin by violaxanthin de-epoxidase (VDE), triggering the zeaxanthin dependent light quenching. VDE is located on the lumen side of the thylakoid and is activated by the reduction of pH caused by photosynthesis. The sequence of VDE has been divided into three domains, a conserved N-terminal domain rich in cysteines, a lipocalin-like domain expected to bind the substrates and a negatively charged C-terminal domain rich in glutamic acid. In this work we have constructed cysteine mutants that revealed the importance of 12 of the 13 cysteines of VDE to the activity. These 12 cysteines were found to form disulphides in a pattern giving two hairpin structures and also increased the thermal stability of VDE.

The active site of VDE does not appear to be exclusively located in the cysteine rich N-terminal domain. The expression of the N-terminal domain without the rest of VDE did not show catalytic activity. The rest of VDE without the N-terminal domain was also not able to catalyse the reaction, but after mixing of these two constructs the activity returned. This shows that the N-terminal domain and the rest of VDE can fold independently and also indicates that the active site is localised in the interface between the N-terminal domain and the lipocalin-like domain. Crosslinking of monomeric VDE could localise the N-terminal domain near the opening of the lipocalin barrel, where violaxanthin is predicted to bind.

The glutamic rich C-terminal domain could be truncated from VDE while the rest of VDE remained active. This showed that the C-terminal domain was not required for the catalytic activity of VDE. The truncation did, however, cause a great loss of activity and a shift in how the VDE activity depends on pH. The C-terminal domain could be linked with the ability of VDE to oligomerise at the pH required for activity. The pH dependent oligomerisation of VDE was lost after truncation of the C-terminal domain. A reduction in pH towards the pH required for optimal activity also causes a strong formation of α -helical structures involved in coiled coils. This formation of secondary structure was also lost after truncation of the C-terminal domain, which is predicted to contain coiled coils. A likely scenario is that the pH activation of VDE involves an oligomerisation event caused by coiled coils at the C-terminal domain. The oligomerisation of VDE

could also be seen using chemical crosslinking at different pH, showing a monomeric state at neutral pH while oligomeric interactions occur at lower pH. Small angle x-ray scattering gave indications of a dimeric symmetry of the oligomeric state, while also revealing an elongated shape of monomeric VDE with the lipocalin-like domain localised in the centre. We also show that the previously proposed symmetric docking of violaxanthin into the dimeric state of the lipocalin-like domain appears less likely compared to a non-symmetric binding, based on the observation that only one side of violaxanthin is converted per substrate binding of VDE.

Populärvetenskaplig sammanfattning

Växter behöver ljus. Ljuset omvandlas genom fotosyntesen till kemisk energi som kan lagras i växten. Mer ljus ger således mer energi men bara upp till en viss gräns. När växten inte längre kan hantera mängden ljus som den utsätts för kommer flaskhalsar i processen orsaka att högenergetiska tillstånd blir mer långlivade. Dessa reaktiva ämnen kan då orsaka skada på närliggande komponenter, vilket kan kräva en kostsam reparation eller till och med död. Växter måste därför kunna reglera mängden ljus som når det fotosyntetiska maskineriet. Detta måste också ske i sådan hastighet att ljusvariationer orsakade på tidsskalen sekunder till minuter, från till exempelvis moln kan hanteras. Då växter inte har förmågan att förflytta sig när de utsätts för starkt ljus har evolutionen tagit fram ett antal alternativ. Riktningen av blad mot eller från ljuskällan och förmågan av de fotosyntetiska komponenterna att skugga varandra är två metoder för att reglera mängden inkommande ljus. Därefter kan upptaget ljus delvis omvandlas till värme, som är en mindre reaktiv form av energi. Exakt hur denna omvandlingsprocess går till är inte känd ännu men vi vet att en av de inblandade komponenterna är molekylen zeaxantin, som bildas tillfälligt då växten utsätts för ljusstress. Zeaxantin omvandlas från violaxantin av enzymet violaxantin de-epoxidas (VDE), som aktiveras då fotosyntesen börjar få svårt att hinna med att hantera det inkommande ljuset.

Detta arbete går ut på att karaktärisera VDE och därigenom ge en djupare förståelse om hur växter och alger kan försvara sig mot ljus. Ett enzyms funktion är väl sammankopplat till dess struktur, vilket gör karaktärisering av strukturen till en viktig del av analysen. Med hjälp av ett antal metoder har information erhållits om strukturen hos VDE och hur denna förändras vid aktivering. Exempelvis kunde vi genom detta visa att VDE molekyler kopplas samman vid aktiveringen, vilket kan vara en viktig del av förmågan att omvandla violaxantin till zeaxantin. Ett enzym består vanligtvis av en kedja av aminosyror. I vissa fall finns interna länkar bildade av aminosyran cystein. Hur dessa cysteiner är länkade inom VDE samt deras innebörd för aktiviteten har studerats. För att analysera vilka delar av VDE som är viktiga för dess aktivitet har dessa delar byts ut och även tagits bort i olika kombinationer. Detta har också gett antydningar om var i VDE som violaxantin binder. Genom att också analysera hur mycket av den bildade slutprodukten samt reaktionens intermediat som förekommer vid olika tidpunkter kunde påvisa att intermediatet lämnar VDE och måste bindas igen för att slutprodukten zeaxantin ska bildas.

List of papers

- I. Violaxanthin de-epoxidase disulphides and their role in activity and thermal stability
Hallin EI, Guo K, Åkerlund HE
Photosynthesis Research 124(2):191-198
- II. Functional and structural characterisation of domain truncated violaxanthin de-epoxidase
Hallin EI, Guo K, Åkerlund HE
Physiologia Plantarum, in press
- III. Molecular studies on structural changes and oligomerisation of violaxanthin de-epoxidase associated with the pH dependent activation
Hallin EI, Hasan M, Guo K, Åkerlund HE
Photosynthesis Research, in press
- IV. Initial characterisation of the closest relative to violaxanthin de-epoxidase
Guo K, Hallin EI, Johansson U, Åkerlund HE
Manuscript

Author contributions

- I. EIH was the main contributor in the design of the study, took active part in construct preparation, performed the sample preparations, performed most of the experiments except ICP-MS, analysed the results and wrote most of the manuscript.
- II. EIH was the main contributor in the design of the study, took active part in construct preparation, performed the sample preparation, performed the experiments, analysed the results and wrote most of the manuscript.
- III. EIH designed the study, took active part in construct preparation, performed the sample preparation, performed the experiments except SAXS, analysed the results and wrote the manuscript.
- IV. EIH took active part in the bioinformatic analyses, took active part in design of experiments, performed the MS preparation/analysis and took active part in writing the manuscript.

Abbreviations

ATP	Adenosine triphosphate
CD	Circular dichroism
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LHC	Light harvesting complex
MALDI	Matrix-assisted laser desorption/ionisation
MGDG	Monogalactosyldiacylglycerol
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NPQ	Non-photochemical quenching
PCR	Polymerase chain reaction
PsbS	Photosystem II protein S
SAXS	Small angle x-ray scattering
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
VDE	Violaxanthin de-epoxidase
VDR	Violaxanthin de-epoxidase related protein

Introduction

Light is essential to plants. Through the process of photosynthesis, light is converted into chemical energy by biochemical machinery evolved billions of years ago. Light is absorbed by chlorophyll bound to protein scaffolds called light harvesting complex (LHC). These scaffolds are forming an antenna to absorb as much light as possible. The absorbed energy is passed on towards a central reaction centre where the energy is used to split water molecules and to pump protons across the photosynthetic membrane. The proton pumping will build up a gradient that is utilised by ATP synthase to generate ATP, which is used to fuel the majority of the reactions taking place inside the cell. The organelle inside the plant cell that account for the photosynthetic activity is called chloroplast (Fig. 1), sharing many similarities with the photosynthetic bacteria cyanobacteria, which is believed to be the origin of the chloroplast. Localised within the chloroplast is the thylakoid, an extensive membrane structure where the main components of the photosynthetic machinery is positioned. It is across this thylakoid membrane that the proton gradient, used for ATP synthesis, is created.

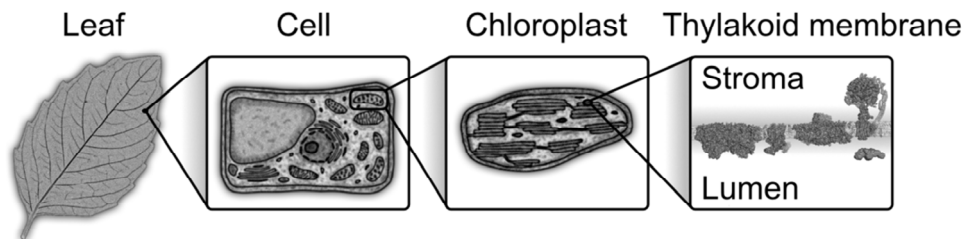


Fig. 1 Illustration of the subcellular localisation of VDE

One could easily think that more light will result in more harvestable energy, which is true within a certain range of light intensities (Tamoi et al. 2006), but above that could cause devastating effects for the organism (Barber and Andersson 1992). When light hits chlorophyll, chlorophyll is excited to a short-lived singlet state. The energy is then transferred towards the reaction centre by resonance with adjacent chlorophyll molecules. However, if the amount of absorbed energy is greater than what the rest of the photosynthetic machinery can handle, this will result in a bottleneck that hinders the energy of the excited state of the chlorophyll to be passed on. The lifetime of the excited state will therefore be prolonged, which increases the risk of formation of a triplet state. The triplet state of the excited chlorophyll is long-lived and has therefore a higher chance of reacting with other nearby molecules. The absorbed light energy stored in the excited state

of the chlorophyll could react with oxygen to form reactive oxygen species. The formed reactive oxygen species will then continue to react with the components of the cell, causing damage to the organism. One of the systems that will be damaged is the photosynthetic machinery itself, resulting in a reduced ability to convert the incoming light and potentially increase the light saturation effect further. This effect is called photoinhibition and will force the organism to repair or rebuild the damaged components.

To recover from the damage caused by photoinhibition is an expensive process and could be avoided if the organism could control the amount of light that it will be exposed to. Plants do not have the ability to move to a shady area when being exposed to intense light. Instead they have evolved alternatives to regulate how much of the light that will be absorbed and how to dispose of the surplus. For plants, the entire leaf could move to adjust its angle towards the light source (Pastenes et al. 2004). The chloroplasts have also the ability to move within the plant cell (Wada 2013). When exposed to low intensities of light the chloroplasts are positioned along the edge of the cell perpendicular to the light source to utilise as much light-exposed area as possible. After exposure to high light intensities, the chloroplasts will move towards the edge of the cell parallel to the light source, which will cause the chloroplast to shade each other and absorb less light. Also, the size of the photosynthetic antennae could be reduced by removing light harvesting complexes or alternatively to scale up the photosynthetic component creating the bottleneck in the reaction pathway (McKim and Durnford 2006). However, the major disadvantage of these methods to withstand the high intensity of light is their speed. The timescale it takes for cloud movements to shade the plant and later to reintroduce full sunlight exposure takes place within seconds to minutes. To both utilise as much light as possible during a cloudy phase and to handle a quick transition to tolerate high light intensities without accumulating too much damage requires a faster alternative. This alternative is called non-photochemical quenching (NPQ) and allows plants and green algae to convert the excess of the absorbed light into heat and through this making the absorbed energy less harmful (Muller et al. 2001).

Photochemical quenching is the process of photosynthesis where light is absorbed and passed on to be converted into chemical energy. The non-photochemical quenching is therefore the process where light is absorbed but does not result in chemical energy. The details of how the energy of the excited state of chlorophyll is converted to heat are not known but have shown to be strongly dependent on three components. Firstly, the pH gradient across the thylakoid membrane, generated by the photosynthetic activity appears to induce the NPQ. Removal of this pH gradient with uncouplers strongly impairs the NPQ. The pH gradient has shown to cause modification and restructuring of the light harvesting complexes

(Breitholtz et al. 2005, Dekker and Boekema 2005), which may alter the local environment of the bound chlorophyll to favour energy release through heat formation. A second component involved in NPQ is zeaxanthin, a xanthophyll localised inside the thylakoid membrane and formed during light stress (Demmig-Adams 1990). The third component of NPQ was found during screening of mutants that displayed a high sensitivity to light variation. One NPQ impaired mutant was lacking PsbS (Li et al. 2000), a membrane bound protein also localised inside the thylakoid membrane. The role of PsbS in NPQ has not been fully determined but may interact with the light harvesting complex (Nield et al. 2000). A second NPQ-mutant had a mutation in violaxanthin de-epoxidase (VDE), a soluble enzyme located on the lumen side of the thylakoids, and could therefore not accumulate the zeaxanthin needed for NPQ (Niyogi et al. 1998).

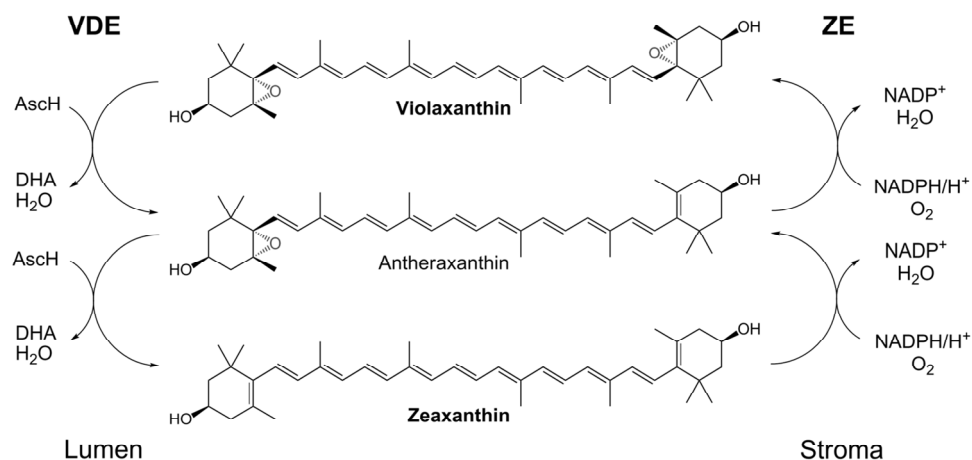


Fig. 2 The xanthophyll cycle

The role of zeaxanthin in NPQ has also not been fully determined. One theory is that zeaxanthin may directly interact with the light harvesting complexes (Formaggio et al. 2001), or indirectly through interaction with other subunits (Pinnola et al. 2013), or by itself altering the properties of the thylakoid membrane to indirectly affect the light harvesting complexes (Szilágyi et al. 2008). The formation of zeaxanthin in plants exposed to strong light is from the pool of violaxanthin, another xanthophyll located within the thylakoid membrane. Violaxanthin is similar to zeaxanthin with the difference of the two epoxides located near the two ends of the carotenoid structure, replacing two double bonds. Both violaxanthin and zeaxanthin are part of the xanthophyll cycle (Fig. 2), which is the conversion of zeaxanthin to violaxanthin and from violaxanthin to

zeaxanthin. The intermediate molecule in this reaction is antheraxanthin, which only contains one epoxide.

A plant, adapted to low intensities of light will have a xanthophyll pool dominated by violaxanthin, but if exposed to high intensities of light this pool will rapidly be converted to zeaxanthin to trigger the zeaxanthin dependent NPQ. This system exists in plants and green algae. A similar, alternative xanthophyll cycle is present in diatoms, which is based on the conversion from diadinoxanthin, a xanthophyll containing one epoxide, to diatoxanthin, where the epoxide has been converted into a double bond (Lohr and Wilhelm 1999). Also, indications of a third and more rare xanthophyll cycle have been reported, involving the de-epoxidation of lutein-epoxide to lutein (Jia et al. 2013).

The epoxidation and de-epoxidation of zeaxanthin and violaxanthin is catalysed by two different enzymes located on the two different sides of the thylakoid membrane, where the xanthophylls are located. On the stromal side of the thylakoid membrane is zeaxanthin epoxidase (ZE) found. ZE is catalysing the epoxidation of zeaxanthin using molecular oxygen and NADPH to form violaxanthin (Eskling et al. 1997). This reaction occurs during the adaption to low light exposure. On the lumen side of the thylakoid is where violaxanthin de-epoxidase (VDE) is located. VDE is catalysing the de-epoxidation of violaxanthin to zeaxanthin using ascorbic acid, which occurs during the adaptation to strong light exposure. When the plant is exposed to strong light the photosynthetic machinery will be working at full activity, rapidly lowering the pH inside the thylakoids by proton pumping (Kramer et al. 1999). This low pH is what triggers VDE to start converting violaxanthin to zeaxanthin, allowing the zeaxanthin dependent NPQ. The de-epoxidation activity of VDE occurs optimally around pH 5.0 - 5.2 (Bratt et al. 1995; Rockholm and Yamamoto 1996) while being inactive near the neutral pH that is present during low or no photosynthetic activity. This pH dependent activation of VDE will cause the soluble VDE molecule to become membrane bound (Gisselsson et al. 2004), which will move VDE closer to its substrate, violaxanthin.

The ability of VDE to bind to thylakoid membranes has been suggested to be dependent on the C-terminal domain of VDE. The C-terminal domain of VDE is containing a high content of glutamic acids (Fig. 3), making this domain negatively charged at neutral pH. A reduction of pH will partly neutralise these residues which might allow VDE to associate with the thylakoid membrane. One concern of this theory is the high content of hydrophilic residues present in the C-terminal domain, which would not aid interactions with a hydrophobic membrane. Truncation of this C-terminal domain will abolish the ability of VDE to bind to thylakoid membranes (Hieber et al. 2002), indicating that the C-terminal domain is

involved in binding to the thylakoid membrane. However, other parts of VDE have also shown to undergo structural change depending on the pH.

```

MALVARSI CVSYDEIAGICNNVSHRNFKKWVQWKNPFLQDDARRNIRFNDRLSCTKFI
GASEKLQHSKSPKSGLISCGWEVNSSKVVSNAVIPKKWNLLKLKVVEVTAIVACTFFVMS
SAQAVDALKTCTCLLKECRIELAKCIANPSCAANVALQTCNNRPDETEQIKCGDLFAN
KVVDEFNECAVSRKKCVPOKSDVGEFPVDPDSVLVKSFNMADEFNGKWFISSGLNPTFDAE
DQQLHEFHLEDGKLVGNLSWRIKTPDGGFFTRTAVQKFAQDPSQPGMLYNHDNAYLHYQD
DWYILSSKIENQPDYVFVYYRGRNDAWDGYGGAFLYTRSATVPENIVPELNRAAQSVGK
DFNKFIRTDNTCGPEPPLVERLEKTVEEGERTIIKEVEQLEGEIEGDLEKVGKTEMTLFLQ
RLLEGFQELQKDEEYFLKELNKEERELEDLKMEAGEVEKLFGRALPIRKLK

```

Fig. 3 The primary sequence of *Spinacia oleracea* VDE. The two signal peptides, removed after translocation, are marked in grey. The lipocalin domain is marked in green, while the two other domains corresponds to the cysteine (red) rich N-terminal domain and the glutamic acid (blue) rich C-terminal domain

The central domain of VDE is a lipocalin-like structure, which mainly consists of an eight stranded β -barrel. The lipocalin fold is conserved in a wide range of organisms from different kingdoms and serves as a transporter of small hydrophobic molecules (Flower 1996). The β -barrel will create a hydrophobic pocket where various substances could be bound. It is therefore predicted that the lipocalin-like domain of VDE will be the position of where violaxanthin binds. One substance found to bind to lipocalin is retinol, a compound with similar structure as one half of zeaxanthin (Godovac-Zimmermann 1988). Lipocalins are, however, rare to function as enzymes themselves and instead will function as carriers of small hydrophobic molecules. Only one lipocalin has so far been found to have catalytic activity, prostaglandin-D synthase (Peitsch and Boguski 1991, Urade and Hayaishi 2000). The regular function of lipocalins would suggest that the lipocalin-like domain of VDE will only hold one side of violaxanthin in place while the de-epoxidation occurs in the other side, in another domain. The violaxanthin molecule is too long to be bound by the lipocalin-like domain alone without the other side of violaxanthin hanging loose. One alternative to this proposed activity mechanism is that the active site of VDE is located within the barrel of the lipocalin-like domain. This theory is supported by the crystal structure of the lipocalin-like domain from VDE, where the lipocalin appears to form a dimer at pH 5 (Arnoux et al. 2009). Within this lipocalin-like dimer it was possible to dock, *in silico*, a violaxanthin molecule together with two ascorbate molecules placed inside the lipocalin barrels (Saga et al. 2010). This docking of both substrates suggests a concerted reaction and would allow the lipocalin-like domain to harbour the active site of VDE. The drawback to this theory is that the lipocalin-like domain shows no VDE activity by itself. N-terminal truncations,

small as only five amino acids have shown to completely remove the VDE activity (Hieber et al. 2002). C-terminal truncations allowed larger fragments to be removed without loss of VDE activity, but complete truncation of the C-terminal domain also resulted in total loss of VDE activity. The loss of activity after the C-terminal truncation is explainable if the function of the C-terminal domain is to allow docking to the thylakoid membrane. VDE might have a limited accessibility to violaxanthin without the docking ability of the C-terminal domain. However, the role of the N-terminal domain remains unclear. Without being part of the active site and not even close to any of the substrates it is still essential to the activity. The N-terminal domain is the most conserved part of VDE and contains a high number of cysteines, 11 of the total 13. These cysteines form a highly conserved pattern in VDE except for the first cysteine which only is conserved among higher plants but not among green algae. What also makes these cysteines interesting is that the presence of reducing agents, such as dithiothreitol, will abolish the VDE activity, which is an indication of that at least one disulphide bond is present and involved in the activity (Yamamoto and Kamite 1972).

A disulphide bond could be directly involved in the catalytic function. One example of this is the function of vitamin K epoxide reductase, where a de-epoxidation is catalysed by a disulphide bond to convert the epoxide to a double bond, same as what occurs in the de-epoxidation of violaxanthin to zeaxanthin (Silverman 1981; Davis et al. 2007). Alternatively, a disulphide bond might be important to maintain a certain structure of the enzyme. The cysteines could also be involved in binding of metals or other cofactors that are required for the activity of VDE. It should be noted that the N-terminal domain of VDE has no related proteins with known functions that could give clues of the function of this domain. However, one other protein shares the conserved cysteine pattern of the N-terminal domain. This protein appears to exist in the same organisms that also contain VDE, following the same photosynthetic organisms. The VDE related protein has not been studied and has simply been named VDE-like or VDE related protein only due to the sequence similarities, but does also show clear sequence differences compared to VDE. This unknown protein has an extended cysteine pattern not present in VDE. Also, the C-terminal domain of VDE appears to be missing. Finding out more about this VDE relative could give clues of the function of the N-terminal domain of VDE.

This work has been focused to study the function of VDE and trying to find out more about the roles of the different domains, deeper understanding of the catalysis and the structural events that trigger the pH dependent activation of VDE. Knowledge of how VDE functions could give important information on how plants and algae are able to optimise their survival ability during strong variations of light exposure.

Materials and methods

DNA cloning and protein expression

Obtaining the required amount of a specific protein to use for different methods of analysis and applications could be difficult and nearly impossible if the protein of interest had to be isolated and purified from the native organism, which may only express this protein in low quantities. A successful alternative could then be to overexpress the protein of interest in a model organism. The simplest alternative of these expression systems is to let the bacteria *Escherichia coli* produce the protein. Other expression systems in eukaryotic organisms will allow post translational modifications of the proteins to mimic a situation that may be more natural to the protein of interest. The drawbacks of the more advanced expression systems are that they will require more preparative work and produce less protein. The prokaryotic expression system only requires the DNA sequence corresponding to the expressed protein sequence inserted in an expression plasmid. This plasmid also contains information required for replication, a selection marker and a promoter for the expression of the protein of interest. The gene sequence of the expressed protein could be isolated from the native organism, amplified and modified through the polymerase chain reaction (PCR) or synthesised chemically. The plasmid containing the gene of the protein to be expressed is transformed into the bacteria through an electrical or thermal shock and then grown in larger quantities in a nutrition broth. When ready, the protein overexpression is activated chemically or thermally depending on the selected promoter. After a certain time the expressed protein can be harvested by breaking open the expression host.

The overexpression of proteins also allows modifications of the sequence that would not be possible from native protein isolation. Certain amino acids in the protein sequence could be replaced to evaluate their involvement in a specific function. This mutation of the expression plasmid can easily be performed using primers with the desired mutation and amplification using PCR. Using similar methods also allow deletions and insertions of larger parts of the gene sequence to further study separate domains of a protein.

The described methods were used to create a plasmid for expression of VDE from *Spinacia oleracea*, without the initial signal peptides. To analyse the importance of the cysteines of VDE, 13 mutants were constructed where each cysteine was replaced by serine, one by one. Additionally, various constructs of domain truncations of VDE were made. Among these, three variations of N-terminal truncations with the three corresponding N-terminal fragments expressed separately. Three truncations of various lengths of the C-terminal domain and one

construct with both the N-terminal and C-terminal domain truncated. All of these constructs were overexpressed using *Escherichia coli* BL21(DE3) as expression host with induction by isopropyl β -D-1-thiogalactopyranoside.

Protein purification

Many of the methods used to characterise proteins require that the protein sample is pure from other proteins and other components that may disturb the measurement. The measured specific properties of the protein of interest will be averaged out if a significant part of the protein sample also contains other proteins. The need of a pure protein sample is therefore of great importance before chemical and biophysical measurements could be performed. The purification of proteins consists of a series of steps to fractionate the sample based on different protein properties. The most commonly used properties for separation are size, charge and hydrophobicity. Specific affinity tags can also be used such as a histidine tag with affinity for nickel ions.

After isolation of the overexpressed protein there is a possibility that the protein was expressed as insoluble inclusion bodies. The methods used to solubilise these inclusion bodies will probably unfold the protein, but the insoluble proteins might have been misfolded from the beginning and therefore require unfolding. The unfolding of proteins can be done with chaotropic denaturants such as urea or guanidinium chloride to interfere with the hydrogen bonds used for maintaining the protein fold. Some purification steps might also require denaturation of the proteins. To regain the protein's native fold the denaturant has to be removed to allow refolding. The removal of the denaturant can be done with different methods such as dialysis for a slow removal, dilution or desalting for a faster approach or on column refolding where the denaturant concentration could be controlled during the whole process. The refolding of proteins does, however, not work for all proteins and may require special conditions to work, such as the presence of chaperones or other agents.

The expression method of VDE reported in this work resulted in formation of inclusion bodies and therefore required refolding. The amount of VDE that managed to fold well during expression was only a minor fraction compared to the amount of insoluble VDE. The advantage of the inclusion bodies is that the majority of the other proteins that belong to the expression host can be washed away by using a series of centrifugation steps. The washed insoluble VDE pellet could then be dissolved with urea before refolding by desalting the sample in two steps. First to remove urea and allowing VDE to refold, then removal of the reducing agent to allow formation of disulphide bonds. The oxidation of VDE was

performed in a redox buffer of glutathione, which resulted in higher recovery of the activity. To remove aggregated protein after the refolding, one or two steps of size-exclusion chromatography were performed before concentration using cut-off filters. The final step is ultracentrifugation to remove the remaining aggregates. This was the purification method that resulted in the highest purity and showed the most VDE activity.

Activity of VDE

The assay used to measure the ability of VDE to catalyse the de-epoxidation reaction of violaxanthin to antheraxanthin and zeaxanthin requires the following components: Violaxanthin, which we isolate from purified spinach thylakoids through organic extraction and purify with high performance liquid chromatography (HPLC). Monogalactosyldiacylglycerol (MGDG), required to solvate violaxanthin and might be involved in the binding of violaxanthin to VDE. MGDG is the most common lipid component of the thylakoid membrane and might therefore give a more natural condition compared to other lipids. The properties of MGDG will cause it to form an inverted hexagonal phase in aqueous solution, meaning that the hydrophobic tails will point outwards towards the solution, which have shown to allow higher VDE activity (Rockholm and Yamamoto 1996). The final required component is ascorbic acid, the cosubstrate of the reaction. Ascorbic acid will donate two electrons and two protons per de-epoxidation and will be converted to dehydroascorbic acid. The VDE reaction is dependent on pH with an optimal activity around pH 5.0 to 5.2. A buffer is therefore required to maintain this pH.

To quantify the final amount of violaxanthin, antheraxanthin and zeaxanthin, the reaction is first stopped by adjusting the pH to neutral, followed by organic extraction to remove the water soluble compounds. The extracted xanthophylls are then analysed using HPLC. The advantage of the HPLC method is that it allows quantification of violaxanthin, antheraxanthin and zeaxanthin. This method could also show potential side products of the reaction. The drawback of the HPLC method is the required amount of preparations and time it takes compared to the spectroscopic method. Yamamoto and Higashi (1978) developed a fast method to measure the activity of VDE that relies on the spectroscopic shift of the absorbance peaks when the de-epoxidation occurs. Zeaxanthin contains a longer conjugated system than violaxanthin and therefore also a different absorbance spectrum. By measuring the difference in absorbance between two specific wavelengths gives a relative value representing the de-epoxidated state of the xanthophylls. This method allows a fast way to estimate the amount of VDE present, but will not give the detailed information that HPLC can present.

Mass spectrometry

The ability to accurately measure the mass of molecules allows a broad set of various analysis techniques. In this work, mass spectrometry was used to identify proteins and for structural characterisation of VDE. These methods start with a digestion of the proteins by proteases. The proteases will cut the protein into peptides at certain amino acid types, giving a mixture of different peptides. To obtain the mass of the different peptides, they initially are ionised by either matrix-assisted laser desorption/ionisation (MALDI) or electrospray ionisation (ESI), then accelerated through an electric field causing separation dependent on the peptides mass to charge ratio, later to reach the detector. The charge of a peptide can be determined by the isotopic pattern seen in the mass to charge spectrum and will therefore give the mass of the analysed peptides. The list of obtained peptide masses are then compared with a theoretical digestion of the protein to find out the composition of the sample.

To reduce the probability of a false match the peptide could be selected for further fragmentation giving a new mass to charge spectrum to match with the theoretical fragmentation of the suggested peptide. Analysing more complex samples with many peptides may require a prefractionation step to allow a larger amount of detectable peptides. This is often performed by reversed phase HPLC to separate the peptides in the sample based on their hydrophobicity. This separation setup could either be connected directly to the electrospray ionisation or eluted on a target plate for MALDI. These methods were used to identify VDE and its truncations during purification and also for further structural analysis of VDE.

Thiol quantification

To measure the oxidation state of cysteines, meaning if they are in reduced form or covalently connected, a compound called Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) was used. This compound contains a disulphide bond which can react with the thiol group in free cysteine. After the disulphide bond in Ellman's reagent is broken the newly formed free compound will give a yellow colour and therefore a spectroscopically quantifiable value relative to the amount of free thiol groups in the sample. The use of a standard curve of plotted absorbances at 412 nm against the concentration of free cysteine is used to measure the amount of thiols that is present in a protein. Quantification of free cysteines in VDE was determined due to the reported connection between activity and oxidation state.

Disulphide connectivity

Which of the cysteines that form disulphides and to which cysteine they are connected to were analysed using mass spectrometry. Digestion of a disulphide containing protein present in its oxidatised state, without the presence of any reduction agent, results in peptides with intact disulphides. These disulphides could either connect two different peptides or be an internal link within one peptide. To screen for peptides containing a disulphide bond, reduced peptide samples were compared with non-reduced samples. The peptide peaks that were present in the non-reduced sample but not in the reduced sample indicate that a present disulphide bond was broken. If a new peak shows up at the position of two Daltons increased mass suggests that the peptide was containing an internal disulphide connection. If there is no peak at this position instead suggests that the disulphide was connecting two peptides, which now might be visible at their corresponding masses. The peptide mass of the peak that disappeared after reduction is matched towards a mass list of theoretical possibilities of peptide combinations made by disulphide formation.

A problem when analysing a protein with a high density of disulphides is that there might not be enough cleavage sites for the protease to separate all disulphides. This can then result in peptides connected to more than one other peptide, creating a cluster of connected peptides difficult to analyse. Therefore, the protease used for the analysis of VDE was thermolysin, a less specific protease giving a larger variation of peptide lengths. This also results in larger peptide variations due to missed cleavage sites, giving a more complex sample to analyse, but at the same time allows the presence of alternative peptide lengths to confirm a specific disulphide connection.

Dynamic light scattering

The use of dynamic light scattering (DLS) allows the size of particles in solution to be determined. During the measurement light passes through the sample. The particles in the sample will then cause the light to be scattered. A detector measures this scattering over a short time period to determine the dynamics of the scattering. The variation of the detected scattering is caused by Brownian motions of the particles and could be used to calculate the hydrodynamic radius using the Stokes-Einstein equation. The hydrodynamic radius measured by DLS is the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. This will cause elongated particles to appear larger than if they had a globular shape. The polydispersity of the sample, meaning how much the particles size varies in the sample, is also determined. This can be used

during protein purification to detect if low amounts of aggregate are present in the sample. DLS was used to measure the size of VDE particles under various conditions, such as with and without reducing agent present, exposed to different temperatures and at different pH.

Small angle x-ray scattering

In a small angle x-ray scattering (SAXS) experiment, the protein sample in solution is exposed to a beam of x-rays. The components of the sample will cause the x-rays to scatter onto a two dimensional detector around small angles. The image of the detected scatter will be averaged and reduced to a one dimensional profile showing the scatter intensity for different angles. After subtraction of the scattering profile of the sample solvent, left is the scattering profile of the protein and solvent interacting with the protein. This profile can be used to obtain low resolution information about the size, shape and structure of the protein. A pair distance distribution can be extracted to get the protein's radius of gyration and maximum diameter. This distribution can also give information regarding if several domains are present and if the protein is globular or elongated.

The scattering profile can also be used to generate low resolution models of the protein. This is done by generating a set of possible bead (DAMMIN) or chain (GASBOR) based models that match the scattering profile. These models are then superimposed and averaged to get an overall structure. If the majority of the generated models are similar to each other this will indicate that the averaged model is more trustworthy. If instead large variation exists between the models this could be the cause of domain flexibility, but could then be further investigated by generation of a larger model set and clustering of these. The disadvantage of the SAXS method when analysing protein structure is the low resolution compared to crystallography, but has the advantage of not requiring crystallisation of the protein and could therefore be studied in solution without possible crystallisation caused artefacts. In this work, SAXS was used to study the low resolution structure of VDE at different pH.

Circular dichroism

Circular dichroism (CD) is a method used to measure the chirality of a sample. Circularly polarized light of both left and right handed orientation passes through the sample. The variation in absorption between the left and right circularly polarized beam gives information about the chirality of the sample component absorbing light at the specified wavelength. This method is useful when analysing

the secondary structure of a protein. A spectrum collected in the far-UV area, where the backbone of proteins absorb light shows a different profile when analysing a system of pure β -sheets, α -helices or random coils. These spectra can be compared with the spectra of other proteins to get a sense of the present secondary structure of that protein. The CD measurement will also indicate how well folded a protein is. A protein with weak secondary structure elements is a sign of an unfolded or flexible structure. CD measurements of VDE in different conditions have been used to analyse the effect of pH and temperature on the secondary structure of VDE.

Thermal stability

The structure of a protein is more or less dynamic. This dynamic variation is dependent on the temperature the protein is exposed to. By, in various ways, measuring the level of structural integrity of the protein at different temperatures gives information about the structure's stability. If different conditions affect a protein's stability it would indicate that the structure itself was affected by this condition and could therefore reveal some information about the protein's structure. During the exposure to increasing temperature the protein will partly lose its structural elements. This unfolding process might be reversible, allowing the protein to regain its structure when the temperature has decreased. Otherwise, if the often hydrophobic inside of a protein is exposed, it has a high chance of aggregating together with other unfolded proteins.

The thermal stability of VDE in reduced and non-reduced form was investigated by DLS, where if a protein starts to unfold and aggregate it will show an increase in particle size. The thermal stability of VDE and truncated constructs at different pH were also studied using CD, where the loss of secondary structure can be measured with increased temperature.

Protein crosslinking

One method to get a rough estimation about internal distances within a protein molecule or within a protein complex is to use chemical crosslinking. This method allows specific amino acids to be linked via a crosslinker. This crosslinker contains two reactive groups separated with a spacer of various lengths. After the crosslinking reaction is performed, the sample can be analysed with SDS-PAGE. If interactions between proteins occur, this complex state could be covalently locked by the crosslinker and seen on the SDS-PAGE gel. This method could be used to investigate the interaction of different proteins or the oligomerisation of

one protein. Mass spectrometry in combination with crosslinking can be used to find out which specific part of the protein that is involved in a protein-protein interaction and also the internal distances within one protein molecule. Chemical crosslinking of VDE was performed to obtain structural information of VDE at different pH.

Results and general discussion

VDE sample preparation

The VDE sample used in the described experiments was obtained through overexpression, yielding inclusion bodies that could be refolded and later purified to give a high purity grade of enzymatically active VDE. Comparing the specific activity of the overexpressed VDE with native VDE, isolated from spinach gives similar results, which indicates that the overexpressed VDE can be refolded into the active state and therefore relevant to study.

Important cysteines

The overexpressed cysteine to serine mutants of VDE showed all very low activity except for one of these mutants (Fig. 4). The mutations caused activity losses of over 99 % for 12 of the cysteines, but mutation of the first cysteine in the VDE sequence showed no activity loss. Instead, this construct was able to give higher activity than the unmutated VDE. The increased activity might be explained by an increased stability over longer incubation times. The unmutated VDE will have at least one free cysteine that may react with another VDE molecule and through this lose or reduce its activity. The result that mutation of the first cysteine does not impair the activity of VDE shows that the specific cysteine properties of this residue are not important for activity. This cysteine is the one that was not conserved in algae VDE, which explains its reduced relevance to VDE activity. Even though all of the other cysteine mutants showed a great loss of activity all of them were still able to catalyse the reaction of violaxanthin to antheraxanthin/zeaxanthin. This means that none of the cysteines are fully essential for the catalysis, but still contributes to the activity in some way. It is therefore less likely that one of the cysteines is directly interacting with the substrates during the catalysis, unless the presence of an alternative residue nearby or by the serine itself can partly replace the features of the removed cysteine.

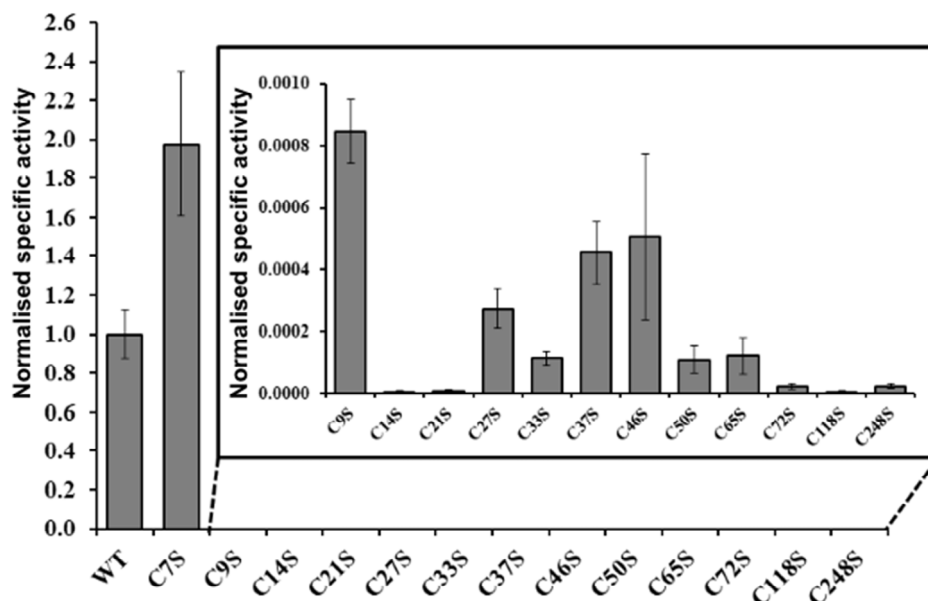


Fig. 4 The specific activity of the cysteine to serine mutants normalised to the specific activity of native VDE (WT). A magnification of the mutants with low activity is shown in a window to visualise the difference in remaining activity (Paper I)

The UV-Vis absorption spectrum of purified VDE did not show any specific features to indicate the presence of any prosthetic groups that the cysteines could have been involved in binding. Any relevant metal ion or complex that could have been a possible cofactor bound to VDE could not be found in concentrations near the molar ratio of VDE. The presence of EDTA during all refolding and purification steps also indicates that no metal is required for VDE activity. The inclusion of EDTA during refolding did instead show a positive effect on the activity, which may be explained by EDTA's ability to remove divalent metal ions from the solution and that these metal ions catalyse the formation of disulphide bonds. If the formation of disulphide bonds occurs too quickly, there is not enough time to allow disulphide mixing to find the correct disulphide pairs. The beneficial effect of EDTA on activity was lost if the oxidative refolding occurred in the presence of a glutathione redox buffer, indicating that the EDTA effect was related to oxidation.

The great loss of VDE activity after 12 out of 13 cysteine mutations shows that these cysteines are somehow involved in VDE activity. A similar study where all of the cysteines of *Arabidopsis thaliana* VDE were mutated one by one to alanines was published recently after this work and could show a similar result of large

activity loss after mutation of all the cysteines except the first cysteine in the VDE sequence (Simionato et al. 2015). Since no indications have been found that the cysteines are involved in binding of a prosthetic group and that the activity is lost under reducing conditions point towards that the cysteines are forming disulphide bonds involved in the activity of VDE.

Disulphide pattern

The odd number of cysteines present in VDE will force at least one of the cysteines to not take part in a disulphide bond, unless the odd cysteine is used to connect two VDE molecules. The quantification of thiols in VDE showed that only one of the cysteines is free, allowing the remaining cysteines to form disulphides. The thiol quantification of the first cysteine mutant did not show any free cysteines, suggesting that the first cysteine of VDE is the free one. This is also the same cysteine mutant that did not show any loss of activity.

By the use of mass spectrometry, the connectivity of the intact disulphides in non-reduced VDE could be determined (Fig. 5). Of the 12 cysteines that possibly could form 6 disulphides in VDE, 5 could be found. The final disulphide bond is expected to join the second and fifth cysteine in the VDE sequence. These cysteines could not be detected either in any reduced state or in any non-reduced state. However, the result of the thiol quantification showed only one free cysteine per VDE molecule and that the free cysteine is the first one in the VDE sequence, suggesting that the remaining two cysteines that could not be found are forming a disulphide bond. This last disulphide connection will together with the other disulphides give a pattern of two 1-4, 2-3 connections, which will appear as two hairpins in the protein structure. After the two hairpins come two other connections, one near the end of the N-terminal domain and a second within the lipocalin-like domain. The final disulphide bond, located within the lipocalin-like domain is confirmed by the crystal structure of the lipocalin-like domain of VDE.

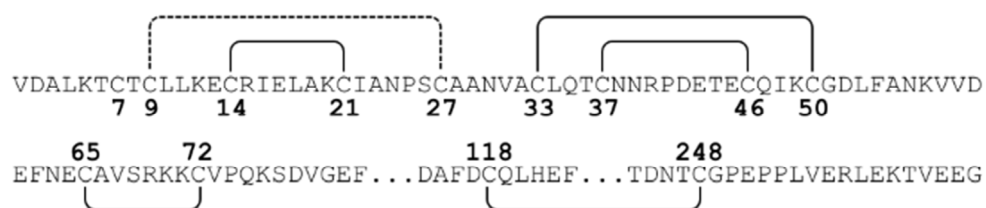


Fig. 5 The primary sequence of the N-terminal domain of spinach VDE and part of the lipocalin-like domain showing the disulphide connectivity (Paper I)

The disulphide pattern and the activity of the cysteine mutants appear to match in the sense that the mutations of two cysteines, forming a disulphide bond, show roughly similar loss of activity. This indicates that the loss of activity after a single cysteine mutation was caused by the lost ability to form a disulphide bond and not by any other specific properties of that cysteine. The matching cysteine mutations and disulphide connections shows that the disulphide bonds have an important role in the function of VDE. The use of disulphide bonds allows proteins to maintain a rigid structure that otherwise would not be stable. The thermal stability of VDE, measured by DLS showed that the reduction of disulphide bonds resulted in a large shift in melting temperature and also appears to increase the size of VDE particles (Fig. 6). Using size exclusion chromatography could also show that reduced VDE appears larger than the non-reduced, active state. These results show that the disulphides are important to maintaining a more rigid structure and probably required for the active state of VDE.

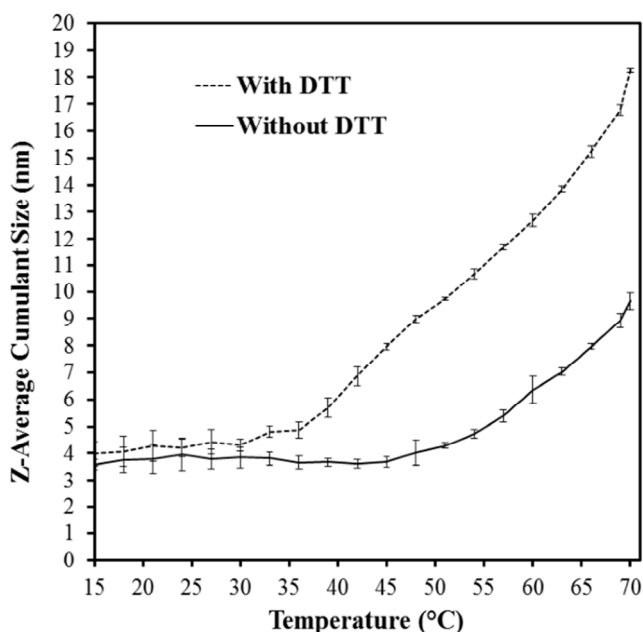


Fig. 6 Temperature denaturation of VDE in reduced (dashed line) and in non-reduced (solid line) form (Paper I)

When comparing the experimentally found cysteine pattern of VDE with the results of prediction software, very few or no connections match. This could be an effect of the many cysteines of VDE which will confuse the prediction algorithm or that the cysteine pattern of VDE is not similar to the other patterns used to build these prediction tools. The structure of the N-terminal domain or any other proteins with similar sequences is unknown. The disulphide pattern could therefore aid future structural studies until the full structure of this domain is known.

Functional domains

The active site of VDE was suggested to be located within the barrel of the lipocalin-like domain by *in silico* docking experiments with violaxanthin and ascorbic acid (Arnoux et al. 2009; Saga et al. 2010). This showed that it was possible to dock both substrates of the VDE reaction within the lipocalin-like domain. However, since the structure of the other two domains of VDE are unknown, potential dockings of the substrates could not be done to the whole structure of VDE and therefore lacks alternative dockings to compare with. The total absence of catalytic activity of the lipocalin-like domain by itself partly contradicts the theory of that the active site is entirely located within the lipocalin barrel.

The previous study of the activity of VDE truncations that showed total loss of activity after truncation of the N-terminal domain or the C-terminal domain (Hieber et al. 2002) gives, however, the appearance of not being able to measure low amounts of VDE activity. Using our methods of sample preparations and activity measurements allow a more sensitive activity measurement, which could show that catalytic activity is possible without the C-terminal domain of VDE. This result shows that the C-terminal domain is not required for the catalytic activity of VDE and therefore not an essential part of the active site. The C-terminal domain would then be involved in other functions of the enzymatic process, explaining the large partial loss of activity. The truncation of the C-terminal domain did not cause a major increase of ascorbate K_M , indicating that this domain is not involved in binding ascorbate.

The C-terminal domain has been shown to be required for VDE to be able to bind to thylakoid membranes (Hieber et al. 2002). Even if this function is lost, the remaining part of VDE could still theoretically perform the catalysis after unspecific collisions with the substrates. The observed shift of relative VDE activity towards higher pH after truncation of the C-terminal domain (Fig. 7) could be explained if the function of the C-terminal domain has a certain pH dependency while the catalytic functions of the rest of VDE have another pH dependency. If these pH dependencies do not overlap, this could result in a shift of how pH affects the activity of VDE. After truncation of the C-terminal domain, the special pH requirement of this domain would not affect the activity any longer and could therefore be the cause of the observed shift of pH dependent VDE activity. If the C-terminal domain allows VDE to bind to the thylakoid membrane, requiring slightly lower pH than the pH that is optimal for the catalytic activity, this would result in a shift of pH dependency, showing relatively higher activity at slightly higher pH. The truncation of the C-terminal domain is also causing a shift of the isoelectric point of VDE. This shift in isoelectric point is similar to the shift of the

pH optimal for VDE activity after truncation of the C-terminal domain. The similarities of these shifts suggest that the activity of VDE requires a certain neutralisation of charge. A construct with higher isoelectric point holds the same charged state at higher pH compared to a construct with lower isoelectric point. The more neutrally charged state might be required in some step of the enzymatic process, for example binding to the thylakoid membrane or an oligomerisation.

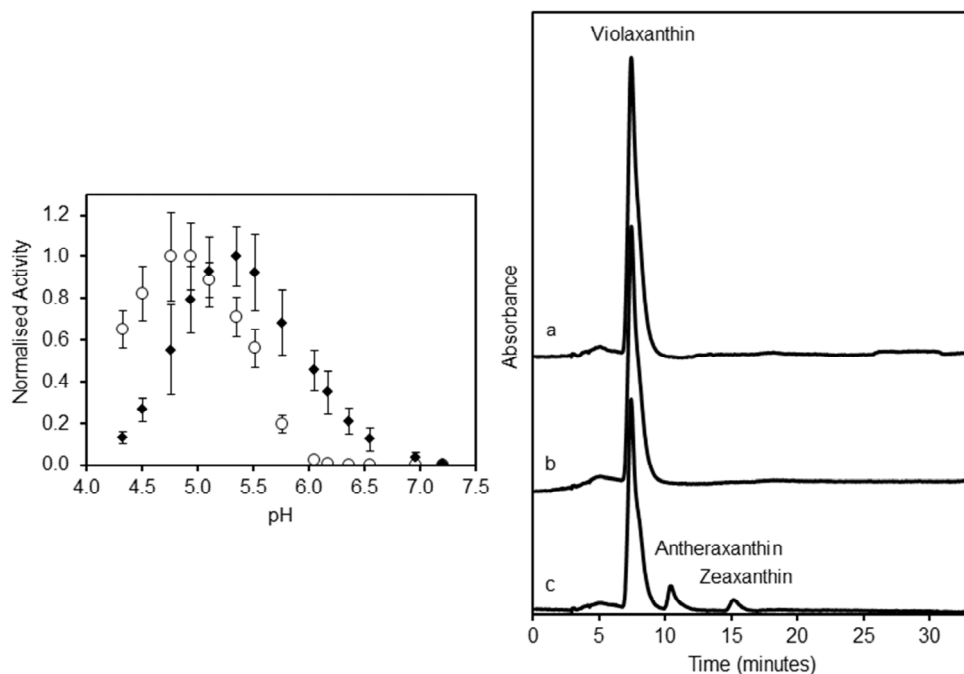


Fig. 7 Left, relative enzymatic activity for native VDE (open circles) and C-terminal truncated VDE (diamonds) measured as a function of pH. Right, HPLC chromatogram (445 nm) of the extracted xanthophylls from VDE activity measurements of (a) isolated N-terminal domain, (b) N-terminal truncated VDE, (c) mixture of isolated N-terminal domain and N-terminal truncated VDE (Paper II)

The function of the N-terminal domain was, however, essential to the catalytic activity of VDE. Truncations of the N-terminal domain resulted in complete loss of activity. The N-terminal domain by itself also gave no activity. But after mixing of these two constructs, the catalytic ability was regained (Fig. 7). This suggests that the active site of VDE is positioned in the interface between the lipocalin-like domain and the N-terminal domain. The theory of that the lipocalin-like domain functions as the holder of violaxanthin while the N-terminal domain contains the catalytic site, is supported by this observation. It is, however, still possible, but

less likely that the entire active site is located within the lipocalin-like domain and that the presence of the N-terminal domain transforms the lipocalin-like domain into an active state. The observation of that the N-terminal domain and the lipocalin-like domain can be mixed after being separately expressed to regain activity shows that these two domains can be folded into the active state separately, which supports the theory that these two domains are truly two domains and not deeply integrated with each other. This knowledge allows the two domains to be analysed separately for future studies.

A non-symmetric conversion

The previous *in silico* docking of violaxanthin into the dimer of two lipocalin-like domains was made such that both epoxides of violaxanthin were placed inside one lipocalin-barrel each (Arnoux et al. 2009). They were also able to dock ascorbic acid inside this barrel, next to the bound violaxanthin (Saga et al. 2010). This would make the lipocalin-like barrel the location of the active site where the de-epoxidation occurs. A consequence of this binding is that both epoxides would be located within an active site at the same time, allowing both epoxides to be converted without the release of the intermediate antheraxanthin. If this is how the de-epoxidation takes place it would not explain the significant concentration of antheraxanthin observed *in vivo*. The concentration of antheraxanthin would in that case be the effect of prematurely released substrate before the full conversion to zeaxanthin has been completed. By measuring the concentration of violaxanthin, antheraxanthin and zeaxanthin at different time points during the VDE reaction *in vitro* we could show that the concentration of antheraxanthin increases rapidly during the initial phase while the increase of zeaxanthin concentration was initially slow and could not even be detected in the early phase of the reaction (Fig. 8). The rate of zeaxanthin formation was increased once a substantial amount of antheraxanthin was present. The concentrations of violaxanthin, antheraxanthin and zeaxanthin at different time points were used to calculate the probability of antheraxanthin to be released from VDE instead of the direct conversion to zeaxanthin. This experiment showed that the probability of antheraxanthin release before conversion to zeaxanthin is either 100 % or near 100 %. The formation of zeaxanthin is caused by the rebinding of released antheraxanthin and not from the direct conversion of violaxanthin. This finding of relative formation of antheraxanthin and zeaxanthin by VDE shows that only one side of violaxanthin is converted and then released.

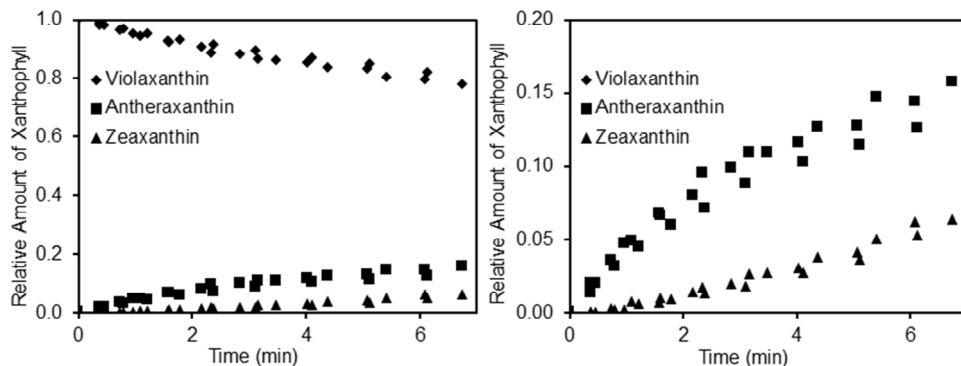


Fig. 8 Early formation of antheraxanthin and zeaxanthin from violaxanthin as a function of reaction time (Paper II)

That VDE only converts one epoxide per substrate binding makes the proposed model of symmetric binding of violaxanthin inside two active sites less likely and instead favours a non-symmetric binding model. In the non-symmetric binding model VDE would instead only bind one side of violaxanthin into the active site while the other side, that will not be converted, will bind to another location. This other binding location could be positioned within another domain of VDE or inside the thylakoid membrane, which then would not require violaxanthin and antheraxanthin to completely dissociate from the membrane during the catalysis. If another domain of VDE binds the side of the substrate that will not be converted this will support the theory where the lipocalin-like domain functions as a holder while the active site where the de-epoxidation occurs is located near the N-terminal domain or in the interface between the N-terminal domain and the lipocalin-like domain.

Previous studies have shown that VDE was able to catalyse the de-epoxidation of various mono-epoxide carotenoids where the other end, opposite to the epoxide end, of the carotenoid could vary and still be catalysed by VDE (Yamamoto and Higashi 1978). If instead the epoxide side was varied by either removing the hydroxyl group or by a change in stereochemistry, VDE was unable to catalyse the substrate. A non-symmetric binding model would only require the side to be converted to have high substrate specificity while the other end of the substrate could be less specific. A symmetric binding of the substrate where both sides have to be bound to the active site would instead be required to accept a broad structural variation of both sides of the substrate, located within the active site, to allow binding and catalysis. That VDE is able to bind β -cryptoxanthin-5,6,5',6'-

diepoxide (Fig. 9), which has the structure of violaxanthin without one of the hydroxyl groups, and catalyse the de-epoxidation of one end but not the other (Goss 2003), is also favouring the non-symmetric binding model. A symmetric model would require the epoxide side without the hydroxyl group to be positioned within the active site in the same way as the epoxide side with the hydroxyl group but still not be catalysed. A non-symmetric model with low specificity of the side not to be catalysed would not be as easily restricted by the other side of the substrate, allowing de-epoxidation of the side with the hydroxyl group but not able to bind the epoxide side without the hydroxyl group.

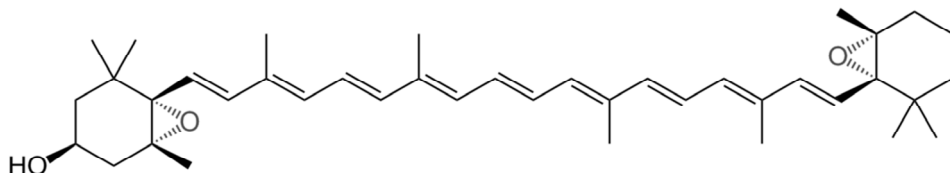


Fig. 9 β -cryptoxanthin-5,6,5',6'-diepoxide

Structural analysis

The only part of VDE with known three-dimensional structure is the lipocalin-like domain. The other two domains of VDE have no sequence resemblance with other proteins with known structure. The predicted secondary structure based on the primary sequence of these two domains suggests an all α -helical structure for both domains. The N-terminal domain is predicted to have five short helices, probably forming a compact bundle due to the presence of five disulphide bonds within this domain. The C-terminal domain is predicted to have two to three longer helices where the longest of them is predicted to form a coiled coil, which is a structural motif of two or more helices that bends around each other due to a repeated pattern of hydrophobic and charged residues.

Using CD measurements of VDE and domain truncated constructs the secondary structure of the different domains could be determined at neutral pH (Fig. 10). The subtraction of the CD spectrum of a domain truncated construct of VDE from the CD spectrum of full length VDE will theoretically give the CD spectrum of the truncated domain, assuming that the truncation did not affect the folding of the rest of the protein. Also, the CD spectrum of a construct with two domain truncations subtracted from the CD spectrum of a construct with one domain truncation leaves the CD spectrum of one domain. Using this method the CD spectrum of the N-terminal domain and the C-terminal domain could be extracted twice by comparing the CD measurements of full length VDE with the two constructs with single domain truncations and the construct with both N-terminal and C-terminal

domains truncated. The CD spectrum of the lipocalin-like domain shows a domination of β structure, in agreement with the structure of a lipocalin. The N-terminal domain appears to have a well-defined secondary structure containing α -helical elements, as the prediction suggested, but may also have some β structure. The CD spectra corresponding to the C-terminal domain also showed indications of an α -helical structure as predicted for this domain. However, the weak CD signal given by the C-terminal domain suggests that the C-terminal domain does not have a well-defined secondary structure.

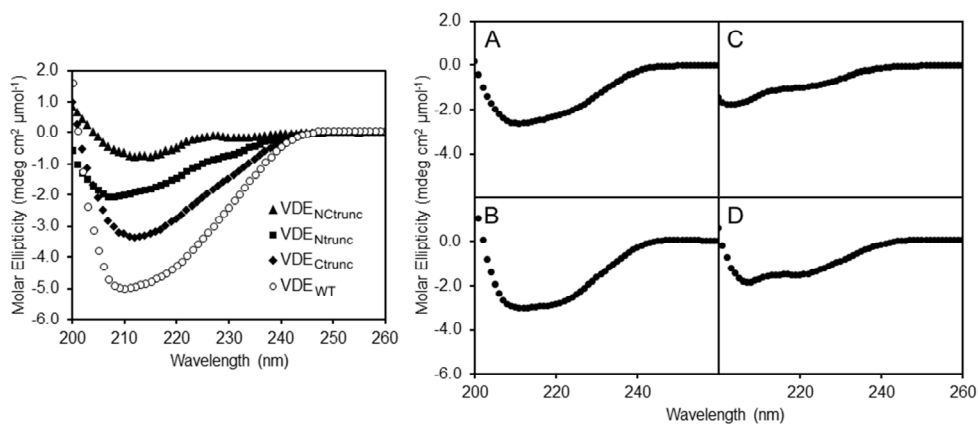


Fig. 10 Left, CD measurements of the VDE and truncated constructs at pH 7.1. Right, difference CD spectra for the N-terminal domain (A, B) and C-terminal domain (C, D) given by the difference of the signal from VDE and truncations. (A) VDE_{Ctrunc} - VDE_{Ntrunc}, (B) VDE_{WT} - VDE_{Ntrunc}, (C) VDE_{Ntrunc} - VDE_{Ntrunc}, (D) VDE_{WT} - VDE_{Ctrunc} (Paper II)

The low resolution model of the lipocalin-like domain of VDE obtained using SAXS (Fig. 11) shows a mostly globular protein with a short elongation on one side. The size of this model fits with the size of the structure of the lipocalin-like domain of VDE obtained through x-ray crystallography. One interesting observation is that the elongated part of the SAXS model overlaps well with the extended β -sheet of the lipocalin-like domain crystallised at pH 5. The largest structural difference between the crystal structure obtained at pH 5 compared with the crystal structure obtained at pH 7 is that the third and fourth β -strands of the lipocalin moves away from the barrel and points outwards. This mobile β -sheet appears to be in the low pH state according to the SAXS model obtained at pH 8. One explanation to the elongated state seen at pH 8 could be that the movement of the β -sheet is flexible and more populated as extended in solution while the crystallisation at pH 7 favoured the non-elongated state.

Lipocalin-like domain

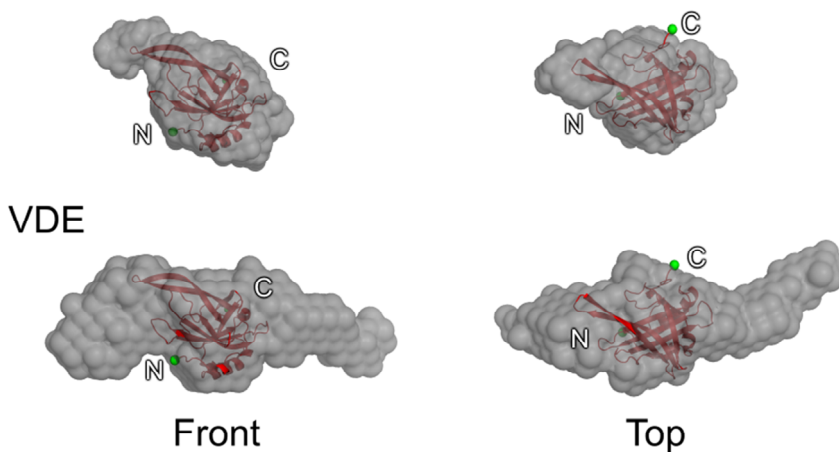


Fig. 11 SAXS models of the lipocalin-like domain and VDE in pH 8 with inserted crystal structure of the lipocalin-like domain (3CQR, Arnoux et al. 2009) from *Arabidopsis thaliana* at pH 5. N-terminal and C-terminal ends are marked with green spheres (Paper III)

The SAXS model of full length VDE shows an elongated structure matching the size of monomeric VDE. The central part of this model is a spherical domain with similar shape and size as the lipocalin-like structure. Next to the central domain is another sphere-like domain of slightly smaller size, while on the opposite side of the central domain is a thin but elongated domain. The central domain of the SAXS model of VDE is predicted to correspond to the lipocalin-like domain due to its fitting size but also based on MONSA calculations (Fig. 12). MONSA can by two given SAXS scatter profiles of a protein and a truncated construct of the same protein calculate a rough 3D model to show the position of the truncated construct. The SAXS scattering profile of the construct consisting only of the lipocalin-like domain together with the scattering profile of full length VDE could be used to position the lipocalin-like domain within the central domain of the SAXS model of VDE with the elongation of the lipocalin-like domain leaning into the sphere-like side domain. This sphere-like side domain of the VDE SAXS model is predicted to correspond to the N-terminal domain due to the many disulphide bonds located within this domain, which would force a more compact structure rather than a more elongated domain. The elongated domain would then correspond to the C-terminal domain of VDE. The C-terminal domain was predicted to contain long α -helical elements, which matches the length of the elongated domain.

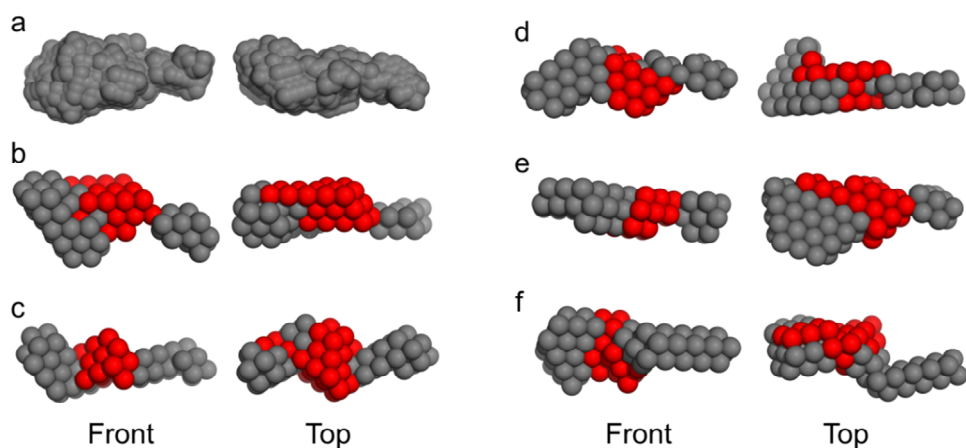


Fig. 12 MONSA generated models showing the location of the lipocalin-like domain within VDE. (a) The averaged and filtered model based on the 10 models generated by MONSA. (b) The five most unique MONSA models with the lipocalin-like domain in red and the rest of VDE in grey (Paper III)

Another indication of the domain orientation shown in Fig. 11 is that chemical crosslinking of VDE at pH 7 resulted in linked peptides connecting different positions of the N-terminal domain to the lipocalin-like domain which places the N-terminal domain near the opening of the lipocalin barrel (Fig. 13). If the elongation of the lipocalin-like domain seen in the SAXS model corresponds to the flexible β -sheet as predicted, the N-terminal domain would be located directly above the opening of the lipocalin barrel shown by the SAXS model of VDE. These indications of the N-terminal domain's position near the opening of the lipocalin barrel support the theory where the active site of VDE is located at the interface between the N-terminal domain and the lipocalin-like domain.

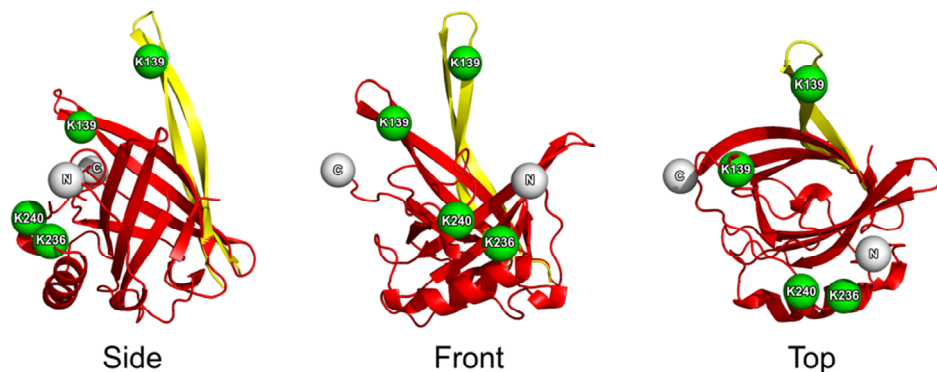


Fig. 13 Crosslinking results showing the lysines within the lipocalin-like domain found linked to the N-terminal domain. N-terminal and C-terminal ends are marked with white spheres. The crystal structure of the lipocalin-like domain from *Arabidopsis thaliana* at pH 7 (3CQN, Arnoux et al. 2009) shown in red and the flexible β -sheet from the structure obtained at pH 5 (3CQR, Arnoux et al. 2009) shown in yellow (Paper III)

Active oligomerisation

The pH regulation of VDE is essential for the plant or algae. The activity of VDE will trigger when to activate the zeaxanthin dependent NPQ to dissipate energy as heat. VDE is therefore regulated to be active at a certain pH, ranging from inactive at neutral pH to an optimal activity between pH 5.0 to 5.2. The simplest model of pH dependent activation would be a protonation of the active site that would allow the reaction to occur. A protonation of the substrate, in this case ascorbate, could also be the pH dependent factor that allows catalysis. Ascorbic acid has been shown to be a more likely substrate to VDE than ascorbate (Bratt et al. 1995). The slightly more complicated scenario would be that a protonation of VDE will cause a structural transformation, forming the active fold of VDE, which also could be combined with active site protonation and substrate protonation.

CD spectra of VDE show a dramatic change when lowering pH towards the active pH (Fig. 14a). These spectra indicate a strong formation of α -helical structures. The relative peak intensity at 208 nm and 222 nm gives a linear correlation from pH 7.1 to pH 5.2 (Fig. 14e), suggesting a single transformation state. At pH 5.0 this correlation stops and the CD spectrum becomes unstable over time, which could be the cause of slow aggregation at this pH. The CD spectrum of the formed structures at pH 5.2 indicates the presence of coiled coils (Fig. 14b). The formation of these structure elements was completely dependent on the C-terminal domain. The construct without the C-terminal domain did not show the same pH

dependent change of the CD spectrum (Fig. 14c), suggesting that the formed α -helical structures were located within the C-terminal domain. The sequence of the C-terminal domain was predicted to give an all α -helical structure and also to contain coiled coils. Both of these predictions match our experimental results. The CD spectrum of the C-terminal domain at neutral pH did show a signature of α -helical structure but the weak intensity of this spectrum indicates that this domain does not have a strong secondary structure and should therefore be more flexible. The high content of glutamic acid might cause charge repulsion limiting the formation of a stable structure. However, the protonation of certain residues may allow formation of the α -helical structure seen with CD. Although the construct without the C-terminal domain does not show any pH dependent change of secondary structure, the thermal stability measurements using CD show a reduced stability at low pH for both full length VDE and the construct without the C-terminal domain (Fig. 14f). This shows that the N-terminal domain and/or lipocalin-like domain also are affected by the low pH, but without causing an alteration of the secondary structure.

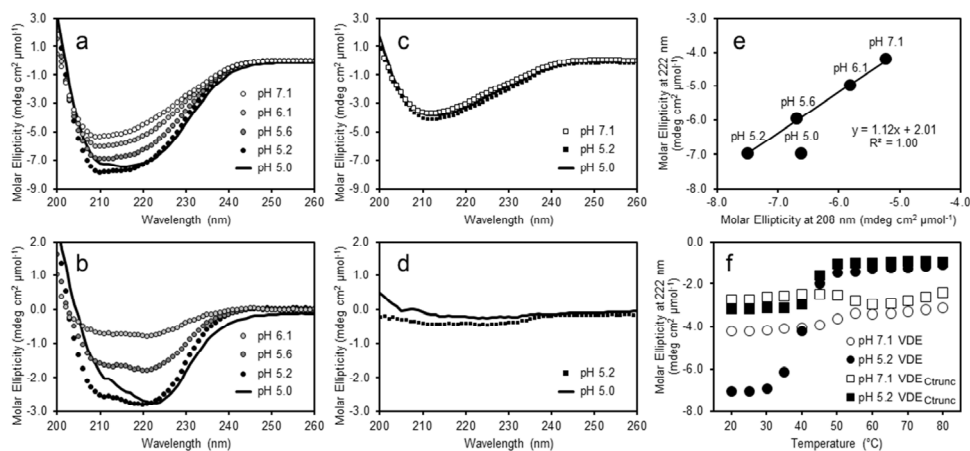


Fig. 14 CD spectra of (a) VDE at different pH. (b) The CD difference spectra for VDE at indicated pH compared to that obtained at pH 7.1. (c) The CD spectra of C-terminal truncated VDE at different pH. (d) The difference spectra for C-terminal truncated VDE at low pH compared to that obtained at pH 7.1. (e) A parametric plot of the CD signal at 222 nm versus the CD signal at 208 nm for VDE. (f) The CD signal at 222 nm of VDE and C-terminal truncated VDE at high and low pH as a function of temperature (Paper III)

The C-terminal domain also appears to be involved in oligomerisation of VDE. The crystal structure of the lipocalin-like domain of VDE showed that a dimerisation occurs at pH 5 but not at pH 7 (Arnoux et al. 2009). Using DLS to measure the particle size of purified VDE at different pH shows that the size

increases to roughly twice the size when lowering pH towards the pH required for activity (Fig. 15). This observation suggests that an oligomerisation of VDE occurs at the pH required for activity. The pH dependent oligomerisation of VDE was also dependent on the C-terminal domain. The construct without the C-terminal domain did not display any pH dependent size increase while the construct without the N-terminal domain did and also appeared to be more sensitive to the low pH. The construct with both the N-terminal and C-terminal domains truncated did not show any pH dependent size increase until a certain pH where the protein aggregated directly.

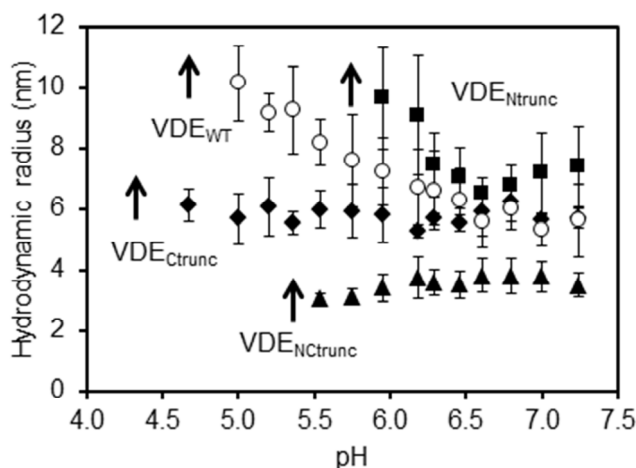


Fig. 15 The hydrodynamic radius for the different VDE constructs obtained from dynamic light scattering measurements. Arrows indicate massive aggregation of VDE_{WT} (*open circles*) at pH 4.7, VDE_{Ctrunc} (*diamonds*) at pH 4.3, VDE_{Ntrunc} (*squares*) at pH 5.8 and VDE_{NCtrunc} (*triangles*) at pH 5.4 (Paper II)

The pH dependent oligomerisation of VDE was also seen using chemical crosslinking (Fig. 16). VDE incubated with a lysine specific crosslinker at pH 7 to 6 resulted in a monomeric band on SDS-PAGE gels, meaning that the formed crosslinks are within one VDE molecule. The same crosslinking reaction repeated at pH 5.5 and 5.0 resulted in oligomeric bands on SDS-PAGE gels, which means that the crosslinks have formed between different VDE molecules, suggesting that VDE oligomerises at these lower pH.

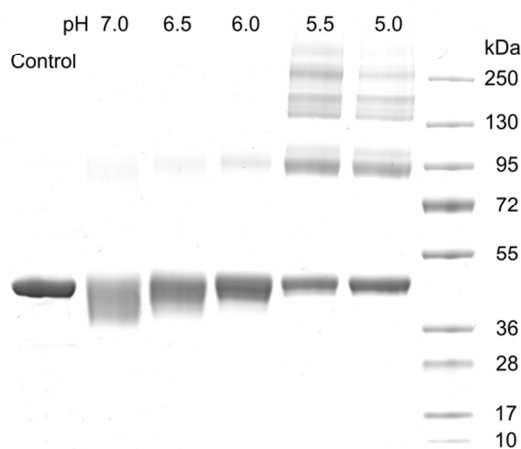


Fig. 16 Coomassie stained SDS-PAGE gel of chemically crosslinked VDE at pH 7 to 5. The lane marked control is the crosslinking reaction without added crosslinker incubated at pH 5.0 (Paper III)

The VDE model from SAXS data showed a non-symmetric shape with the size of monomeric VDE when measured at pH 8 (Fig. 11). If instead measured at pH 5.6 the SAXS data suggests an increase in size and generates a model with dimeric symmetry where it looks like one of the domains of VDE is causing the dimeric interaction (Fig. 17).

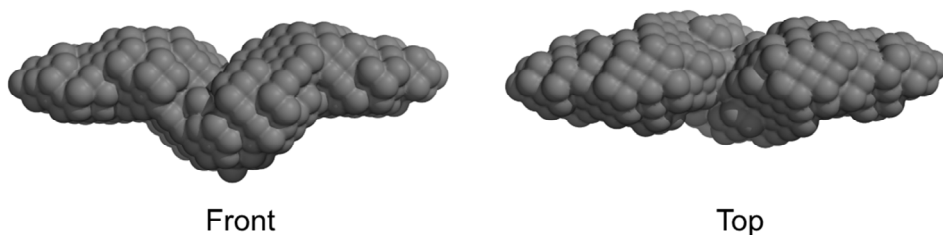


Fig. 17 The SAXS model of VDE at pH 5.6 (Paper III)

The C-terminal domain was shown to be required both for the pH dependent oligomerisation and for the secondary structure formed at low pH. It is therefore reasonable to believe that these two events are connected. The coiled coil structure formed at low pH is a common motif for protein oligomerisation (Greenfield 1999; Lupas and Gruber 2005), which proposes a model where the low pH causes formation of α -helical structure within the C-terminal domain, which allows oligomerisation through the formation of coiled coils. The C-terminal domain has

been shown to be required for the ability of VDE to bind thylakoid membrane (Hieber et al. 2002). This docking ability might not be caused by the direct interaction of the C-terminal domain with the membrane itself but could instead be an effect of the oligomerisation. The interface of VDE that will bind to the thylakoid membrane might be formed after the oligomerisation. This behaviour of a protein-membrane interaction depending on the formation of an oligomeric structure has been reported for other proteins (Cornell and Taneva 2006). This is explained by weak membrane binding properties of the protein in monomeric state, but the combined binding interface of a dimer is strong enough to dock the protein to a membrane.

The hydrophilic properties of the C-terminal domain would make it less expected to be the domain that will dock to a membrane but could still be possible. The activity of VDE when violaxanthin was inserted in different lipid membranes showed that the size ratio between the lipid tail and head group was the important factor for efficient VDE activity and not the specific headgroup (Latowski et al. 2004), indicating that VDE does not bind to a specific headgroup when docking to a membrane and instead interacts with the hydrophobic environment of the membrane. One explanation to the C-terminal domain's involvement in the membrane docking is that this domain allows oligomerisation and that it is the oligomeric state that will bind the thylakoid membrane.

The SAXS model of VDE at pH 5.6 shows a structure with dimeric symmetry where one of the side domains appears to cause the interaction between two VDE molecules (Fig. 17). This domain is expected to correspond to the C-terminal domain which at low pH has formed an α -helical structure interacting with the corresponding structure of another VDE molecule to form coiled coils together. However, it should be remembered that the pH required for optimal activity is slightly lower than the pH required for oligomerisation. The oligomerisation might continue to form trimers, tetramers or higher oligomeric states that will perform the catalytic activity. Alternatively, the dimeric state is the active oligomeric state and why lower pH gives higher activity could be explained by either a higher dimer/monomer ratio or protonation of other residues not involved in oligomerisation but necessary for the activity. The SAXS measurements of VDE at pH 5.4 to 5.0 showed a continued increase of the size of VDE, similar to the behaviour seen with DLS, which may be the cause of minor aggregation. The collected SAXS data at pH lower than 5.6 did, however, not show any alteration of the shape and still no indications of trimeric, tetrameric or higher oligomeric symmetries. The crosslinking of VDE did also not show any shifted ratio towards higher oligomeric states at pH 5.0 compared to 5.5, suggesting that the oligomeric state required for activity already was formed at pH 5.5.

The unknown relative

The closest relative to VDE is the protein named VDE-like/related protein (VDR), which appears to coexist within the same organisms that contain VDE. VDR share the conserved cysteine pattern present in VDE (Fig. 18). Sequence alignments and secondary structure predictions show that VDR seems to have the N-terminal domain and the lipocalin-like domain of VDE. This suggests that an overall structure is shared between VDE and VDR. However, that VDR contains conserved regions within the shared domains that are not conserved in VDE is an indication that VDR and VDE are not functionally identical. Even if VDE and VDR potentially could catalyse the same reaction they might be active at different occasions, which would make VDR an isoenzyme of VDE. A signature of VDR is the lack of the C-terminal domain of VDE. The VDR sequence ends after the lipocalin-like domain and should therefore lack any features given by the C-terminal domain of VDE, such as the oligomerisation or membrane binding ability that the C-terminal domain in VDE is predicted to account for, unless other parts of VDR could compensate for this domain. The N-terminal sequence before the conserved cysteine pattern is longer for VDR compared to the corresponding region in VDE. Either the N-terminal domain of VDR is longer compared to VDE or VDR has a longer N-terminal signal peptide for translocation. The length of this signal peptide would, however, make it much longer than other known signal peptides. The conserved regions of this signal peptide also speak against that it would be cleaved off. One possible explanation is that this peptide could be involved in other functions after cleavage such as regulatory crosstalk.

The function of VDR could be something unrelated to the function of VDE. VDR is present in eukaryotic photosynthetic organisms, predominantly expressed in photosynthetic tissue and predicted to be translocated to the chloroplast. These are all indications of that VDR is involved in photosynthesis. Initial characterisations of an *Arabidopsis thaliana* VDR knockout were made to show that the mutation was not lethal. The plant was able to survive and showed reduced growth compared to wild type *Arabidopsis thaliana*, but did not show any other phenotypic features that could reveal a potential function of VDR so far.

Summary

One of the main findings of this work regarding the characterisation of VDE was the importance of the many cysteines to the enzymatic activity. Only one cysteine could be replaced by a serine without loss of activity. This cysteine was not conserved in algae VDE and was the only cysteine not involved in disulphide bonds. The connectivity of these disulphides could be determined to find a pattern giving two hairpin formations. These disulphides seem to play a role in the activity of VDE due to the often pairwise loss of activity for the respective cysteine mutant pairs. The reduction of these disulphides resulted in lost catalytic activity and loss of thermal stability. The disulphides may be involved in redox regulation, similar to other photosynthetic components.

The N-terminal domain where five of the total six disulphides are located was not able to catalyse the VDE reaction by itself, neither did the rest of VDE, but if these two constructs were mixed together, the catalytic activity was regained. This shows that the two domains can fold independently to form the active state of VDE, suggesting that the active site should be located in the interface between the N-terminal domain and the lipocalin-like domain. One theory is that the lipocalin-like domain binds violaxanthin and holds it in place so that the N-terminal domain can perform the catalysis. We could show that the N-terminal domain is located near the opening of the lipocalin barrel, where violaxanthin is predicted to bind.

The C-terminal domain was not required for VDE activity but instead seems to be involved in oligomerisation of VDE due to formation of coiled coil structures at low pH. The oligomerisation occurs when the pH is reduced towards the pH required for VDE activity but required the C-terminal domain for this function. The expected dimeric state of VDE could be modelled using SAXS, which also could show the monomeric elongated shape of VDE at neutral pH.

By measuring the formation of antheraxanthin and zeaxanthin over time during the VDE reaction showed that only one side of the substrate was converted per binding. This suggests a non-symmetric binding model of the substrate, which contradicts the previously presented symmetric binding of violaxanthin to the lipocalin-like domain.

Future perspective

The next step in structural analysis of VDE would be x-ray crystallography of the whole protein. A great effort has been put into the crystallisation of VDE but has not resulted in crystals of the required size and quality. One solution to structural analysis of a multidomain protein is to crystallise the separate domains individually. We have shown that the N-terminal domain and the rest of the protein can be folded into the active fold by themselves, allowing these domains to be studied separately. The structure of the different domains could later be merged using the SAXS data of full length VDE in solution. If the different domains also are unable to crystallise, one alternative is structural studies using NMR due to the small size of these truncated domains.

A functional analysis of VDE with the goal to specify the binding site of violaxanthin was initiated. We managed to synthesise an azide containing violaxanthin substrate that after exposure to UV-light in the presence of VDE caused VDE to migrate differently on a SDS-PAGE gel compared to if the sample was not exposed to UV light. This indicates that the substrate had bound covalently to VDE and could potentially with further analysis using mass spectrometry locate the binding site of violaxanthin.

More studies of the pH dependent activation of VDE could reveal more information of the oligomeric interface. One potential method to analyse which residues of VDE that interacts with the other VDE molecule is hydrogen-deuterium exchange mass spectrometry. This method could also be used to find the binding site of the substrates as well as the interface of VDE that binds to the thylakoid membrane. The membrane binding ability of VDE is something that also could be analysed using tryptophan residues as fluorescent probes reporting how deep certain positions of VDE are inserted into the membrane phase.

A more farfetched goal is to find out the function of the unknown VDE relative to hopefully reveal a common function of the N-terminal domain and the need for the many disulphides. This could also give information regarding the evolutionary history of VDE.

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