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Purification and Characterization of Violaxanthin De-epoxidase, an Enzyme of the Xanthophyll Cycle

A Dissertation Submitted to the Graduate Division of the University of Hawaii in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

In

Botanical Sciences
(Plant Physiology)

August 1995

By

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ACKNOWLEDGEMENTS

I would like to acknowledge the financial support of Harry Yamamoto over the years through the DOE and USDA grants he administered. I also thank Dr. Y for his tutelage, under which I grew in many ways. I wish to thank Sam Sun for the wise and caring counsel he gave. I thank the rest of the committee, Mike Harrington, Neil Reimer, and Tom Humphries, for their input too at various times during this project. I am indebted to Ann Hirata who prepared numerous partially purified VDE isolations. I thank the Hawaiian Sugar Planters Association for use of their lab and FPLC. I’m thankful for the friendship and help of people in the department: Stefan, Christian, Doris, Nihal, Sunethra, Nancy, Gail, Bob, Narendra, … and even Adam.

I thank my parents Don & Doris Rockholm for their love and support my life through. I thank my parents-in-law, Aminiasi & Sainiana Waqanivalu for their prayers and their allowing me to take their daughter away to live the life of grad students. I thank this daughter, Matelita, from the bottom of my heart- her love and faith kept me going, and they still do. Finally, I am grateful to Jesus Christ, who made it ALL possible.
ABSTRACT

Violaxanthin de-epoxidase (VDE) catalyzes the conversion of violaxanthin to zeaxanthin in chloroplast thylakoids in the presence of ascorbate and an acidic lumen. In higher plants zeaxanthin plays a role in photoprotection by mediating non-radiative (heat) dissipation of light energy in the light-harvesting complexes of photosystem II (PSII) whenever the light intensity exceeds a plant's capacity for carbon-fixation. Because of VDE's central role in this process, its purification and characterization are of interest. VDE had been partially purified from *Lactuca sativa* var. Romaine by differentially extracting sonicated thylakoids at both neutral and acid pH's, followed by size exclusion chromatography. I have now purified VDE by anion-exchange chromatography (Pharmacia Mono Q column) and a unique lipid-affinity precipitation step to one major polypeptide detectable by two-dimensional SDS-PAGE. VDE at pH 5.20 associates with monogalactosyl diacylglycerol (MGDG), the principal thylakoid lipid, forming a complex that can be precipitated by ultracentrifugation. In contrast to MGDG, very little VDE precipitated in the presence of eight other lipids, indicating a specific association of VDE for MGDG. The uniqueness of VDE's affinity for MGDG is further exemplified through a negative result: established protein purification procedures using various surfactants to form reverse micelles failed to isolate VDE.
The purified enzyme has an apparent molecular mass of 43 kD and a pI of 5.4.

The $K_M$ values of VDE for its substrates were 0.352 μM for violaxanthin and 8.54 mM for ascorbate. Neutral red, 9-aminoacridine, and dibucaine (substances used to determine pH intracellularly and/or selectively uncouple the lumen proton gradient) directly inhibited VDE. VDE's amino acid composition and a number of partial amino acid sequences from VDE were determined (its N-terminus and four tryptic fragments). Various diverse characteristics of the purified enzyme are also reported, including how $T^\circ$ and BSA affect VDE activity and stability as well as the absence of mobility shifts on polyacrylamide gels.

Polyclonal antibodies were generated to purified VDE (in conjunction with Katrin Hinderhofer) which inhibited the de-epoxidase reaction and reacted principally to the 43 kD polypeptide on a Western blot.
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ABBREVIATIONS

Ab       antibody
ATP      adenosine 5'-triphosphate
BHA      butylated hydroxyanisole
BHT      butylated hydroxytoluene
BSA      bovine serum albumin
CAPS     3-{cyclohexylamino}-1-propanesulfonic acid
CHAPS    3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate
CTAB     dodecyltrimethylammonium bromide
DGDG     digalactosyl diacylglycerol
DEAE     diethylaminoethyl
dtt      dithiothreitol
EDTA     ethylenediamine tetraacetic acid
FPLC     fast protein liquid chromatography (Pharmacia)
HPLC     high performance liquid chromatography
I_{50}\% concentration of substance inhibiting 50% of enzymatic activity
IEF      isoelectric focused
kD       kilodalton
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>LHC</td>
<td>light harvesting complex</td>
</tr>
<tr>
<td>LSB</td>
<td>Laemmli sample buffer: 250 mM Tris-Cl, pH 6.80 + 2% SDS + 20% glycerol + 5% β-mercaptoethanol</td>
</tr>
<tr>
<td>MGDG</td>
<td>monogalactosyl diacylglycerol</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>9-AA</td>
<td>aminoacridine</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonionic detergent P-40</td>
</tr>
<tr>
<td>NPQ</td>
<td>non-photochemical quenching of chlorophyll fluorescence</td>
</tr>
<tr>
<td>OG</td>
<td>n-octyl-β-D-glucopyranoside</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>capryl alcohol</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFD</td>
<td>photon flux density</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidyl-DL-glycerol</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidyl-L-serine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>PS I or II</td>
<td>photosystem I or II</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature - 25°C</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SMN</td>
<td>50 mM MOPS, 400 mM sorbitol, 10 mM NaCl, 16 mM sodium ascorbate at pH 7.20</td>
</tr>
<tr>
<td>TCA</td>
<td>( \text{CCl}_2\text{COOH} )</td>
</tr>
<tr>
<td>TEMED</td>
<td>( N,N,N',N'\text{-tetramethylethylenediamine} )</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TOMAC</td>
<td>trioctyl methyl ammonium chloride</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>( \text{NH}_2\text{C(CH}_2\text{OH})_3\text{HCl - electrophoresis grade} )</td>
</tr>
<tr>
<td>Triton X-45</td>
<td>octyl phenoxy polyethoxyethanol</td>
</tr>
<tr>
<td>V</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>VDE</td>
<td>violaxanthin de-epoxidase</td>
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<tr>
<td>Z</td>
<td>zeaxanthin</td>
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In a given day, at some point, most plants will receive more sunlight energy than they can process through photosynthetic carbon reduction. "Plant evolution has... had to tread a path between maximizing light interception for photosynthesis and minimizing the potential for damage arising from the over excitation of the photosynthetic apparatus." (Long et al. 1994). Plants may minimize the potentially deleterious situation of over excitation in a number of ways: 1) they may regulate the amount of light intercepted, 2) change their capacity for photosynthesis, or 3) dissipate excess excitation energy through a variety of processes.

In the first category above, some plants can affect the amount of light intercepted by changing the leaf orientation relative to the solar beam (paraheliotropic movements), and presumably chloroplast movement occurs in all plants to both reduce light absorbance in an individual chloroplast and to provide more uniform light distribution. Longer term responses altering the amount of light intercepted by the plant include changes in leaf reflectance (a non-reversible change), and changes in the amount of chlorophyll per unit leaf area (Ludlow & Björkman 1984, Powles 1984, Björkman & Demmig-Adams 1993). Plants also respond to high incident light by increasing their photosynthetic capacity for carbon fixation, the second category above. This is also a longer term adaptation but the
most effective of the three categories. However, even plants with the highest photosynthetic capacities still utilize all regulatory mechanisms.

The third category of plant responses to an excess of excitation energy involves a number of proposed mechanisms within the chloroplast. "The fact that such a wide array of photoprotective processes have evolved is fully consistent with the assumption that overexcitation of the photosynthetic system is very harmful and must be avoided." (Björkman & Demmig-Adams 1993) Examples of such intrachloroplastic mechanisms include state 1- state 2- transitions, and the Mehler reaction (Powles 1984). In the former, the light-harvesting complexes (LHCs) are phosphorylated and migrate from photosystem II to associate with photosystem I, thereby limiting overexcitation of photosystem II and stimulating cyclic electron flow (Bennett 1991). In the Mehler reaction reduced ferredoxin passes its electron to molecular oxygen rather than NADP⁺. Thus the Mehler reaction enables electron transport to proceed (instead of any more injurious side reactions) despite a lack of oxidized NADP⁺. In addition, the carotenoid pigment zeaxanthin (Z) has been suggested to be a major means by which a plant avoids damage from excess light energy (Demmig et al. 1987). The mechanism by which zeaxanthin acts in this capacity is in dispute though it "... seems likely that the xanthophyll cycle [by which Z is formed] is a first line of defense against PSII overexcitation during absorption of light in excess of photochemical utilization." (Long et al. 1994). Consequently the investigations which
follow are of an important enzyme, the one responsible for the formation of zeaxanthin in thylakoids, namely violaxanthin de-epoxidase.
A. Role of Carotenoids in Photosynthesis

Karnaukhov observed in his 1990 review that the structures and chemistry of carotenoids were far better known than their function. Berzelius (1837) first named the yellow pigments in fallen pear leaves as "Blattgelb" in his native German or "Xanthophylls" in Greek (both names mean "yellow leaf" or "yellow foliage"). Specifically, these pigments were the transparent yellow crystals he derived from an alcohol extraction of fresh "lemon yellow" leaves in autumn which was subsequently dried, that which solubilized with ether and allowed to crystallize. (The term "xanthophyll" has since been restricted to the oxygenated class of carotenoids.) By 1907 the chemical formulas of a number of carotenoids were determined (reviewed in Davies 1976 and Karnaukhov 1990). There are now nearly 600 carotenoid structures and their glycosides determined (Armstrong et al. 1990). The variations of carotenoid structure are based on seven different end groups (structures shown in figure 1), of which only four are found in higher plants (Bramley et al. 1993).

The annual production of carotenoids worldwide is estimated to be 100 billion kilograms (Siefermann-Harms 1985) and reputed to be the most widespread group of...
End groups (R, R'):

Figure 1 - Basic structure of the carotenoids. The central hydrocarbon chain is shown above and the seven different end groups below. Only $\beta$, $\varepsilon$, $\kappa$, and $\psi$ are found in higher plant carotenoids (Bramley et al. 1993).
pigments (Bramley & Mackenzie 1988). All photosynthetic prokaryotes and eukaryotes synthesize carotenoids, as well as certain fungi, yeasts, and nonphotosynthetic bacteria (Armstrong et al. 1990). Carotenoids have been commonly referred to as "accessory pigments", yet the absence of any extant photosynthetic organism without them suggests, in fact, they are essential pigments (Cogdell & Gardiner 1993, Yamamoto & Bassi 1995). However, there is room for discussion as to what the essential role(s) of carotenoids may be. Many "diverse biological functions within the cell" have been attributed to carotenoids (Kamaukhov 1990) and so I herein review the better attested of those functions.

Xanthophylls, and in particular lutein, outnumber other non-chlorophyll pigments within the LHC's for the photosystems of higher plants. Each major LHC apoprotein binds between 7 and 14 chlorophyll molecules along with 2 to 3 xanthophylls (Siefermann-Harms 1985, Thornber et al. 1993). HPLC makes it possible to accurately determine the relative amounts of each pigment, but because the various pigments do not bind covalently (and carotenoids are removed more easily than chlorophylls from apoproteins), the pigment:protein stoichiometry cannot be so easily determined. In a recent review Yamamoto & Bassi (1995) settle on 7 chl a, 5 chl b, and 2 lutein molecules per LHC II (the LHC associated with photosystem II) complex. Recent findings from electron crystallography (Kühbrandt et al. 1994) build a case for a structural role of lutein in LHC II as the two luteins form a cross-brace between helices of LHC II in the membrane. However, these lutein molecules are also in van der Waals contact with seven
chlorophyll molecules and thus close enough for direct energy transfer to chlorophyll, as
there is also spectral overlap which would enable such transfer to occur (Yamamoto &
Bassi 1995).

Plumley & Schmidt (1987) showed xanthophylls to be essential for the
reconstitution of functional LHC which had been completely denatured. Functionality of
LHC was assessed by comparisons of the native- and reconstituted-complex absorption
and CD spectra as well as by the complexes' fluorescence excitation and emissions
spectra. They concluded the xanthophyll requirement was specific and not due to any
nonpolar properties. Generally a mix of two of the pigments of either lutein, neoxanthin,
or violaxanthin were necessary for reconstitution, but lutein by itself facilitated reassembly,
albeit with low yields. Cammarata & Schmidt (1992) extended the reconstitution studies'
by using partially purified cloned LHC II translation products. In the absence of either
chlorophyll or lutein there was no reconstitution, whereas in the absence of V
reconstitution proceeded "suboptimally". They also found two regions in LHC using
deletion mutagenesis the loss of which had a pleiotropic effect on pigment binding and
stability (AA #50-57 on the stromal side and AA #204-212 on the lumenal). The
Arabidopsis thaliana "aba" mutant contains low V and neoxanthin levels yet evidenced a
measure of stable LHC assembly (Rock et al. 1991, 1992). However, mutants contained
half the usual amount of granal stacking. Xanthophylls undoubtedly do have a structural
role.
The direct participation of carotenoids in energy transfer in photochemistry was already considered "well-established" by 1955 (Griffiths et al. 1955). Xanthophylls contribute an estimated 43% of the absorbance between 390 and 540 nm in LHCs though comprising but 23% of the pigment (Siefermann-Harms 1985). Xanthophyll to chlorophyll a energy transfer efficiency supposedly approaches 100% in tested algal and bacterial systems (Siefermann-Harms 1987). *Lactuca sativa* has the highest reported violaxanthin:chlorophyll a ratio in LHC of higher plants (1:10). Energy transfer in a lettuce LHC II preparation has been measured at 100% by means of action spectra for chlorophyll a fluorescence (Siefermann-Harms 1985) and so it follows that xanthophylls must also be involved in energy transfer in higher plants.

Frank et al. (1994) used femtosecond time-resolved, fast-transient optical spectroscopic analyses of xanthophylls to demonstrate that the excited singlet energy state ($2^1Ag$) of both V and A is above the lowest excited singlet state of chlorophyll a ($S_1$) and consequently may act in the capacity of light-harvesting pigments. Their results support Siefermann-Harms (1985), who in her review stated the plausibility that xanthophylls, or at least epoxidized ones, act as primary donors of light energy. Earlier Frank et al. (1991) had concluded the carotenoids in bacteria carried out light-harvesting by singlet to singlet energy transfer to bacteriachlorophyll in the antenna proteins. Gilbro et al. (1993), by studying the fluorescence spectroscopy in two series of carotenoids (both natural pigments as well as synthetic minicarotenoids), concluded that a mechanism of
Dexter electron energy exchange is utilized in this singlet-singlet transfer. Such a mechanism requires that there be contact and overlap between donor and acceptor spectra but not necessarily that the coupled states be dipole allowed. If this is the case, as they suggest, carotenoids could “act as an electronic wire between the antenna and the RC [reaction center].”

One unicellular yellow-green algae, a *Nannochloropsis* sp. (Eustigmatophyceae), contains a large amount of violaxanthin and chlorophyll a but no chlorophyll b or c. The ratios of chl a:V have been reported as 0.78 and 1.2 (Chystal & Larkum 1987, Livne et al. 1992). Chystal & Larkum (1987) have shown that for these algae, xanthophylls play a major light-harvesting role, extending the range of effective energy capture below 540 nm. Their work was the first demonstration of a major light-harvesting role of violaxanthin in algae.

The carotenoids in the reaction center of the photosystems are thought to have a different primary function than those carotenoids in the LHC. In contrast to the LHC, within the reaction center the non-oxygenated class of carotenoids, the carotenes (and in particular β-carotene), predominate. The carotenes in the reaction center probably protect against triplet chlorophyll the longer lasting form of excited chlorophyll (on the order of tens of microseconds) and therefore able to interact with molecular oxygen beginning the process of photooxidation. Carotenoids quench chlorophyll a triplets 100 to 1000 times faster than such chlorophyll reacts with oxygen (Frank & Cogdell 1993).
Carotenoids outside of the reaction center also may protect photosynthetic systems against singlet oxygen (the product of triplet chlorophyll and molecular oxygen). Singlet oxygen is toxic to cells, able to peroxidize lipids, destroy membranes, and react with nucleotides or proteins to cleave and/or inhibit them (Foote 1968, Cogdell 1988, Rau 1988). However, when carotenoids react with excited triplet chlorophyll or singlet oxygen, the energy is simply quenched, "deactivated" and dissipated harmlessly as heat. (Beddard et al. 1977 suggests that these reactions occur by electron transfer, where Cogdell 1988 suggests they are by a triplet-triplet exchange reaction.) The low-lying triplet state of carotenoids makes it possible for them to quench triplet chlorophyll as well as singlet oxygen (Cogdell & Gardiner 1993, Frank et al. 1994).

The two energy functions of carotenoids are opposite in their directionality: they either help bring in light energy to the photosynthetic reaction center or they discharge energy from the photosystem. "There is a time for everything,... a time to scatter... and a time to gather" (Ecclesiastes 3: 1,5). Energy is "scattered" when it occurs in the potentially dangerous forms of triplet chlorophyll or singlet oxygen, forms which may occur even in plants with a high photosynthetic capacity. In the full sun, plants acquire light energy in excess of what they can use in photosynthetic electron transport, the situation is exacerbated when a plant is stressed in any way. An idealized graph of incident light versus that portion of the energy utilized in photosynthesis is depicted in figure 2. When the photosynthesis rate is maximal, any additional light is entirely in excess, therefore, at
Figure 2 - The calculated response of an ideal C₃ leaf at 25 and 10°C to varying incident photon flux (I). $I_a$ is light absorbed by the leaf; $I_o$ is that light energy which is utilized. In this steady-state model of photosynthetic carbon metabolism, a leaf absorbance of 0.9 is assumed (Long et al. 1994).
high fluences, the amount of excess light is a large proportion of that absorbed. Besides carotenoids, other deactivators of singlet oxygen include catalase, superoxide dismutase, glutathione reductase, phenolics, and ascorbate (Demmig-Adams 1990). Carotenoids act from both within the reaction center (β-carotene) and, as we shall discuss, from somewhere outside it (zeaxanthin).

Some researchers question the proposed photoprotective role of carotenoids quenching triplet chlorophyll. Nechushtai et al. (1988) presented evidence that the capacity of carotenoids to quench chlorophyll $\alpha$ triplet states was already exceeded at low light intensities. Instead, they proposed the heat dissipation of excess light energy to be the result of triplet-triplet chlorophyll $\alpha$ annihilations.

Nevertheless, most would agree with the summary: "while carotenoids have two major functions in photosynthesis, namely acting as accessory light-harvesting pigments and preventing photooxidation, it is the only the latter of these that is essential for a plant's survival." (Young & Britton 1990 and similarly in Griffiths et al. 1955).

**B. Carotenoid Biosynthesis**

The topic of carotenoid biosynthesis has been recently reviewed by both Goodwin (1993) and Sandmann (1994). Carotenoids are condensation products of isoprenoids (the first three condensations are shown in figure 3) whose chromophore properties are derived from a system of conjugated double bonds (Davies 1976). The first carotene, phytoene, is the condensation product of two molecules of geranylgeranyl diphosphate
Figure 3 - Conversion of Δ^1-Isopentenyl pyrophosphate (IPP) to geranylgeranyl pyrophosphate (GGPP). The first step is the isomerization of IPP to dimethylallyl pyrophosphate (DMAPP). Three IPP's are then sequentially added to DMAPP by prenyl transferases (Goodwin 1993).
(GGPP). The steps to then produce β-carotene first involve four desaturations (to yield lycopene) and then two subsequent cyclization steps (figure 4).

In higher plants the biosynthetic steps yielding β-carotene take place within the chloroplast. Britton (1990) points out, however, that there are conflicting opinions as to whether all the necessary precursors (especially mevalonic acid) are also synthesized within the chloroplast or whether they are imported. Sandmann (1994) states that “all steps from phytoene synthesis to cyclization of lycopene take place at a membrane-integrated multienzyme complex”. Britton (1990) believes that this purported complex is organized specifically with or at least associated with electron transport components, since all carotenoid biosynthetic activity is lost when the membranes are solubilized.

Subsequent synthesis of either carotenes or xanthophylls from β-carotene, however, occur at different sites. Bramley & Mackenzie (1988) cite kinetics and herbicide inhibition studies as evidence for this. Siefermann-Harms et al. (1978), using xanthophyll pigment fluxes as evidence, hypothesized a xanthophyll exchange between chloroplast envelope and thylakoid while synthesis occurred at one site only. In any case, the xanthophyll to carotene ratio is three times greater in envelopes than in thylakoids.

Britton (1990) stressed that in any study of chloroplast development the important variables are age and environmental conditions. He also noted that carotenoid biosynthesis may be expected to vary with species, variety, and even from cotyledon to the true leaves on the same plant. Justification for these warnings was the finding of
Figure 4 - Sequence of synthetic steps from geranylgeranyl pyrophosphate to \(\beta\)-carotene. Arrows indicate the formation of double bonds which alternate from the left to right of center (Sandmann 1994).
phytoene synthase in the stroma of Capsicum but associated with the envelope in Spinaceae (Bramley et al. 1993). Furthermore, zeaxanthin epoxidase activity has been found in a range of locales, recently it was found associated with a LHC II preparation from winter rye (Gruszecki & Krupa 1993a,b) whereas activity has also been reported in the envelope of spinach chloroplasts (Costes et al. 1979). However, such variability is not the exclusive domain of carotenoids biosynthetic enzymes- histones too have been found in purified PS II fractions (Arvidsson et al. 1992). With regards to zeaxanthin epoxidase, there may be more than one enzyme catalyzing this reaction, i.e., one involved in the initial biosynthesis of violaxanthin and another in its cyclic conversion back to violaxanthin (Britton 1993).

The control of carotenoid biosynthesis is expected to be a highly regulated process, needing to occur in the proper context and timing during chloroplast development as well as in response to stress (Herrin et al. 1992). Regulation must involve the coordinate expression of a number of proteins from a number of different biosynthetic pathways including for chlorophyll and carotenoid pigments, the apoproteins which bind them and their lipid components (Britton 1993). Grumbach (1984) reported phytochrome control for the production of cyclic carotenoids in radish- a low level of red light stimulated synthesis of such carotenoids. Sandmann (1994) reviewed evidence for at least three carotenoid biosynthetic enzymes being photoregulated: phytoene desaturase, phytoene synthase, and GGPP synthase. The last enzyme mentioned was regulated at the
transcriptional level via instability of its mRNA. When the green alga *Dunaliella bardawil* was light-stressed its carotenogenesis was accelerated (Levy et al. 1993). In *Dunaliella* under such circumstances, a carotenoid biosynthesis related gene (*cbr*) has been shown to be transcriptionally and translationally activated. This algal *cbr* was shown to be homologous to the early light-induced proteins (Elips) of higher plants which have also been found to be made in response to light-stress conditions (Adamska et al. 1992, Levy et al. 1992).

**C. Xanthophyll Cycle**

The xanthophyll cycle components are (3S,5R,6S,3’S,5’R,6’S)-5,6,5’,6’-diepoxy-5,6,5’,6’-tetrahydro-β, β-carotene-3,3’-diol (better known as violaxanthin), (3S,5R,6S,3’R)-5,6-epoxy-5,6-dihydro-β, β-carotene-3,3’-diol (antheraxanthin), and (3R,3’R)-β, β-carotene-3,3’-diol (zeaxanthin, all of the xanthophyll cycle pigments are shown in figure 5). Together they comprise from 10 to 40% of the total carotenoids in leaves (depending on species and growth condition). Sapozhnikov et al. (1957) first noted that strong light caused violaxanthin to decrease and lutein to increase in the four species of plants he tested. Sapozhnikov & Bazhanova (1958) found the same relationships of V and lutein when light treatments were administered to isolated chloroplasts (from “horse bean”). Blass et al. (1959) investigated a pair of unicellular green algae and found the V concentration altered in the light only for *Scenedesmus obliquus* (Turpin) Kuetying but not for *Chlorella pyrenoidosa*. Blass et al. (1959) also presented a model on the
Figure 5 - The xanthophyll cycle pigments. The arrows relate the manner in which the pigments are enzymatically converted (after Yamamoto 1979).
possible interrelationships of the xanthophylls which in hindsight can only be described as hopelessly convoluted.

Yamamoto et al. (1962) corrected Sapozhnikov, by demonstrating that the lutein concentration does not change with exposure to light. Rather light occasions a nearly stoichiometric decrease in violaxanthin concentration matched by a concomitant increase in zeaxanthin, and is accompanied by a transient rise in antheraxanthin. Yamamoto et al. (1962) also documented some reversion (though incomplete) of these pigments in the dark and that the light reaction was enzyme-mediated. De-epoxidation and epoxidation were more accurately described as light-induced dark reactions by Yamamoto et al. (1967). Nonenzymatic conversion of V to Z is also possible. Schubert et al. (1994) purified Z by HPLC from *Chlorella pyrenoidosa* and when exposed to high light (10,000 μE x m⁻² x s⁻¹) and air for 20' at 50°C, V formed.

Hager (1969) showed that de-epoxidation of V could be triggered without light by adding ATP. Another manner of inducing the de-epoxidation of violaxanthin within darkened chloroplasts was found to be lowering the pH of the suspension to values <5.0. Yamamoto et al. (1971) demonstrated the induction of de-epoxidation with ascorbate and by monitoring zeaxanthin formation through dual-wavelength spectrophotometry at 505 nm with a reference wavelength of 580 nm. Yamamoto et al. (1972) reported that PS I probably mediated VDE activity as acidification from H⁺ transport alone was sufficient to drive de-epoxidation. PS I sufficiency was also shown by Ladygin & Shirshikova (1988)
who used *Chlamydomonas* PS II mutants in which Z accumulated to three times the wild type concentration. (Their result may further indicate that the absence of PS II may make Z’s presence more necessary.) Yamamoto & Kamite (1972) found DTT inhibited the 505 nm spectrophotometric change related to Z formation. Siefermann & Yamamoto (1974) confirmed that the 505 nm absorbance changes were indeed correlated with a decrease in V concentration in isolated chloroplasts using thin layer chromatography (TLC). De-epoxidation activity was found to be present in both grana and stromal lamellae (Siefermann & Yamamoto 1976).

The epoxidation reaction was also investigated. The oxygen from water was initially thought to be the source of the epoxy groups in V (Sapozhnikov 1961), but later the epoxy groups in V and antheraxanthin were shown to derive from molecular oxygen through the use of $^{18}$O$_2$ (Yamamoto & Chichester 1965). The epoxidase was found to be membrane bound and not inhibited by DTT. However, 5 mM of the copper chelator salicylaldoxim did inhibit the epoxidase (Hager 1980). The epoxidase continued to be active at low light levels after VDE had shut off and so able to reverse the “gains in Z” (reviewed in Siefermann-Harms 1977).

Figure 6 shows the transmembrane model of the xanthophyll cycle by Siefermann & Yamamoto (1975a). The monoepoxide A is the intermediate for both the forward and reverse reactions. The figure also presents ascorbate as a reactant of de-epoxidase. Ascorbate is present in vivo, but Yamamoto (1979) did not categorically rule out the
Figure 6 - The transthylakoid model of the xanthophyll cycle. The intermediate antheraxanthin is not shown. (after Siefermann & Yamamoto 1975a)
reduction of V was by another reductant. The diagram depicts the pigments as free in the lipid matrix of the thylakoid membrane. However, the minor chloroplast proteins of 24, 26, and 29 kDa (CP’s 24, 26, and 29 respectively) have been shown to bind >80% of the V associated with PS II. Furthermore, the V of these complexes was reportedly (though I found the evidence wanting) convertible to Z (Dainese et al. 1992). Consequently, it has been hypothesized that the "xanthophyll cycle pigments are dissolved in lipids that are tightly bound to the complexes [CP’s 24, 26, 29]" (Yamamoto & Bassi 1995). Certain data suggests that Z is less tightly bound than V in thylakoids and possibly more mobile there. (However, Z is not as freely exchanged with the envelope as V, Sieffermann-Harms et al. 1978.) Thayer & Björkman (1992) found preferential loss of Z during solubilization of thylakoid membranes using 6% n-nonyl β-D-glucopyranoside, 3% decyl β-D maltoside, and 1% SDS with subsequent separation on Deriphat-PAGE gels. Sieferman-Harms (1984) found 60% of the Z formed by a light treatment of spinach thylakoids to be in the "free pigment" fraction on IEF/digitonin-PAGE.

The number and range of supposed functions attributed to the xanthophyll cycle has been vast. Sapozhnikov (1973) thought it probable that the cycle participates in oxygen transport, as part of an O₂ liberating system. Yamamoto (1979) pointed out that the cycle operates at two orders of magnitude slower than photosynthesis, and for that reason it probably had no direct involvement in photosynthesis (such as in photophosphorylation or oxygen evolution). Yamamoto (1979) thought the cycle did not
act as a photoprotector but instead as a regulator of photosynthesis by altering membrane properties (also Sieferman-Harms 1977).

Hager (1980) seemed to suggest that the major function of the xanthophyll cycle lay in its use of reducing equivalents. Using up ascorbate was thought to improve the ratio of ATP to NADPH produced by photosynthetic electron transport and preventing "overreduction" of the thylakoid which would inhibit linear electron transport. By the xanthophyll cycle’s use of ascorbate it may thereby also be involved in regulating carbon metabolism and/or the glutathione reduction cycle. Earlier, Hager & Perz (1970) suggested V's binding to VDE (without necessarily reacting) could trigger electron transport changes, changes in membrane potential, ion transport changes, and enzyme activation/deactivation, simply by altering the ratio of free to bound V.

Krinsky (1979) reviewed the evidence for carotenoids with 9 or more conjugated double bonds being effective quenchers of singlet oxygen. De-epoxidation of V could then be considered a reversible means of forming a more effective photoprotector against oxidation, as Z has two more conjugated double bonds than V (11 vs. 9). Gruszecki (1985) found Z slowed down the photosensitized destruction of phosphatidyl choline emulsions at pH 5.0 whereas V had the same effect in solutions at neutral pH. Gruszecki (1985) also suggested that both these xanthophylls functioned in a photoprotective role and that one of the functions of the xanthophyll cycle was to provide a pool of effective photoprotectors in response to light intensity. Such a role was further evidenced by
violaxanthin and zeaxanthin (again with different pH optima) slowing the photooxidation
of chlorophyll $a$ in liposomes (Gruszecki & Sielewiesiuk 1986). Schubert et al. (1994)
states that exothermic trans-cis isomerizations of Z are the means by which plants
harmlessly relax chlorophyll triplet states after they have been transferred first to Z.

Demmig et al. (1987) suggested that the xanthophyll cycle functioned to provide a
different means of dealing with light energy in excess of what could be used in
photochemistry and so provide "photoprotection". In their study, photoprotection was
seen to occur earlier, on energy absorbed by photosynthetic pigments and still capable of
being used in photosynthesis. In the manner spoken of by Krinsky or Gruszecki, however,
photoprotection was against absorbed energy no longer capable of photosynthesis but
rather in a form potentially lethal to the plant.

Demmig et al. (1987) stated the photoprotective role belonged to Z alone (and not
V as implicated by Gruszecki 1985 and Gruszecki & Sielewiesiuk 1986), and that
increased Z concentration correlated with increased dissipation of excess light energy.
Such excess energy dissipation would reduce the amount of energy available to form
reactive triplet oxygen. Demmig et al. (1987) exposed different plant species to
"photoinhibitory light levels" and measured their pigment levels and fluorescence
parameters. (Room temperature fluorescence of PSII chl $a$ accounts for less than 4% of
absorbed light energy but can be used as a non-invasive monitor of photosynthesis,
measuring, for example, the quantum yield of photochemistry or used as a reflection
regarding the level of stress a plant is receiving; reviewed in Krause & Weis 1991.)

Demmig *et al.* (1987) found that those plants which had a higher capacity to form zeaxanthin correlated with those which experienced less photoinhibitory damage to PS II reaction centers. They also found that "sun plants" were immune to photodamage up to the point where an elevated light level is reached that resulted in no additional zeaxanthin formation.

The work of Demmig *et al.* (1987) has proven to be a turning point in understanding the xanthophyll cycle and focusing research. Despite most of the field following their hypothesis, other suggestions as to the function of the xanthophyll cycle have continued to be made. Karnaukhov (1990) defended Sapozhnikov’s initial idea that the cycle functions as a liberator of oxygen. He also critiqued the xanthophyll cycle model depicted in figure 6 as inadequate because it was derived from an *in vitro* system using isolated (and damaged!) chloroplasts; so the model "very likely ... disregard[s] some of [the chloroplast’s] properties in intact cells".

Gruszecki (1991) characterized the monomer and aggregated forms of V and concluded that V functions as an antenna for light harvesting. His subsequent work with Havaux *et al.* (1991) photoacoustically monitored thermal energy dissipation *in vivo*. This research purportedly severed the link Demmig *et al.* (1987) established for Z formation by measuring energy dissipation when the xanthophyll cycle was blocked. Consequently, Havaux *et al.* (1991) hypothesized Z worked as a photoprotector in strong light
specifically of membranes, since the xanthophyll pigments were thought to be relatively free in the membrane (Krinsky’s as well as Gruszecki’s initial assumption). In Havaux & Gruszecki (1993) they amplified their suggestion that Z protected lipids from oxidation, saying that Z acted like cholesterol by decreasing membrane fluidity. Havaux & Gruszecki’s (1993) experiments involved the reoxidation of plastoquinone and PS II thermal stability at different Z levels in potato thylakoids. The ability of Z to reduce membrane fluidity in vivo hinges upon its being free in the membrane. If this is so, a membrane containing free Z may also be less permeable to small molecules such as oxygen, further protecting the photosynthetic apparatus and membrane.

Most recently Quiñones & Zeiger (1994) manipulated the Z content in corn coleoptiles by various means (red light, red light followed by darkness, or incubation with DTT). They found that the Z content of the coleoptile was proportional to the degree of the coleoptile’s bending in response to blue light. They then presented a good case for Z having the attributes of an ideal blue light photoreceptor: “i) Z is present in low concentration, ii) it is the photoproduct of a cyclic reaction that regenerates its precursor in the dark, and iii) its concentration in red light-treated tissue increases in parallel with blue light sensitivity.”

It is still too early to tell if Quiñones & Zeiger’s (1994) paper will forge a new trail of xanthophyll cycle research as Demmig et al.’s (1987) clearly did. There now exists a large body of evidence documenting the involvement of the xanthophyll cycle in
dissipating excess light energy. However, the mechanism of Z's involvement in the dissipation is much debated.

After their landmark 1987 paper, Demmig et al. (1988) and Demmig-Adams et al. (1989) demonstrated that Z concentrations in intact leaves were linearly related to the amount of radiationless dissipation of heat (seemingly the safest way for a plant to rid itself of excess light energy). They suggested zeaxanthin created "quenching centers" which biophysically favored interaction with antenna chlorophyll so that the light energy would not be transferred to the reaction centers.

Demmig-Adams et al. (1990) related Z formation to high-energy state (ΔpH-dependent) fluorescence quenching (quenching which relaxed within a few minutes upon darkening of the sample). High-energy state quenching was the largest component of all non-photochemical fluorescence quenching (NPQ, which has been defined as light energy used in a way other than for photosynthetic carbon reduction). Since a high energy state was required for this "zeaxanthin quenching", they suggested that the thylakoid membrane underwent a conformational change so that zeaxanthin contacted excited chlorophyll, causing a non-radiative decay of the chlorophyll's (excess) energy.

Gilmore & Yamamoto (1991) isolated Z formation from other effects of light (but not those effected by DTT) by using a single photon flux density (PFD) for their experiments and different quantities of DTT to vary the concentration of Z. A linear relationship was maintained between Z concentration and the amount of NPQ. This
relationship in osmotically shocked chloroplasts existed irrespective of whether conditions mandated cyclic or linear electron flow. Gilmore & Yamamoto (1992) then showed that this NPQ dependence on Z could be mediated by ATP (its hydrolysis forms a ΔpH). By ATP inducing NPQ in the same manner as light, they clearly showed that quenching occurred apart from the formation of any light-induced carotenoid triplets. Consequently, Z’s involvement in increasing NPQ is definitely separate from protection against toxic photoactive products.

Gilmore & Yamamoto (1993) proposed that "all high-energy dependent quenching was xanthophyll dependent" since they found that antheraxanthin (A) contributed to quenching as effectively as Z. Adding the concentration of A to a model predicting quenching, accounted for variance previously unexplained using the concentration of Z alone. The model suggested that both "de-epoxidized xanthophylls, along with some ΔpH-dependent conformational changes result in static quenching in the pigment bed [as opposed to the reaction center of PS II]." Subsequently Gilmore et al. (1994) have shown that epoxidation (Z to A and V) reversed fluorescence quenching in the presence of transthylakoid ΔpH.

Neubauer (1993) found that intact chloroplasts incubated with high concentrations of DTT had only 20% of the maximal NPQ of the control and that Z formation was almost completely inhibited. The fluorescence quenching which was present correlated with the concentration of A. In fact, "... no reversible, ΔpH-dependent quenching can be
detected if A is also absent." This is in agreement with Gilmore & Yamamoto’s 1993 model.

Thayer & Björkman (1990) found that the xanthophyll cycle pool size was roughly four times bigger in 10 species of sun-grown sun plants than in 9 species of shade-grown shade tolerant plants (both on the basis of leaf area or on chlorophyll content). Other xanthophyll pigments, namely lutein and neoxanthin, also varied but without any consistent pattern of difference. Demmig-Adams & Adams (1992a) compared the carotenoid composition of nine annual crop plants, several species of perennial shrubs and vines, as well as deep-shade leaves from seven rainforest species. They found that all of the sun leaves contained considerably more \( V + A + Z \) than did shade leaves. This difference held whether sun and shade species were compared or if the comparison were between sun and shade leaves of the same species as Thayer & Björkman (1990).

Demmig-Adams & Adams (1992a) likewise did not find any consistent difference in lutein or neoxanthin concentrations between samples. Additionally, Demmig-Adams & Adams (1992a) found that those species with lower photosynthetic rates de-epoxidized a greater portion of their xanthophyll pool at noon in the full sun. Furthermore, Adams et al. (1992) found the time that maximum levels of \( Z \) were reached was different for leaves with different orientations, however, these levels all coincided with maximum incident light on the individual leaf. Finally, Johnson et al. (1993) correlated the relative shade tolerance of 19 British plant species with the total xanthophyll cycle carotenoid content. Essentially
they looked at the same relationship from a different angle. They found the greater the shade tolerance, the smaller the xanthophyll pool.

Demmig-Adams & Adams (1993) found a link between the xanthophyll cycle and protein turnover in the high-light tolerance of sun leaves. Either could be inhibited (with DTT or chloramphenicol respectively) without irreparable damage occurring, however, when both were interfered with, a "strong and irreversible depression of photochemical efficiency" occurred. This result was in apparent disagreement with Greer et al. (1991) who found photoinhibitory damage from chloramphenicol alone. However, the level of damage observed by Greer et al. (1991) was on par with what Demmig-Adams & Adams (1993) defined as a level not inhibiting the recovery from high-light induced decreases in photochemical efficiency.

Another link between the xanthophyll cycle and protein turnover has been found in various atrazine-resistant mutants of Conyza canadensis (L.) Cronq. (horseweed). These plants have been shown to have greater susceptibility to photodamage, a higher turnover of the D1 protein of PS II, and by an altered xanthophyll cycle pigment composition (Váradi et al. 1994). Consequently it has been speculated that the mutation leading to atrazine-resistance either has pleiotropic effects or that the altered D1 protein results in a delayed pH decrease which changes xanthophyll composition and effectiveness (Váradi et al. 1994).
The importance of the xanthophyll cycle perhaps may be seen in a yellow-green mutant of wheat (*Triticum durum* L. cv. Cappelli). Antonielli *et al.* (1990) found the mutant contained less of all the photosynthetic pigments while having a larger xanthophyll pool than wild type. In high light, a greater proportion of their xanthophyll pool was de-epoxidized than in the wild type. With these compensations, the mutant produced a normal yield in the field.

Tuvenson & Sandmann (1993) used a transformed *Escherichia coli* able to synthesize the carotenoids phytoene, β-carotene, β-cryptoxanthin, zeaxanthin, and the corresponding glycosides to test transformant survival in the presence of α-terthienyl plus near-UV light (300-425 nm). α-terthienyl targets the membrane and has as its chief damaging product singlet oxygen (\( ^1\text{O}_2 \)). (The DNA used to transform the *E. coli* came from a 15.6 kb fragment from the bacterium *Erwinia herbicola*.) Control *E. coli* and those transformed bacteria only able to carry out carotenoid biosynthesis through to lycopene (a desaturated but acyclic carotene) died, whereas *E. coli* able to perform the entire carotenoid pathway from *E. herbicola* survived. *E. herbicola* colonizes plant surfaces and although non-photosynthetic, To *et al.* (1994) suggests its ability to synthesize these carotenoids are essential to its survival in the midst of phototoxic agents produced in its occasional high-light natural environment. Tuvenson & Sandmann (1993) concluded that the membrane must be an important target for lethal photoreactivity and
that the protection afforded by the carotenoids could either be from their reacting with a triplet photosensitizer or $^{1}$O$_2$.

Peter Horton has considered the xanthophyll cycle as an amplifier of the true plant defense mechanism (and one working in a structural capacity) rather than as the key regulated plant photoprotector against excess energy (a contention also supported in Briantais 1994). Horton & Ruben (1992) studied the closely related kinetics of fluorescence quenching and light-scattering changes (the latter thought to be indicative of conformational changes in the membrane). From these studies (also Ruban & Horton 1994) they proposed an organisational change in the thylakoid, such as aggregation of LHC’s, was responsible for NPQ. Horton et al. (1994) presented a model where a light-induced protonation in LHC (or in "another localized domain") and Z formation were the processes which formed a quencher of excess energy. They understand the importance of the xanthophyll cycle being that the concentration of Z in the membrane might influence LHC protonation. Horton & Ruben (1992) did not rule out the fact that Z might participate in a quenching pigment species with chlorophyll somehow related to xanthophyll agglomerization (which would be in addition to LHC clumping), but they do have a bias against parsimony of theory (and perspicuity of expression!): "...just because carotenoids are colored molecules it does not mean that they necessarily have a direct role photophysically." (Horton et al. 1994)
Ruban et al.'s (1993) study on Z aggregation (in vitro) found its aggregation gave a
small rise in absorption in the 520-540 nm region, which was the absorption range
correlated with energy dissipation. However, no evidence was presented that this
absorption change upon Z aggregation was the same signal as measured in chloroplasts.

Ruban et al. (1993) has reasoned that Z, since it had nearly planar end-groups, could
aggregate more readily than A or V which have end-groups perpendicular to the rest of
the molecule. In fact, Pfundel & Bilger (1994) noted from a large number of studies the
absence of a large absorbance shift in difference spectras of in vivo quenching, such
should be typical if there were such aggregation. Horton's group may not consider the
xanthophyll cycle central to protection against excess light energy, but even they state, "...
there is abundant evidence that [the xanthophyll cycle] is involved in the photoprotective
dissipation of excitation energy in the thylakoid membrane" (Johnson et al. 1993).

On the opposite end of an "importance spectrum" regarding the xanthophyll
cycle's role in photoprotection is Adams & Demmig-Adams (1995). They measured
fluorescence parameters in periwinkle (Vinca minor) and another broad-leaved evergreen
(Euonymus kiautschovicus) during the winter. They observed energy dissipation
developed slower at lower temperatures but also that it reached a greater steady state.
Mornings following a cold day and night saw the leaves exhibiting a sustained depression
of photosynthetic efficiency and retaining high concentrations of Z + A which were
formed the previous day. They again associated the xanthophyll cycle with
photoprotective thermal energy dissipation, and implicated VDE as the control point for such when "light becomes excessive".

Levy et al. (1993) proposed that Cbr (a carotene biosynthesis related protein) binds Z to form photoprotective complexes within the LHC. Long et al. (1994) reviewed possible mechanisms of avoiding PSII damage and stated that ΔpH and the xanthophyll cycle are assumed to act synergistically in some way. They concluded that the xanthophyll cycle is "a, or the, major initial mechanism... of defense against PS II overexcitation...." (Long et al. 1994).

As a summary for this section I turn to Demmig-Adams & Adams (1993) compilation of the various possible mechanisms for Z-associated energy dissipation:

1) Z interaction by direct energy transfer with triplet excited chlorophyll $^3\text{Chl}^*$

2) Z interaction by charge transfer with singlet excited chlorophyll

3) A structural change in the thylakoid allowing some unidentified dissipation process to proceed

4) Z de-excitation of singlet oxygen

Cases can and have been made for all of the above. Before a consensus mechanism can be agreed upon, more information needs to be obtained, specifically, the location and structure of the xanthophylls in the LHC complex, especially during the occurrence of NPQ.
D. Carotenoid Protein Purification

Scientists have long endeavored to purify proteins associated with carotenoids. The first reported success from green tissue was by Nishimura and Takamatsu (1957), who isolated a protein associated with β-carotene. Schimmer & Krinsky (1969) solubilized the diadinoxanthin de-epoxidase from *Euglena gracilis*. A protein that principally bound V (65-80% of the pigment was V, the remainder being neoxanthin) was isolated by Powls & Britton (1976) from the green alga *Scenedesmus obliquus*. The apoprotein stabilized when complexed with the pigment. The authors speculated that the protein-pigment complex might be (in a higher plant analog) the natural substrate for VDE. The amount of V complexed by this protein was, however, <1% of the alga’s total. The protein was purified by anion exchange and size exclusion chromatography until it “was shown to be homogenous by PAGE after staining with napthalene black”.

Holt & Krogmann (1981) isolated some carotenoid-containing proteins (16 kD) from 3 genera of cyanobacteria. The easily solubilized pigment-proteins had an association which was relatively stable, although a second round of gel filtration separated the pigment from protein. Masamoto et al. (1987) also used cyanobacteria to isolate a “highly enriched” fraction containing a carotenoid and a 42 kD protein. They salt-washed thylakoid membranes that had been solubilized using n-octyl-β-D-glucopyranoside (OG) and dodecyl-maltoside, and then fractionated the sample on a sucrose gradient. In order
to further remove other materials, the sample subsequently passed through an anion-exchange and affinity column both in the void volumes.

Dogbo et al. (1988) purified and characterized phytoene synthase. Armstrong et al. (1989) reported the first DNA sequence of a carotenoid gene from any organism. The genes first isolated coded for proteins involved in the very early steps of carotenoid biogenesis from *Rhodobacter capsulatus*, a purple nonsulfur photosynthetic bacteria. Henrysson et al. (1989) isolated and characterized the minor chlorophyll a/b binding apoprotein, which also binds 0.5-1 V per protein (CP 29). Then Armstrong et al. (1990) isolated some further carotenoid biosynthetic genes both from *Rhodobacter* and *Erwinia herbicola*, and found evidence they produced structurally similar enzymes (41.7% amino acid identity with additional conservative amino acid substitutions in the phytoene dehydrogenases).

Cervantes-Cervantes et al. (1990) identified a 58 kD membrane protein associated with the accumulation of carotenoids in ripening sweet pepper (*Capsicum anuum*). Milicua et al. (1991) claimed to have isolated to "electrophoretic homogeneity" (they did not document this) a 290 kD carotenoprotein from carrot roots. Homogenized roots were stirred for a day with 1% Triton X-100 before the sample was precipitated with ammonium sulfate, dialyzed, concentrated, and chromatographed using anion exchange (DEAE-cellulose) and size exclusion (Sephadex G100). The yellow protein contained mostly phytoene (77.8%). All of the carotenoids present with the purified protein were in
different proportions from the root as a whole, indicating a specificity in carotenoid association. Marwell et al. (1992) isolated an envelope membrane particle from pea (*Pisum sativum* L.) using 0.5% Deriphat 160 which reportedly maintained the carotenoid-protein association. Yet they shied away from concluding that the carotenoids in the envelope membranes occur as pigment-protein complexes. The isolated particle was composed of V and at least one other carotenoid, and five different-sized polypeptides, none of which reacted to Ab’s generated to other known chloroplastic proteins.

Engle et al. (1991) purified and characterized from *Prochlorothrix* (a photosynthetic prokaryote) a Z-binding protein complex, which also contained lipopolysaccharide. Both Z and its associated apoproteins (56 + 58 kD) accumulated to a greater extent in the organism (2.3 fold more) under high-light conditions. The protein complex was both water-soluble yet membrane-associated as immunochemistry revealed only a labeled cell surface. This result suggests that the Z-binding complex in *Prochlorothrix* has a photoprotective role as the cell membrane is not involved in photosynthesis. On the other hand, Katoh et al. (1993) also isolated a supramolecular assembly of xanthophylls and proteins coming from the brown algae *Petalonia* which were thought to be involved in photosynthetic light harvesting. These assemblies they termed xanthosomes, and were isolated from thylakoids using 0.8% decanoyl sucrose and n-decyl-β-D-glucopyranoside. The complex consisted of a single species of polypeptide (19.6 kD) associated with three molecules of fucoxanthin, one chlorophyll c and four
molecules of chlorophyll a. Electron microscopy and western blots both evidence xanthosomes in association with the photosynthetic reaction center P700 and were concluded to function in a photosynthetic role.

Pecker et al. (1992) cloned and sequenced a phytoene desaturase (PDS) from tomato. Transformed *E. coli* expressed active PDS. The tomato protein (transcripts of which increased 10 fold from mature green to orange fruit) was found to be highly homologous to the PDS of cyanobacteria and algae but not to the PDS from purple bacteria or fungi. Indeed, it was a fragment of the green algae *Dunaliella* PDS gene which identified the tomato gene from a cDNA library.

Hundle et al. (1992) were also able to express a functional carotenoid biosynthetic enzyme, zeaxanthin glucosyltransferase, from *Erwinia herbicola* in transformed *E. coli*. The addition of a glucose moiety to Z increases its solubility in water from 12.6 to 100 ppm; the addition of a second adds another 700 ppm to Z's solubility. Recently, To et al. (1994) sequenced the 7 kb fragment from *E. herbicola* which contained at least 5 genes yielding products responsible for converting farnesyl pyrophosphate to β-carotene and then Z to Z-β-diglucoside.

Fraser et al. (1993) recovered active recombinant phytoene desaturase (PDS) of *Synechococcus* after purifying the *E. coli* protein and then reactivating the enzyme. Reactivation was accomplished by removing the urea from the sample buffer using a desalting column and then allowing the protein to refold in the presence of DTT. The
success rate of achieving functional carotenoid biosynthetic enzymes from the cloned
genes has been rather phenomenal compared to that of other enzymes.

E. Prospectus for Carotenoid Protein Isolation

In the section above, I reviewed the isolation of a number of carotenoid-associated
proteins (to varying levels of purity) from higher plants using protein purification
approaches as well as by fishing in the higher plant gene pool using cyanobacterial and
algal homologs. Both will continue to be an essential approaches in the study of
carotenoid proteins. Additional successes in isolating the homologous higher plant genes
may well come as there are further advances in isolating the carotenoid biosynthesis genes
from cyanobacteria and algae. The limitation, however, to this second approach may be
the divergence of the later steps in carotenoid biosynthesis in higher plants. On the other
hand the protein purification approach faces its own problems in that the post-phytoene
biosynthetic enzymes are thought to form membrane-bound multienzyme complexes.

Commenting on the difficulty of purifying any of these Hundle et al. (1992) noted "No
homogenous protein fraction catalyzing individual post phytoene reactions have been
isolated thus far." VDE is a post-phytoene carotenoid biosynthetic enzyme, though there is
no evidence one way or the other that it exists in a multienzyme complex and VDE is not
necessarily membrane-bound (see below).

Chapman et al. (1991) reviewed strategies for the isolation of thylakoid membrane
proteins. They repeated a commonly stated observation that membrane proteins were
more difficult to purify than essentially hydrophilic ones. They concluded that compromise
with regards to the purification may be best at times since "It might not be possible to
achieve full success in purification as well as high yield, retention of structure and
preservation of function." Further, and somewhat against intuition, they noted that
hydrophobic columns have few applications for separating intrinsic membrane proteins.
Perhaps the differences in hydrophobicity are diminished in the presence of the
detergents (or organics) necessary to initially solubilize the proteins.

Weselake and Jain (1992) discussed immunoaffinity chromatography, subunit
affinity chromatography, immobilized metal ion affinity chromatography, affinity labeling
and the use of detergents for purifying plant proteins. These topics were taken up as the
latest protein purification strategies, though they saw the future of plant protein study
being bright in the use of Arabidopsis mutants where the gene for a given protein would
be identified first. Yet they also shared their motivation for working in protein purification:
the satisfaction coming from developing a new step which results in a homogenous
protein preparation.

The use of detergents mentioned up to this point has been for their ability to
gently solubilize membranes or for their use with sequestered proteins to maintain their
structure and function. However, Wolbert et al. (1989) used detergents for protein
purification in a different way: forming reversed micelles with them in an organic solvent
and then selectively transferring proteins to their inner hydrophilic core. Wolbert et al.
(1989) utilized trioctylmethylammonium chloride (TOMAC) and nonylphenol pentaethoxylate (Rewopal) as the surfactants, octanol as the cosurfactant, and isooctane as the continuous phase. They then studied the uptake profiles of 19 different proteins and found partitioning based on the protein’s size, isoelectric point, and distribution of surface charges. Most proteins retained their native conformation and were in an active form upon transfer back to a physiological buffer.

Both Dekker (1993) and Dwyer (1993) have reviewed the use of reversed micelles as a means of purifying proteins. Proteins can be differentially extracted from an aqueous phase to the reversed micelle and then subsequently recovered from the organic phase by a reverse transfer to a second aqueous phase. The important variables in effecting transfer were found to be pH, temperature, ionic strength, and surfactant type.

F. VDE Isolation

Hager (1969) and Hager & Perz (1970) were the first to partially purify VDE. Chloroplasts were isolated from spinach (Spinacea oleracea L.), suspended in pH 6.8 buffer and sonicated. The centrifuged extract passed through a DEAE column using a high molarity of sodium phosphate buffer (240 mM) and was precipitated using ammonium sulfate. This precipitate was resuspended in order to apply the sample to a 65 cm size-exclusion column (Sephadex G100).

Yamamoto & Higashi (1978) optimized the above protocol for lettuce (Lactuca sativa v. Romaine) and added another sonication step. Preceding the extraction of
thylakoids by sonication at pH 7.2, a pH 5.2 sonication was performed and the resulting supernatent from centrifugation discarded. As in the isolation procedure from spinach above, the neutral sonication extract was applied to an anion exchange column (DEAE A25). Likewise, the flow-through was collected and concentrated before size exclusion chromatography was carried out (2.5 x 80 cm Sephadex G100). The fractions containing VDE were then located using an activity assay.

Recently, Hager & Holocher (1994) published another effort to isolate VDE. Repeated freeze-thaw treatments of isolated thylakoid membranes (up to seven cycles of liquid nitrogen and a 29° C water bath) released active enzyme to the media. The amount of VDE released declined if the buffer was below pH 6.5 and VDE was not released at all from the thylakoids at pH 5.2. Prior illumination of the leaves also reduced the amount of VDE recovered by the freeze-thaw treatments.

Hager (1975) estimated the mass of VDE to be 54 kD. Yamamoto & Higashi (1978) used the elution of reference proteins to calibrate a size-exclusion Sephadex G200 column and estimated VDE’s molecular weight to be 60 kD. Hager & Holocher (1994) correlated VDE activity with the appearance (using their freeze-thaw technique) of a 58 kD band on SDS-PAGE.

Yamamoto et al. (1971, 1972) found that the enzymatic action of VDE in chloroplasts, induced by ascorbate, led to large changes in a difference spectra at 505 nm. They looked for such a change since the product of VDE absorbed light at longer
wavelengths than the reactant, and hoped to see an absorbance shift as the reaction proceeded. Following the kinetics of this absorbance change has become a standard means of determining VDE enzymatic activity in vitro (Yamamoto 1985). Yamamoto et al. (1972) unsuccessfully attempted to reverse de-epoxidation through the removal of the reactant ascorbate or by adding chloroplast extract. Hager (1980) stated that VDE cannot catalyze the reverse epoxidation of Z "under any circumstances".

Yamamoto and Kamite (1972) concluded VDE had a disulphide bond essential for activity. They reacted isolated chloroplasts with dithiothreitol (DTT) and the absorbance increase at 505 nm indicative of VDE activity was inhibited. Since DTT did not affect NADP photoreduction, the inhibition of VDE was deemed specific. Besides a disulphide bond, other components found necessary for in vitro VDE activity (apart from thylakoids) were ascorbate and monogalactosyldiglyceride (MGDG).

Hager (1969) found that in chloroplasts VDE could be triggered with ATP. He also looked at the pH optimum for VDE in isolated chloroplasts and found it to be <5. However, in adding a solution of his partially purified VDE (and additional V) to the chloroplast, the pH optimum shifted to 5.3. He speculated (from VDE’s pH optimum) that the stimulatory effect of light on VDE lay in its induction of a transthylakoid ΔpH which lowered the pH in the vicinity of VDE and consequently activated the enzyme.

Pfundel & Dilley (1993) also examined the effect of pH on VDE in isolated chloroplasts. In pea chloroplasts, the rate constant of Z formation significantly altered
between pH 5.8 and 6.3. They reasoned that such a pH effect demonstrated regulation of Z formation. However, in contrast to Hager (1969), they could not acquire VDE activity data below pH 5.2 because the membranes precipitated. Moreover, VDE activity was likewise undetectable above pH 6.3. Long-term kinetics experiments (65') determined VDE activity to have two components (Pfündel & Dilley 1993). VDE's biphasic kinetics were explained by assuming V existed in separate pools, the rapidly convertable pool of V being about twice as large as the slower converting one. Both kinetic components were pH-dependent, but Pfündel & Dilley (1993) could not determine whether the rate limiting step of the reaction belonged to the catalytic properties of VDE or to a pH-dependent V diffusion process.

Sapozhnikov (1973) found the $T_{\text{max}}$ of VDE to be 42°C in the presence of a threshold amount of light. He speculated that this result was really due to an inactivation of the reverse epoxidation reaction at this temperature. Sapozhnikov (1973) also measured the VDE in tropical plants as having higher temperature optima than their temperate counterparts. Bilger & Björkmann (1991) measured the dependence of V de-epoxidation rate in cotton (chilling-sensitive Gossypium hirsutum L.) and Malva parviflora L. (chilling-resistant) leaves at lower temperatures. They found the $Q_{10}$ at 15°C to range between 2.2-2.6 for various Malva leaves, whereas it was as high as 4.6 in cotton leaves. They also found that Malva leaves grown in early spring had 4x the VDE rates at 15°C and 2x the rate at 30°C as leaves grown in summer at those same temperatures.
Furthermore, *Malva* spring leaves had 30-70% faster VDE activity than cotton grown at a constant 30°C. Thus, leaves resistant to photodamage had a more robust VDE response in conditions that are more vulnerable because of the threat of chilling.

Sapozhnikov (1973) did not find VDE activity in etiolated corn seedlings until there had been 1-2 hours of greening. However, Pfündel & Strasser (1988) found VDE activity in etiolated leaves of bean (*Phaseolus vulgaris* L. var Commodore). Etiolated leaves vacuum-infiltrated with pH 5 buffer and ascorbate occaissioned Z formation whereas leaves infiltrated with a pH 7 buffer or with pH 5 buffer, ascorbate and DTT exhibited no change in Z composition. They measured both spectral changes at 505 nm and compositional changes of pigments on TLC. However, a detectable difference between samples required a few hours of dark incubation. Nevertheless, the results seemed to indicate that assembled chlorophyll-pigment proteins were not necessary for VDE activity.

Hager (1975) reported the $K_m$ of VDE for V to be 10.6 $\mu$M; Yamamoto & Higashi (1978) reported it to be 0.049 $\mu$M when maintaining an optimum MGDG:V ratio (28:1). They also estimated a molar ratio of MGDG to VDE of 6:1 in the native state. Hager (1980) reported his student Perz’s (1970) dissertation results that the $K_m$ of VDE for antheraxanthin (A) to be 10.9 $\mu$M and for diadinoxanthin 160 $\mu$M. He also found the cis-stereoisomers of V and neoxanthin as well as synthetic epoxides of various carotenes were unreactive with VDE (also Yamamoto & Higashi 1978). Yamamoto & Higashi (1978) measured the relative reactivity of VDE on A to be 5.25 x that of V, for diadinoxanthin.
2.25 x greater than for V, for lutein epoxide 1.33 x, for β-cryptoxanthin epoxide 0.92 x, but for neoxanthin 0.10 x. Neubauer & Yamamoto (1994) reported VDE to have a $K_m$ for ascorbate of 3.1 mM.

Hager (1980) surveyed the plant kingdom for evidence of the xanthophyll cycle (and consequently of VDE or a homolog's activity). He found its occurrence widespread and concluded: "One can see that the xanthophyll cycle has been never forgotten during the phylogenetic development of the plants and this indicates that it probably has an important function." The essence and mechanism of that function are still in the process of being clarified, as they relate to the dissipation of excess light energy. At present, many researchers are investigating the xanthophyll cycle and its effects using fluorescence and spectrophotometric means. It is hoped that this further purification and characterization of VDE itself will greatly further understanding of the xanthophyll cycle.

G. Hypotheses

Based on a review of the literature and preliminary experiments, I made the following hypotheses:

1. VDE may be further isolated by a standard application of protein purification techniques developed in the years since a partial purification was accomplished by Hager & Perz (1970) and Yamamoto & Higashi (1978).
2. VDE enzymatic activity is specifically associated with a polypeptide of approximately 43 kD molecular mass.

3. The properties of VDE will be measurably different from previously determined ones (eg. $K_m$ of substrates and effects of inhibitors) ascertained from chloroplast preparations or partially purified VDE preparations.
CHAPTER II

PURIFICATION AND PARTIAL PEPTIDE SEQUENCES

OF VIOLAXANTHIN DE-EPoxidase

A. Introduction

Violaxanthin de-epoxidase (VDE), an enzyme of the xanthophyll cycle localized in
the chloroplast lumen, catalyzes the conversion of violaxanthin (V) to antheraxanthin (A)
and zeaxanthin (Z) in the presence of an acidic lumen and ascorbate. In vivo, de-
epoxidation is induced when a high transthylakoid ΔpH develops in plants exposed to
light intensities exceeding their photosynthetic capacity. The accumulation of A and Z,
along with the transthylakoid ΔpH, mediates non-radiative dissipation of light energy
(NRD) in the antennae (reviewed by Björkman & Demmig-Adams 1993). This NRD is an
alternative energy pathway that diverts energy from PS II, effectively down-regulating PS
II's efficiency. Although a large body of evidence has accumulated documenting the
relationship between the presence of the de-epoxidized xanthophyll-cycle pigments (A and
Z) and a plant's ability to dissipate excess light energy as heat, critical details about the
biochemistry, physiology, and the quenching mechanism are unclear (Horton et al. 1994,
Pfündel & Bilger 1994, Yamamoto & Bassi 1995). Consequently the hope is that the
purification and characterization of VDE will greatly further understanding of the xanthophyll cycle.

Previously VDE was partially purified from spinach (Hager & Perz 1970) and lettuce (Yamamoto & Higashi 1978). Although far from pure, these preparations allowed the characterization of several properties of the de-epoxidase. Using such a VDE preparation it was found that purified V alone was a poor substrate for VDE, however, maximal VDE activity was recorded when V was resuspended with monogalactosyldiacylglyceride (MGDG), the major lipid of chloroplast thylakoids (Yamamoto et al. 1974). Moreover, VDE itself also appeared to require MGDG for activity (Yamamoto & Higashi 1978). The de-epoxidase was stereospecific against 3-hydroxy-5,6-epoxy carotenoids that were in a 3S, 5R, 6S configuration and where the polyene chain was all trans (Yamamoto & Higashi 1978). The partially purified de-epoxidase was estimated to be 54 kD (Hager & Perz 1970) or 60 kD (Yamamoto & Higashi 1978) by non-denaturing gel electrophoresis or size exclusion chromatography respectively.

This chapter documents the purification of VDE to a single major component of 43 kD, as detected by two-dimensional IEF/SDS-PAGE. The critical final step in the purification depends on the apparent specific association of VDE with MGDG into a precipitable complex. Because of this specificity, this new method was termed "lipid affinity precipitation." I also report some partial amino acid sequences, first of the N-terminus as well as of several sequences from peptides obtained by tryptic digestion.
B. Materials & Methods

1. VDE activity assay

VDE enzymatic activity was determined following Yamamoto (1985). I found the most consistent activities were obtained by assaying 50 μl or less of protein samples. The absorbance at 505 nm relative to 540 nm ($\Delta A_{505-540}$) was monitored over time using a SLM dual-wavelength spectrophotometer (DW 2000). Enzymatic activity was determined from the initial angle of the $A_{505-540}$ absorption change and using the difference extinction coefficient for Z and V of 63 mM$^{-1}$cm$^{-1}$. From a printed graph (17.9 x 24.9 cm) and a scale 5 minutes vs. 0.023 absorbance units, the initial angle ($\theta$) was converted to units of enzymatic activity per ml solution by the formula

$$\left(\tan \theta \times 6.4207 \times 10^{-3} \text{ A min}^{-1}\right) \div 6.3 \times 10^{2} \text{ μM}^{-1} \text{cm}^{-1} \times 1 \text{ cm} \div (X \text{ ml}).$$

Where X was the volume of the VDE sample assayed. A unit of VDE activity has been defined as 1 μmol violaxanthin de-epoxidized per minute (Yamamoto 1985).

2. VDE purification

The initial steps of VDE purification essentially followed Yamamoto & Higashi (1978), through to size-exclusion chromatography on Sephadex G100. To begin with, typically seven or more heads of market or freshly harvested field-grown *Lactuca sativa* var. Romaine were trimmed and deribbed yielding between 700 and 1000 g of leaf parts. All steps were done in a 2°C room or on ice. The Sephadex column was equilibrated and run with 20 mM Tris-HCl + 100 mM sodium chloride, pH 7.20. Optimum separations
occurred when the flow rate was 0.45 ml/min or slower. Fractions containing VDE were identified by VDE activity assay. VDE eluted from the Sephadex column approximately 185 ml after sample application. Fractions containing more than 0.3 U/ml VDE enzymatic activity were pooled for anion-exchange chromatography on a Mono Q column with the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia).

The buffers used in the FPLC system were filtered through a 0.22 µm membrane and extensively deaerated. The Mono Q column was cleaned according to the manufacturer’s protocol after every 3rd sample run in order to maintain precision in the chromatography separations. Before the application of a 2 ml sample to the Mono Q column, Sephadex fractions were concentrated using a membrane filter with a 30,000 NMW cutoff (Centriprep 30 - Amicon) and then the insoluble material removed by microfuging 10’. However, obtaining a superloop for the FPLC (which allowed repeated loadings of 10 ml samples) later in this work made feasible the direct application of Sephadex fractions to the column, thereby bypassing this concentration step. The column was loaded at rates up to 1.5 ml/min, but then the separation would be carried out at a flow rate of 0.4 ml/min. To remove more weakly bound proteins, the column was run for 15’ with 20 mM Tris-HCl, pH 7.20 + 150 mM NaCl. This was followed by a ramp between 180 and 190 mM NaCl (in 20 mM Tris-HCl, pH 7.20) over the course of 30’ during which time VDE eluted. Three minute fractions were collected and VDE was again located by activity.
Lipid-affinity precipitation was carried out on individual Mono Q fractions that contained significant VDE activity. The Mono Q fraction (1.2 ml) was mixed with 10 μl of 3.45 mM purified MGDG and 10.8 ml of 0.2 M sodium citrate, pH 5.20 in an ultracentrifuge tube (Seton #5030). These mixtures were incubated at 2°C for 30', inverting the tubes periodically. Afterwards they were centrifuged at 83,000g for 2.5 hours at 3°C (Beckman L8-70M Ultracentrifuge). The pellets were air-dried then solubilized in 62.5 μl of 0.5% n-octyl-β-D-glucopyranoside (OG). The solubilized pellet was either directly assayed for VDE activity or run on SDS-PAGE as OG did not interfere with either procedure. Other lipids tested for their ability to precipitate VDE were cardiolipin, digalactosyldiacylglycerol (DGDG), phosphatidic acid (PA), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), and phosphatidyl serine (PS).

3. MGDG purification

Chloroplasts were isolated at 2°C from 250 g of pre-chilled and trimmed spinach (Spinacea oleracea L.) leaves by homogenizing in 250 ml of SMN buffer (50 mM MOPS, 400 mM sorbitol, 10 mM NaCl, 16 mM sodium ascorbate at pH 7.20) in a prechilled blender (Waring model 7011G) on "high" for 1 minute. The homogenate was squeezed through a 36 μm nylon mesh cloth and the filtrate was centrifuged at 5700g and 2°C for 20'. The chloroplast pellet was suspended in 125 ml of SMN.
Total chloroplast lipids were extracted according to Allen & Good (1971) and Bligh & Dyer (1959) with slight modifications. MGDG was separated from the total lipid extract following Siefermann-Harms et al. (1987). Under reduced light, the chloroplast suspension was blended in 450 ml of cold chloroform:methanol (1:2) in a Waring blender equipped with an explosion-proof, protected motor. Another 150 ml of cold chloroform was added and blended for 30 seconds before finally blending in 150 ml of 0.73% NaCl. This mixture was filtered through a 1 liter sintered glass funnel, which had been layered with glass wool, and then poured into a 2 liter separatory funnel. The solution sat for 5 minutes before the lower chloroform phase was collected and evaporated in a rotary evaporator (Büchi RE 120) to 20 ml. The concentrate was then loaded onto an acid-washed florisil (17% H$_2$O) column (30 x 2.4 cm) equilibrated with water-saturated chloroform. Most of the chlorophyll and carotenoid pigments eluted between 10 and 15% acetone in chloroform (350 ml total). MGDG eluted from the column at 15% or more acetone in chloroform.

The purified lipid was assessed by TLC as well as by the VDE activity assay. Oven-dried silica TLC plates (Fisher Redi/Plate 06-600A) were developed in 65:25:4 chloroform:methanol:water and visualized under UV light after spraying the plate with a solution of 0.003% Rhodamine 6G in 1 N NaOH. The fraction giving the purest MGDG spot ($R_f \approx 0.75$) on TLC and the highest VDE activity was used for experimental VDE activity determinations and for lipid affinity precipitation.
The MGDG concentration was quantified using a galactose assay. An aliquot of lipid was added to 1 ml of 2% phenol and 4 ml of concentrated $\text{H}_2\text{SO}_4$, vortexed, and allowed to stand 15'. The $A_{480}$ was determined and the concentration of MGDG calculated from a standard line constructed using known amounts of galactose.

4. Violaxanthin purification

Important considerations for the purification of carotenoids are discussed in Davies (1976) and Britton & Young (1993). Chloroplasts were prepared from spinach (Spinacea oleracea L.) as described above. The isolated chloroplasts were extracted twice with 200 ml of methanol; resuspended first with the help of a sterile dacron swab and then centrifuged at 5700g, for 20'. The supernatents were pooled and saponified with 15% w/v KOH with occasional swirling for one hour in the dark at 2°C. The saponified extract was washed into an equal volume of fresh anhydrous ethyl ether before adding two volumes of water. After settling, the lower, aqueous phase was discarded. The ether phase was washed with 250 ml water six times. The carotenoid ether extract was concentrated to approximately 5 ml on a rotary evaporator.

The carotenoids were separated on unactivated silica TLC plates developed with 1:3 acetone:hexane. Typically violaxanthin ($R_F$ of approximately 0.19) comprised the third most intense band, after $\beta$-carotene ($R_F$ of 0.88) and lutein ($R_F = 0.27$). The violaxanthin band was scraped off into acetone. The pigment was extracted by swirling the acetone and decanting it into a centrifuge tube and centrifuging at 1000g for 5' at
room temperature. The supernatent was decanted a second time and recentrifuged. An absorbance spectra was then taken. Only pigment with absorption maxima at 441 and 471 nm in a ratio of less than 1.05 (an indication of violaxanthin purity) was used. A 10 μM violaxanthin solution was made by diluting the solution with MeOH so that the $A_{441}$ value at 1.5 absorption units (zeroed at 550 nm). The violaxanthin remained stable indefinitely at -70°C.

5. Reversed micelle protein purification

There are a number of protocols for protein purification using reversed micelles (Dekker 1993). Two of these were tested on VDE. The first was the procedure of Hilhorst et al. (1983) where 12 ml of 200 mM CTAB in 1:4 octanol:isoctane was added to 0.57 ml of VDE protein sample. This mixture was extensively vortexed and ultracentrifuged (83,000g, 2.5 hours, 3°C) before the pellet was resolubilized in 62.5 μl 0.5% OG.

The second reversed micelle procedure tested was that of Wolbert et al. (1989). Reversed micelles were first formed by mixing together 2 ml isoctane + 8 mM trioctylmethylammonium chloride (TOMAC) + 2 mM Triton X-45 + 0.1% octanol. Two milliliters of a VDE sample at pH 10.0 were then added and gently mixed on ice for 4’. The solution was subsequently centrifuged for 2’ at 750g, 2°C and the aqueous layer removed. The organic phase was extracted with 2 ml of 500 mM potassium phosphate, pH 6.90. After vortexing this mixture, it settled and the aqueous layer was then checked for VDE enzymatic activity as well as concentrated for one-dimensional SDS-PAGE.
Controls for both procedures were carried out on different protein solutions: non-fat dry milk (Carnation), Romaine lettuce chloroplasts, lysozyme (ICN), alcohol dehydrogenase (Nutritional Biochemical), and *Achromobacter lyticus* lysyl endopeptidase (Wako).

6. Sample preparation for SDS-PAGE

Sephadex or Mono Q fractions were concentrated by membrane filtration to the stop volume of the Centricon-30 (Amicon), approximately 35 µl. Concentrated samples were mixed with 23 µl of 2x Laemmli sample buffer (LSB; 0.5 M Tris-HCl pH 6.8 + 4% SDS + 20% glycerol + 10% β-mercaptoethanol) before electrophoresis. For electrophoresis of equal amounts of protein, protein determinations were first done and appropriate sample volumes loaded on the gel.

Protein concentration was determined using a modified Lowry method designed to sample small volumes containing Tris-HCl, sodium citrate, or detergent (Bensadoun & Weinstein 1976, Waterborg & Matthews 1984).

7. SDS-PAGE

Procedures for one- and two-dimensional SDS-PAGE were carried out according to Laemmli (1970) and as modified by Moissyadi & Harrington (1989). Typically, for one-dimensional SDS-PAGE, 15 µl of a concentrated sample was mixed with 10 µl 2X LSB and loaded into a gel well using a 25 µl blunt-ended syringe. Slab minigels were run in minielectrophoresis chambers (self-made).
Tube gels were run in an electrophoresis chamber (Bio-Rad model 150A) loaded with up to 35 μl of sample mixed with 15 μl IEF sample buffer (9.9 M urea + 4% NP-40 + 5% ampholytes + 100 mM DTT). The ampholyte mixture incorporated ampholines from the pH range 4.5 - 6.5 as well as 3.5 - 10 in a 1:1 ratio.

8. SDS-PAGE gel staining and blotting

Gels were stained with Coomassie Blue (0.1% Coomassie Blue R-250, 50% methanol, 10% acetic acid) for at least 20' with gentle shaking on an orbital shaker and destained in 5% methanol + 9% acetic acid for at least 30', also with gentle shaking. Balled-up Kimwipes were used to absorb solubilized stain. Before drying, gels were given a final water rinse of 5' with gentle shaking.

Various silver and copper staining procedures were tried (Merril et al. 1981, Sammons et al. 1981, Lee et al. 1987) before Harrington's (1987) was settled on, which for consistency was the most reliable. Milli Q grade water (Millipore) was essential for clear, colorless backgrounds in silver staining.

Proteins on gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, 0.45 μM, Millipore) according to LeGendre and Matsudaira’s (1989). Complete transfer was obtained at 100 ma (constant current) for 70' for polypeptides below 65 kD using a Mini Protean II (Bio-Rad) blotting unit at 4°C.

Prior to the excision of a polypeptide from the PVDF membrane for the purpose of amino acid analyses, the membrane was briefly stained with Coomassie Blue. After 5'
the membrane was destained with 50% methanol + 10% acetic acid for 30', rinsed with water a couple of times, and air-dried. The VDE band was subsequently cut out and analyzed by the Biotechnology - Molecular Biology Instrumentation Facility (University of Hawaii) for amino acid composition and N-terminus sequence, and the Beckman Center (Stanford University Medical Center) for sequences of the N-terminus and peptides generated by tryptic digestion.

C. Results

1. VDE purification

Previously, VDE was partially purified from *Lactuca sativa* var. Romaine by extracting sonicated thylakoids at different pH's and chromatographing the soluble material at pH 7.20 by size exclusion on an 80 cm Sephadex G100 column (Yamamoto & Higashi 1978). Figure 7 is a two-dimensional IEF/SDS-PAGE of the (concentrated) peak fraction of VDE activity eluting from the Sephadex column following Yamamoto & Higashi's (1978) procedure. According to the $A_{280}$ chromatogram of material eluting from the Sephadex column, a relative minima of material elutes concurrently with VDE activity; nevertheless, as seen by the gel, this peak fraction contains a plethora of polypeptides. Even so, by examining successive column fractions on a 12.5% acrylamide gel, a polypeptide band between 48-52 kD appeared to be associated with VDE activity (figure 8).
Figure 7 - Two-dimensional IEF/SDS-PAGE of a peak VDE activity fraction (1.835 U/ml) separated through to the Sephadex G100 stage of VDE purification. The ampholytes used in isoelectric focusing were an equal mix of two ranges, the pH 3-10 and pH 5-8. The SDS-PAGE gel was composed of 12.5% acrylamide and the gel was stained with Coomassie Blue R-250. The molecular mass of the markers is indicated to the left in kD.
Figure 8 - One-dimensional SDS-PAGE of successive Sephadex G100 fractions. The VDE activity of a given fraction's contents is shown above the respective gel lane. The purported VDE band is marked with an arrow. The 12.5% acrylamide gel was stained Coomassie Blue R-250.
Further purification of VDE was achieved by anion-exchange chromatography (Pharmacia FPLC Mono Q column). Elution of VDE activity roughly corresponded to a peak of $A_{280}$ absorbance on the chromatogram from the Mono Q column (figure 9). When sequential fractions eluting from the Mono Q column were concentrated and separated on a one-dimensional SDS-PAGE (figure 10A), VDE activity corresponded to the appearance of the same band earmarked by Sephadex chromatography. However, subsequent gels made it clear that the estimated molecular weight was nearer to 43 kD, since the band lined up with the 43 kD marker on 10% acrylamide gels, an acrylamide composition yielding optimal resolution for polypeptides in the range 31 to 54 kD. (12% acrylamide maximizes resolution in the 21-36 kD polypeptide range.) The staining intensity of numerous polypeptide bands from the gel shown in figure 10A was assessed using a Bio-Rad Model 620 Video Densitometer. The optical density of the four most prominent bands were plotted against VDE activity. It was clear that a relationship existed between the appearance of the band of approximate molecular mass 43 kD and VDE enzymatic activity. This relationship was statistically significant with an $r^2 = 0.953$ and was the only band for which a significant linear correlation existed, as visual inspection ruled out other polypeptide bands which were not scanned by the densitometer.

Further purification was not achieved by rechromatographing through Mono Q or by further chromatography on hydrophobic interaction columns (Alkyl Superose or Phenyl Superose - Pharmacia), size separation columns (Superose 6 or Sephacryl S200 -
Figure 9 - $A_{280}$ chromatogram of material eluting from the Mono Q anion-exchange column running on a FPLC system (Pharmacia). The x-axis is the fraction number and the NaCl content of the running buffer is shown by (—) in mM x 10 using the left y-axis. The $A_{280}$ is shown by (—) as a percentage of a 0.02 A full scale. The VDE activity of the fractions is superimposed as a bar graph (—) using the right-hand y-axis.
Figure 10A - Successive fractions from Mono Q were concentrated and run on 10% acrylamide one-dimensional SDS-PAGE stained with Coomassie Blue. The staining intensity of a number of bands was determined using a densitometer (Bio-Rad Model 620). The staining of two of these polypeptides in each of the fractions is plotted as a bar graph in figure 10B. A line graph overlay indicates the VDE activity in each of the same fractions.
Figure 10B - OD of SDS-PAGE bands and VDE activity overlay in successive Mono Q fractions.
Pharmacia), or a cation-exchange column (Mono S - Pharmacia). Neither could active
VDE be extracted from non-denaturing acrylamide gels. Standard methods were,
therefore, of no avail in purifying VDE further.

Subsequently a novel lipid affinity precipitation was developed that achieved the
desired purification. Because VDE binds to thylakoids (at pH 5 but not at pH 7), the
question was, would MGDG, the major thylakoid lipid and the one necessary for VDE
activity, bind VDE specifically at pH 5 and form complexes large enough to be
precipitated by ultracentrifugation? A pellet did result when this experiment was done.
The pellet was resolubilized with the non-ionic detergent n-octyl-β-D glucopyranoside (OG)
and found to have high VDE activity.

Figure 11 shows a number of Coomassie-stained two-dimensional IEF/SDS-
PAGE’s of the OG-solubilized lipid affinity pellet from separate VDE purifications. The
gels clearly document the presence of a single polypeptide species. A silver-stained gel of
this same stage (figure 12) showed VDE as a negatively stained spot and other proteins
present in trace quantities.

Figure 13 shows a one-dimensional SDS-PAGE, each lane containing the peak
VDE-activity fraction from the different stages of protein purification. Each lane contained
3.525 μg of total protein as determined beforehand by a modified Lowry assay. Only the
polypeptide in line with the 43 kD marker increased in staining intensity with progressive
VDE purification.
Figure 11 - Two-dimensional IEF/SDS-PAGE of the lipid-affinity pellet resolubilized with 0.5% OG. Gels represent peak VDE activity from separate enzyme isolations purified through the Mono Q stage and subsequently complexed with MGDG and ultracentrifuged. The sample was isoelectrically focused on a tube gel using a 50:50 mixture of amphoines of the 3.5 to 10 pH range and 4.0 to 6.5. The 10% acrylamide slab gel was stained with Coomassie Blue.
Figure 12 - As figure 11, however, the slab gel was stained with silver nitrate.
Figure 13 - Different stages of VDE purification run on 10% acrylamide one-dimensional SDS-PAGE and stained with Coomassie Blue. Each sample lane contained 3.525 μg of total protein.
Table 1 is the purification table for a typical VDE purification. The specific activity of lipid affinity purified VDE was more than 44,000 times greater than in chloroplasts and 17.1 times more than size-exclusion chromatography. In the end, 2 μg of VDE was purified after starting with 5 kilograms of lettuce.

Purified VDE was blotted onto PVDF membrane for both N-terminal and amino acid sequence determination. Table 2 shows the partial amino acid sequences for the N-terminus of VDE and various tryptic fragments, numbered by their elution from capillary electrophoresis chromatography and not necessarily in their order of appearance in the protein. The N-terminus sequence agreed with the sequence determined for the polypeptide band which correlated with VDE enzymatic activity eluting from the Mono Q column. Table 3 shows the amino acid composition of a VDE blot in mole percentages. The two acidic residues comprise the two highest percentages of the determined amino acid residues. VDE can also be seen to contain a rather large portion of nonpolar residues.

2. Characteristics of the lipid affinity precipitation

Purified MGDG in buffer at pH 5.20 selectively associated with VDE so that the complex pelleted when ultracentrifuged (83,000g, 2.5 hours, 3°C). This precipitation was pH-dependent; at pH's 7.20 and 10.0 no VDE precipitated in the presence of MGDG. The extent of VDE precipitation was dictated by the amount of MGDG present. A control lacking MGDG at pH 5.20 did not precipitate any VDE when ultracentrifuged. Thirty five
Table 1 - Purification table of VDE. Stages are as defined in materials & methods. A unit (U) of VDE activity is defined as 1 µmol violaxanthin de-epoxidized per minute (Yamamoto 1985).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total Protein (µg)</th>
<th>Total Activity (U)</th>
<th>VDE Specific Activity (U/mg)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romaine Lettuce Chloroplast Preparation</td>
<td>6.75 x 10^6</td>
<td>626.4</td>
<td>2.075 x 10^{-2}</td>
<td>1</td>
</tr>
<tr>
<td>Soluble Lumenal Proteins @ pH 7.20</td>
<td>1.20 x 10^4</td>
<td>128.1</td>
<td>11.691</td>
<td>564</td>
</tr>
<tr>
<td>Size Exclusion Chromatography (Sephadex)</td>
<td>1.71 x 10^3</td>
<td>91.2</td>
<td>53.43</td>
<td>2575</td>
</tr>
<tr>
<td>Anion Exchange Chromatography (Mono Q)</td>
<td>14.6</td>
<td>50.3</td>
<td>760.209</td>
<td>36, 644</td>
</tr>
<tr>
<td>Lipid Affinity Pellet</td>
<td>2.02</td>
<td>5.5</td>
<td>915.371</td>
<td>44, 123</td>
</tr>
</tbody>
</table>
Table 2 - Partial amino acid sequences for VDE and the picomole yields on which the determination is based. The tryptic fragments were numbered on the basis of their elution from capillary electrophoresis chromatography and not necessarily on their order in VDE. Determinations made from an immobilon blot of the 43 kD polypeptide by The Beckman Center at the Stanford Medical Center.

<table>
<thead>
<tr>
<th>Tryptic fragment #9</th>
<th>N-terminus</th>
</tr>
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<tbody>
<tr>
<td>VOALKTCACLLKE</td>
<td>V D A L K T C A C L L K E</td>
</tr>
<tr>
<td>Tryptic fragment #11</td>
<td>L N D G F F T Y</td>
</tr>
<tr>
<td>Tryptic fragment #15</td>
<td>S P T L P E S I I P N L Q T/(Q)</td>
</tr>
<tr>
<td>Tryptic fragment #21</td>
<td>(A) V Q (T) F V Q D P (T) -- P -- A L</td>
</tr>
</tbody>
</table>

51 35 54 47 18 17 -- 25 -- 17 29 8.5 --

16 4.9 7.9 6 6.8 5.9 3.7 2.1

8.1/4.1 3/5.6 5 5.8 4 3.2 -- 1.9 1

39 44 29 38 37 30 10 16 24 15 11 7.5 4.2 0.8/3

1.9 1.7 1.3 0.8 1.3 0.7 1.1 0.6 1.3 0.4 -- 1.1 -- 0.3 0.3
Table 3 - Amino acid composition of Violaxanthin De-Epoxidase in mole percentages. Determined from Immobilon P blot of 43 kD polypeptide by University of Hawaii’s Biotechnology - Molecular Biology Instrumentation Facility.

<table>
<thead>
<tr>
<th>Basic</th>
<th>Acidic</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg (R)</td>
<td>3.222</td>
</tr>
<tr>
<td>his (H)</td>
<td>0.805</td>
</tr>
<tr>
<td>lys (K)</td>
<td>4.479</td>
</tr>
<tr>
<td></td>
<td>asp (D) 8.011</td>
</tr>
<tr>
<td></td>
<td>glu (E) 8.896</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Uncharged</th>
<th>Non-polar</th>
</tr>
</thead>
<tbody>
<tr>
<td>asn (N)</td>
<td>N.D.</td>
</tr>
<tr>
<td>cys (C)</td>
<td>N.D.</td>
</tr>
<tr>
<td>gln (Q)</td>
<td>N.D.</td>
</tr>
<tr>
<td>gly (G)</td>
<td>4.802</td>
</tr>
<tr>
<td>ser (S)</td>
<td>2.976</td>
</tr>
<tr>
<td>thr (T)</td>
<td>5.437</td>
</tr>
<tr>
<td>tyr (Y)</td>
<td>2.153</td>
</tr>
<tr>
<td></td>
<td>ala (A)   4.400</td>
</tr>
<tr>
<td></td>
<td>ile (I)   3.041</td>
</tr>
<tr>
<td></td>
<td>leu (L)   6.098</td>
</tr>
<tr>
<td></td>
<td>met (M)   1.072</td>
</tr>
<tr>
<td></td>
<td>phe (F)   3.911</td>
</tr>
<tr>
<td></td>
<td>pro (P)   3.990</td>
</tr>
<tr>
<td></td>
<td>trp (W)   N.D.</td>
</tr>
<tr>
<td></td>
<td>val (V)   4.434</td>
</tr>
</tbody>
</table>
nanomoles of MGDG precipitated the maximal VDE. Increasing concentrations of MGDG precipitated smaller amounts of VDE: 484 nmol precipitated less VDE than 346 nmol < 242 < 155 < 104 < 69 < 35 (data not shown). Only a limited range of VDE concentrations from Mono Q fractions were lipid affinity precipitated, but on the basis of the determinations above, the optimal molar ratio of MGDG to VDE for the precipitation of VDE was 128:1 (mol:mol).

Other factors also affected the extent of lipid affinity precipitation. Evaporating the methanol in which the MGDG was solubilized (before adding buffer and protein sample) reduced the VDE precipitated. Measured by the amount of VDE activity precipitated, sonication was a less effective means of preparing the components of the lipid affinity precipitation compared with periodic inversions of the ultracentrifuge tubes during a half hour incubation (data not shown).

The presence of xanthophyll cycle pigments affected the results of lipid precipitation. At higher MGDG levels than typically used (173 nmol), violaxanthin (0.4 nmol) slightly increased (although not significant statistically) the amount of VDE precipitated (figure 14). However, with 35 nmol MGDG, the presence of 0.5 nmol violaxanthin led to the precipitation of 78.7% of the VDE with MGDG alone. In contrast to these small variations in the presence of V, the presence of the same concentration of zeaxanthin, the de-epoxidized product of violaxanthin, sizably decreased the amount of
Figure 14 - Bar chart of VDE activity in the pellet of various ultracentrifuged mixtures in 200 mM citrate buffer, pH 5.20. 14.4 μM MGDG and 0.33 μM of indicated pigment were used in each tube to be ultracentrifuged. Two standard deviations (95% confidence interval) are indicated above each bar, a similar distance also extends below each mean.
VDE precipitated: MGDG + Z precipitated only 39.3% of the VDE activity precipitated by MGDG + V.

Figure 15 compares the amount of VDE activity precipitated by nine different lipids when a purified VDE sample was ultracentrifuged in citrate buffer at pH 5.20. The order of the lipids ability to precipitate VDE under these conditions (from high to low) was MGDG, DGDG, PC, PE, PG, PS, cardiolipin, PI, and PA. MGDG pelleted 3.7 times more VDE than the second highest lipid, DGDG, and 37.6 times more than phosphatidic acid, the lowest VDE-precipitating lipid. These results support the naming of VDE purification using MGDG as "lipid affinity precipitation".

Further evidence of the specificity of the lipid affinity precipitation of VDE comes from negative results using two other protein purification procedures also utilizing lipids. The reversed micelle procedure of Hilhorst et al. (1983) mixed CTAB in organic solvents to sequester an entire set of active NADH-regenerating enzymes. Wolbert et al. (1989) used a different reversed micelle system with an organic phase consisting of TOMAC and nonylphenol pentaethoxylate as surfactants, octanol as cosurfactant and isoctane as the continuous phase. Using this system they extracted from solution 11 of the 19 proteins tested (and detected transfer for a number more). Wolbert et al. (1989) proposed a formula to determine the pH at which (above its pl) a given protein would transfer into the reversed micelles. The protein could then be reextracted later into a fresh neutral buffer. Both of these procedures were followed using control proteins and in an attempt to isolate
Figure 15 - Bar chart of VDE activity recovered by resolubilizing the lipid complex with 0.5% OG after ultracentrifugation. 2.9 μM of each lipid was present with 200 mM sodium citrate buffer, pH 5.20 and a VDE sample. 95% confidence levels are indicated (n= 4 for each lipid).
VDE. Both procedures successfully isolated milk and chloroplast proteins, as well as purified alcohol dehydrogenase, lysozyme, and lysyl endopeptidase. In our hands, Wolbert et al.'s (1989) procedure failed to isolate BSA, as previously reported. Furthermore, each reversed micelle procedure isolated a different spectra of chloroplast proteins from Romaine lettuce. However, in no case was VDE activity recovered, nor was a 43 kD polypeptide band detected on a silver-stained acrylamide gel using semi-purified VDE material (no band larger than 30 kD appeared on the gel - results not shown).

D. Discussion

1. VDE purification

The isolation of VDE from spinach described by Hager & Perz (1970) yielded a solution containing a 40-50 fold enrichment of VDE activity over soluble chloroplast proteins. They checked the purity of this enzyme purification using native-gel disk electrophoresis. This gel was not shown but was described as containing, in addition to the band they associated with the enzyme, "merely two other pollutions which are hardly perceptible" (my translation).

Yamamoto & Higashi (1978), using Romaine lettuce, refined Hager & Perz's (1970) purification procedure, and added a sonication step of the thylakoids at pH 5.2 (VDE was membrane-bound at this pH). Yamamoto & Higashi (1978) reported their further efforts to purify VDE were unsuccessful. Yamamoto & Higashi (1978) also used native-gel disk electrophoresis to check the purity of their VDE sample. They detected
only a single band in their VDE preparation, yet cautiously concluded that they had
achieved a VDE fraction that was "highly purified, although probably not homogenous."

The two-dimensional IEF/SDS-PAGE depicted in figure 7 was the fraction
containing peak VDE activity eluting from the Sephadex G100 column using the
purification procedure described by Yamamoto & Higashi (1978). The VDE sample was
readily seen as being far from pure, yet with trenchant observation a band was picked out
that appeared to be associated with VDE activity (figure 8). VDE has now been further
purified to one major polypeptide detectable by two-dimensional SDS-PAGE by making
use of new protein isolation technology and a novel final purification step. The
polypeptide identified as VDE after Sephadex G100 chromatography in figure 8
corresponded to the polypeptide obtained at this final purification step.

The anion-exchange chromatography of VDE carried out on a Mono Q column
removed significant amounts of extraneous proteins (A_{280} chromatogram figure 9 and
purification table 1), nevertheless, numerous impurities yet remained (figure 10A). The
staining intensity of a number of polypeptide bands in successive Mono Q fractions were
assessed by a densitometer and graphed along with VDE enzymatic activity (two of these
are shown in figure 10B). Only the 43 kD polypeptide correlated with VDE activity, and
the linear relationship was statistically significant (r^2 > 0.95). After this stage, however,
further purification efforts by standard protocols met with little success (attempts detailed
in Results). Consequently, another purification step was developed, one that may
ultimately help in solving other protein purification problems.

Instrumental in the development of the lipid affinity precipitation step was the
observation that at acidic pH (5.2) VDE could not be solubly extracted from chloroplast
thylakoids (Yamamoto & Higashi 1978). Hager & Holocher's (1994) work verifies the
reversible association of VDE with the lumenal side of the thylakoid membrane.
Additionally important in the development of the lipid affinity precipitation step was the
importance of MGDG to VDE activity. Its presence enabled the maximal rate of VDE
activity in a partially purified VDE sample (Yamamoto et al. 1974), and Yamamoto &
Higashi (1978) considered MGDG to be both a functional component of the de-epoxidase
binding site as well as a "solvent" for the reactant V. Furthermore, MGDG is the principal
thylakoid lipid (Allen et al. 1966), MGDG was shown to be essential in restoring
photosynthetic electron transport using isolated components (Siefermann-Harms et al.
1982, 1987), and MGDG reactivated another carotenoid biosynthetic enzyme, purified
recombinant phytoene desaturase (Fraser 1993). MGDG was known to aggregate as a
hexagonal (Hn) phase (an arrangement whereby the head groups aggregate to form
cylinders with the hydrophobic tails radiating outward and mixing with the fatty acid tails
of other cylinders) when hydrated at physiological temperatures (Cullis et al. 1983). Thus,
for the purification step MGDG was mixed with the peak VDE sample off of Mono Q in a
citrate buffer at pH 5.20. The complexes which formed were subsequently pelleted by ultracentrifugation.

Samples from different lipid affinity precipitations were run on two-dimensional IEF/SDS-PAGE and a single polypeptide spot appeared after staining with Coomassie Blue R-250 (figure 11). A separate VDE lipid affinity precipitation was run on two-dimensional IEF/SDS-PAGE and stained with silver nitrate. A whitened spot developed in the region where VDE typically ran (figure 12). The staining of this gel was representative of the negative staining of VDE by the various silver staining techniques tried (mentioned in Materials & Methods). As silver is a more sensitive stain, additional minor spots in figure 12 reveal the presence of trace components in addition to the 43 kD polypeptide. The lipid affinity pellet presents a highly purified preparation of VDE but cannot be considered homogenous.

The purification table (table 1) and figure 13 both document the increased purification of VDE. The purification table reflects this through increases in the specific activity of VDE as the purification proceeds. The same can be seen in figure 13 which contained equal amounts of protein loaded in each lane: the VDE band was detectable in all stages of isolation and intensified as the purification proceeded. The Sephadex stage (where the procedures of Yamamoto & Higashi 1978, as well as Hager & Perz 1970 ended) had a specific activity 860x higher than that of a crude chloroplast isolation. This
was a significant enhancement though not quite as great as Hager & Perz (1970) who reported a 1200 fold increase of VDE activity using the same manipulations.

The determination of VDE activity from a chloroplast suspension cannot be directly compared to later purified enzyme preps. This is because there are membrane barriers to the assay reagents (Neubauer & Yamamoto 1994), as well as endogenous MGDG, ascorbate, and V extant. Be that as it may, by the conclusion of the purification the increase in specific activity over what was previously achieved (Sephadex stage) was greater than 17 fold.

The molecular mass of VDE has previously been estimated to be 54 kD (Hager 1975) and 60 kD (Yamamoto & Higashi 1978). Recently, Hager & Holocher (1994) correlated VDE activity to a polypeptide of 58 kD in spinach thylakoids. However, the earlier works do not show the data upon which their conclusion is based and the gels which Hager & Holocher drew upon to make this association show more protein components than that contained in the gel run from material eluting from the Sephadex column (figure 2). I find it questionable what Hager & Holocher (1994) describe as a "clear correlation" between VDE activity and the 58 kD polypeptide. It appears that no single polypeptide band in their preparation has an unambiguous relationship with VDE activity. While a polypeptide of apparent molecular mass 58 kD also appears in VDE preps from Romaine lettuce (figures 7-9), examination of the SDS-PAGE gel containing successive Mono Q fractions, (figure 10A) showed that the correlation observed by Hager &
Holocher (1994) did not exist here. The 58 kD polypeptide eluted before the peak of VDE activity in Mono Q chromatography (figure 10B). Furthermore, the 58 kD polypeptide was found in scarcely detectable quantities in the lipid affinity pellet despite a large increase at this stage in VDE specific activity. These results do not exclude the possibility that spinach VDE is significantly larger than the homologous enzyme in Romaine lettuce. Whereas there have been surveys of the plant kingdom for the presence of the xanthophyll cycle (reviewed in Hager 1980, Demmig-Adams & Adams 1992b, Demmig-Adams & Adams 1993), studies regarding the relative homology of the de-epoxidizing enzyme have not yet been done.

2. Characteristics of the lipid affinity precipitation

There are a number of interesting characteristics of lipid affinity precipitation. The first is that the precipitation is pH-dependent, occurring at pH 5.20 but not at either neutral or alkaline pHs. The pH optimum for VDE activity is 5.2 (Hager 1969, Yamamoto 1979), and VDE binds to the thylakoid at pH 5.2 so that it could not be solubly extracted (Yamamoto & Higashi 1978). These results indicate VDE’s binding to the thylakoid is directly to the lipid matrix and the predominant component of it, MGDG. It seems likely that VDE complexed so well with the lipid at pH 5.20 because there was little net charge on the protein (VDE’s pI = 5.42).

The optimal molar ratio of lipid to VDE which precipitates the protein was 128:1. In contrast, there was no optimal MGDG concentration with regards to the extent of de-
epoxidation found; above a concentration of 10 \( \mu \text{M} \) there was a plateau of de-epoxidation extent (Yamamoto et al. 1974). There was an optimal amount of MGDG found to be necessary to measure the maximal rate of VDE activity (Yamamoto et al. 1974). The critical ratio in the latter case was of MGDG to \( V \) and was much lower (28:1) than for precipitating VDE. Yamamoto et al. (1974) concluded this ratio of MGDG:V was important to VDE activity by solubilizing and orienting \( V \) for de-epoxidation. Whereas, for VDE purification, the MGDG:VDE ratio is important to precipitate VDE. The MGDG concentration found to precipitate the maximal amount of active VDE was 2.9 \( \mu \text{M} \), this compared with the 30 mM of MGDG used in the VDE assay. The 2.9 \( \mu \text{M} \) of MGDG is a concentration leading to just 70% of the maximal VDE rate and about 60% of the available \( V \) being de-epoxidized after the standard reaction time of 4 minutes (Yamamoto et al. 1974).

The presence of \( Z \) reduced the amount of VDE which MGDG precipitated in conjunction with \( V \) more than 60% (figure 14). The fact that these results were obtained using MGDG concentrations higher than was optimal indicated that \( Z \) was not simply competing with VDE for MGDG lipophilic sites. The propensity of VDE to dissociate from its product \( Z \) was either greater than VDE's affinity for MGDG or \( Z \) in some way adversely affected the affinity of VDE for MGDG.

MGDG was the most effective lipid tested for precipitating VDE (Figure 15). From 3.7 to 37.6 times more active VDE was recovered using MGDG than the eight other
Yamamoto et al. (1974) also found a MGDG specific requirement for when assessing the extent of de-epoxidation. A partially purified enzyme, was used to find that the lipids phosphatidyl inositol, phosphatidyl glycerol, and sulfoquinovosyl diglyceride gave just less than 50% the extent of de-epoxidation that MGDG did. Phosphatidyl choline and digalactosyl diacylglycerol yielded values up to 60-70% that of MGDG. The fatty acid composition of the MGDG did not seem an important consideration in de-epoxidation extent (Yamamoto et al. 1974). These results suprisingly show, however, there was less difference in de-epoxidation extent using a lipid other than MGDG than there was in lipid precipitation using something other than MGDG. One would anticipate a greater specificity for an enzyme reaction rather than in a precipitation complex. DGDG was second best (though by a long shot) at precipitating VDE as well as ranking second in terms of maximal extent of VDE activity. DGDG is also the second largest lipid component of the thylakoid membrane, after MGDG. The four lipids least effective in precipitating VDE also comprise a negligeble portion of the thylakoid.

MGDG uniquely associates with VDE, and MGDG is also a unique lipid in its own right. It holds the distinction of being the "most abundant polar lipid in nature" by virtue of its being the dominant lipid in the thylakoid, which is the most extensive membrane system in the world (Allen et al. 1966, Gounaris & Barber 1983). MGDG is synthesized in the chloroplast envelope (where DGDG dominates) and transferred, presumably by lipid vesicles, to the thylakoid (Douce & Joyard 1990). There is a high degree of unsaturation
in MGDG, in some cases the di-linolenoyl species (C18:3) makes up as much as 90% of the MGDG present. Consequently, MGDG functionally provides a fluid environment for photosynthetic diffusional processes. Perhaps too MGDG's unique shape of a small head group and the hugely unsaturated hydrophobic tail facilitates the optimal packing of intrinsic proteins in the bilayer (Cullis et al. 1983). Physically, MGDG preferentially forms a hexagonal lipid structure (H$_{II}$) which has been described by Gournaris & Barber (1983) as "an arrangement consisting of water cylinders in a lipid matrix with the hydrocarbon chains radially oriented outwards from the central axis of the cylinders."

The H$_{II}$ cylinders which MGDG forms contain a 2 nm aqueous channel. Consequently I wondered whether VDE associated with the polar head groups of MGDG in the aqueous middle of the channel or preferentially with the exposed fatty acid chains. If it were the former, the lipid precipitation step might be replicated to some extent using reversed micelles. Therefore I tested two established protein purification protocols (Woll et al. 1987, Dekker 1993) which incorporated the use of reversed micelles. The components differed, but both methods used surfactants and buffer mixed in an organic solvent such that reversed micelles formed, trapping some of the buffer within and orientated with their hydrophobic tails facing the organic phase. Proteins transferred to the aqueous core of the micelle "with some degree of selectivity" upon mixing when the pH and salt content were adjusted so that the surfactant head group and protein were oppositely charged and optimally attracted (Dwyer 1993).
These reversed micelle methods, however, do not work universally in protein purification. Wolbert et al. (1989) found their reversed micelle system significantly extracted 11 of the 19 tested proteins from solution and detectably transferred a few more. I too successfully used two different reversed micelle protocols (Hilhorst et al. 1983, and Wolbert et al. 1989) to isolate different proteins from solution. It was interesting that the two sequestered a different array of chloroplast proteins from Romaine lettuce leaves, however, VDE was not transferred to the reversed micelles of either procedure. Neither VDE activity nor a polypeptide band on SDS-PAGE corresponding to VDE was recovered. The fact that lipid precipitation of VDE occurred preferentially with MGDG (a H₂lipid) and near the pl of VDE, and could not be replicated by reversed micelle procedures indicated that the protein was probably associating with the lipid matrix. These negative results at VDE recovery from the reversed micelle protein purifications further indicated that lipid affinity precipitation was a novel purification step.

The use of lipid affinity precipitation in the purification scheme of VDE yielded a purer preparation than chromatography using various hydrophobic interaction columns, including those run using detergent. However, MGDG precipitated a limited number of different polypeptides from a solution of proteins extracted from thylakoids and the chloroplast lumen in neutral high ionic-strength buffer. Lipid precipitation by MGDG is thus restrictive, although not exclusive to VDE. Consequently, the use of MGDG (in buffers near a protein’s pl?) for lipid precipitation may be an approach finding further
fruitful application in the purification of other membrane or membrane-associated proteins, perhaps especially thylakoid proteins.

3. Partial peptide sequence

The amino acid composition of VDE (table 3) is unusual. The two acidic residues are the amino acids representing the largest mole percentages in the protein. The two major amino acids are typically glycine, alanine, or leucine in the five carotenoid biosynthetic genes of *Erwinia herbicola*, the three oxygen evolving enzymes (OEE) of *Chlamydomonas reinhardtii*, and various higher plants plastocyanins, rubiscos (large and small subunits), and different chlorophyll a/b binding apoproteins (GenBank). (The pIs of the above proteins range from 4.86 in plastocyanin to 10.01 in dycopene cyclase.) Only in the case of OEE3 (pI = 5.54) is there a composition of aspartate and glutamate comparable to that in VDE. Nevertheless, the major amino acids of OEE3 are still alanine and leucine, and the protein has an equal amount of basic and acidic amino acids. VDE contains twice as many acidic as basic amino acids. Though the nonpolar amino acids are the largest group after the acidic residues in VDE, compared to the proteins listed above, VDE contained about 50% fewer nonpolar amino acids than these.

Table 2 shows the partial amino acid sequence of the N-terminus of VDE as well as of various tryptic fragments. All the sequences shown in table 2 are unique in the GenBank data base. Consequently VDE is a newly described protein. Furthermore, the sequence information in table 2 has been used to construct nucleotide primers for a
polymerase chain reaction (PCR) using a cDNA library made from developing Romaine lettuce leaves as template. Already a PCR product has been obtained containing three of the amino acid sequences determined for VDE and shown in table 2 (Bugos & Yamamoto, personal communication). The PCR product was then used to identify a full length clone which coded for an additional amino acid sequence presented in table 2 (fragment #9 has not been found encoded by this clone) and which was found to exhibit VDE activity using an *E. coli* expression system. These results remove any doubt as to the veracity of this purification - the 43 kD polypeptide isolated from chloroplasts ultimately by lipid affinity precipitation is indeed VDE.
A. Introduction

In thylakoids, violaxanthin de-epoxidase (VDE) catalyzes the conversion of violaxanthin (V) to zeaxanthin (Z) in the presence of ascorbate and an acidic lumen. Demmig et al. (1987) proposed that Z helped plants limit the deleterious effects of excess light energy. They found both a correlation between Z content and amount of non-radiative dissipation of light energy as well as determined that those plants which had a higher capacity to form Z experienced less damage to PS II. Stemming from their proposal, a large body of evidence has accumulated documenting the relationship between the presence of the de-epoxidized xanthophyll-cycle pigments (antheraxanthin, A, and Z) and a plant’s ability to dissipate excess light energy as heat (reviewed in Demmig-Adams & Adams 1992b). There remains much debate regarding the mechanism by which Z (and A) work, the relative merits of the hypotheses has recently been assessed by Pfundel & Bilger (1994). However, there exists consensus that these xanthophyll pigments are involved in photoprotection (principally of PS II) when light is beyond what the plant can use photochemically.
In this chapter I will determine anew the $K_M$ of VDE using a purified enzyme sample, measure other of VDE’s biochemical properties, and characterize a VDE polyclonal antibody’s reactions.

B. Materials & Methods

1. Sample material and gel methods

Starting material for the biochemical characterizations was a VDE sample purified through to the Mono Q stage (see chapter II). The VDE activity assay and gel techniques are also as described in chapter II. Non-denaturing-PAGE was run according to the technique presented in the Hoefer Scientific Instruments catalog (1990).

To determine pI from two-dimensional SDS-PAGE, multiple purified VDE and control tube gels (empty of protein sample) were run. The control gels were cut in the equivalent region bracketed by VDE on the experimental gels. These cut pieces were then incubated 24 hours in 100 mM CaCl$_2$ with gentle shaking which allowed the ampholytes to diffuse out of the gel. The pH of this solution was then read using a microtip pH probe (Fisher #13-620-297). The measured pH was that to which VDE migrated after extensive isoelectric focusing and where there was no net charge on the polypeptide, in other words, the pI of VDE.

2. Glycan immunoassay

A glycan detection immunoassay was carried out by running duplicate one-dimensional SDS-PAGE gels with lanes containing different stages of VDE purification.
One gel was stained with Coomassie Blue and the contents of the other blotted onto a PVDF membrane, both as described above. A digoxigenin glycan detection kit (Boehringer Mannheim #1142372) was then used to assay the presence of any glycoproteins on the membrane.

3. Western blot

A PVDF membrane blot was prepared and western blot immunodetection was carried out as in Sun (1989). The only modification being a second Blotto/NP-40 (5% Carnation Non-fat Dry Milk, 0.01% Antifoam A emulsion, 0.001% Merthiolate, 150 mM NaCl, 20 mM Na$_2$HPO$_4$, pH 7.2, and 0.1% NP-40) wash of 30' after reaction of the goat anti-rabbit antibody.

4. Antibody purification and affinity column

Rabbit anti-VDE polyclonal antibody was purified with Pharmacia’s E-Z-Sep™ kit (#17-1449). The solvent of the purified polyclonals was first exchanged with phosphate buffered saline (PBS; 10 mM Na$_2$HPO$_4$, pH 7.4, 150 mM NaCl) before an affinity column was made of them using Pierce’s AminoLink® Plus Immobilization kit (#44894).

C. Results

1. Purified enzyme properties

The pl determination was based on numerous two-dimensional IEF/SDS-PAGE gels, a sampling of which were previously shown in figures 11 and 12. The ampholytes used in these gels were an equal mix of pH 4.0-6.5 and 3-10. The pl value of the purified
VDE polypeptide was $5.42 \pm 0.04$. The $K_M$'s of VDE for ascorbate and violaxanthin were calculated from the Lineweaver-Burk plots shown in figure 16. The graph in figure 16A showed the $K_M$ for ascorbate to be 8.54 mM. The $K_M$ of VDE for violaxanthin calculated from the graph in panel B was 0.352 μM.

A glycan detection immunoassay of a one-dimensional SDS-PAGE gel of different stages of VDE purification indicated that VDE was neither glycosylated nor associated with any glycosylated lipid. Furthermore, there was no indication that this condition changed with further purification - samples from each of the major stages of purification (Sephadex size exclusion chromatography, Mono Q anion exchange chromatography, and lipid affinity precipitation) yielded the same negative result.

Table 4 shows the effects of various detergents on VDE activity. The percentage of VDE activity relative to control was measured over a range of concentrations for each detergent. The detergent was added to VDE in citrate buffer and stirred two minutes before the de-epoxidase reaction was initiated with ascorbate. CTAB completely inhibited the reaction even down to 0.002% (v/v), whereas by a detergent concentration of 0.001-0.002% (v/v), all of the other detergents tested 60% or greater of the control activity. OG yielded the highest VDE activity at the highest detergent concentration tested (0.1% w/v), 75.3% of control. After OG, the next least interfering detergent of VDE activity was CHAPS, which at 0.1% (v/v) had 29.2% of the control VDE activity.
Figure 16A - Lineweaver-Burk plots of VDE for ascorbate using standard VDE activity assays (Yamamoto 1985). Ascorbate was varied while concentrations of violaxanthin, VDE, and MGDG were kept constant. For figure 16B the violaxanthin was varied while maintaining ascorbate, VDE, and MGDG. The violaxanthin used for these assays had a ratio of $A_{442}:A_{472} = 1.033$ and the VDE an activity of 5.11 U ml$^{-1}$. 

\[ Y = 0.1928 + 1.6469 (X) \]
\[ r^2 = 0.9928 \]
\[ K_M = 8.54 \text{ mM} \]
Figure 16B - Lineweaver-Burk plot of VDE for violaxanthin.

\[ Y = 0.0998 + 0.0351(X) \]

\[ r^2 = 0.9044 \]

\[ K_m = 0.352 \mu M \]
Table 4 - Percentage of control VDE activity in the presence of different concentrations of detergents. Standard VDE activity assays were run with a given percentage of the specified detergent added and mixed two minutes before the de-epoxidase reaction was initiated using ascorbate (--- not determined). Control VDE activity was 0.644 U ml⁻¹.

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The polypeptides in semi-purified VDE samples separated well on a non-denaturing gel, but the addition of substrate violaxanthin did not alter the mobility of VDE (results not shown). Neither did the combination of V with VDE, change the absorption spectra of V. Additional chemicals were tested to see if they effected the mobility of VDE on a one-dimensional SDS-PAGE gel. However, the addition of either 4 mM EDTA or 4 mM CaCl$_2$ did not change the $R_f$ of VDE (results not shown).

VDE exhibited different sensitivities to various protein detection methods. This has already been noted in the previous chapter regarding the silver-staining of VDE in SDS-PAGE (discussion on figure 12). VDE stained negatively with silver nitrate on SDS-PAGE gels but positively with Coomassie blue or with copper staining. Lowry and Bicinchoninic acid protein assays determined similar values regarding VDE concentration. However, the micro-Bradford method, which is based on the protein binding the same dye used in the Coomassie staining of gels, determined the protein concentration to be just 14.2% that of the other two assays.

2. Characteristics of polyclonal antibody to VDE

There were four attempts to generate antibodies to VDE. The first was in December 1989 using as antigen peak VDE activity fractions eluting from the Sephadex column. Mouse monoclonal antibodies were generated. Antibodies from various cell lines reacted principally to a 59 and a 49.5 kD band on a Western blot. These polypeptides were present in the fraction used as antigen in only trace quantities since their peak of
elution occurred in fractions far removed from peak VDE activity. The second attempt at monoclonal antibody generation (March 1991) used as starting material fractions containing peak VDE activity from the Mono Q anion-exchange column stage of purification. The purer antigen did not prevent multiple cross-reactivity from again occurring. A third attempt (September 1993) was through rabbits for polyclonal antibody generation and the antigen was a synthetic peptide matching the first 12 residues of mature VDE’s N-terminus coupled to keyhole limpet hemocyanin (KLH). Unfortunately, the Ab’s so generated did not inhibit VDE activity at all, and consequently of no help relating a polypeptide band on SDS-PAGE with VDE enzymatic activity. Finally, a measure of success was achieved generating polyclonal antibodies (November 1993) using as starting material excised SDS-PAGE pieces of the 43 kD polypeptide (VDE samples purified through the Mono Q stage). (The VDE sample was prepared by Katrin Hinderhofer.)

Figure 17 shows the effects of varying the concentration of this final batch of polyclonal antibodies (after the Ab’s had been purified) on VDE activity. Increasing antibody concentrations decreased VDE activity. As a control, VDE activity was checked in the presence of denatured Ab (boiled) and found to be greater than that of VDE mixed with intact Ab by a factor ranging from 1.51 to 4.31 (as the concentration of the Ab’s ranged from 31.6 to 505.8 µg/3 ml). However, it was clear that even the denatured antibodies had a sizeable effect on VDE activity. Pre-immune sera was also tested for its
Figure 17 - VDE activity in the presence of purified rabbit polyclonal anti-VDE Ab. The lower graph represents intact Ab, the upper, denatured. Standard VDE activity assays were conducted except Ab was added before VDE and the solution stirred 7' before initiating the de-epoxidase reaction with ascorbate. Violaxanthin used for these assays had an $A_{440}:A_{470}$ ratio of 1.023 and the VDE an activity of 2.750 U ml$^{-1}$.
effect on VDE, after it too went through the same Ab purification protocol. In concentrations above 130.1 μg/ml, the inhibition to VDE activity by the pre-immune sera was actually greater than that of the Ab’s for VDE (data not shown).

The order in which reactants were added was critical in determining the extent of Ab inhibition on VDE activity. When 30 μg of Ab were added before VDE for an activity assay with MGDG and V at pH 5.20 and incubated 5’, the activity was 37.4% of the control. If that same amount of Ab were added after VDE, the activity was then 81% that of control.

An affinity column was made using purified November 1993 polyclonal antibodies. Figure 18 shows a Western blot after affinity column chromatography. The first lane contained a VDE-active sample eluted from the affinity column followed by a blank lane and the third lane the flow-through. The starting material applied to the affinity column was from the Sephadex size-exclusion chromatography stage of VDE purification. Consequently a large number of polypeptides were present in the flow-through and this sample was concentrated for SDS-PAGE. Whereas the strongest antigen reaction occurred with the VDE band, there was some cross-reactivity to other polypeptides. At least there was no reactivity towards the blotted molecular weight markers, a reaction which actually did occur using one of the monoclonal Ab cell lines (!). A range of titrations, of both the VDE-specific rabbit polyclonals as well as with the goat anti-rabbit IgG (Southern Biotechnology Associates, Inc) were reacted with another Western blot. The titration
Figure 18 - Western blot of a one-dimensional SDS-PAGE containing the elute and flow-through from an affinity column for VDE. The sample applied to the column had been purified through to the Sephadex stage and was concentrated before loading onto the gel. Elution from the affinity column was by 100 mM sodium borate (pH 9.2). Dilution of rabbit anti-VDE was 1:100, dilution of goat anti-rabbit IgG 1:4000, and color development was for 3'. Electrophoresis and blotting procedures detailed in chapter II.
ratios with the clearest reactions were 1:200 for rabbit anti-VDE Ab and 1:4000 for goat anti-rabbit.

D. Discussion

1. Purified enzyme properties

VDE has been implicated to be the control point for de-epoxidation and to be active specifically "when light becomes excessive" (Adams & Demmig-Adams 1995). Previously some of the biochemical properties of VDE were determined using partially purified enzyme preparations. I repeated these determinations using VDE purified through the Mono Q stage and found the $K_M$ of VDE for V to be 0.352 $\mu$M and the $K_M$ for ascorbate 8.54 mM (figure 16). Yamamoto & Higashi (1978) calculated the $K_M$ for V (using VDE purified through to the Sephadex stage) to be as low as 0.049 $\mu$M, and 0.145 $\mu$M when a higher ratio of MGDG:VDE was used. Hager (1975) reported VDE's $K_M$ for V in spinach to be 10.6 $\mu$M, without supplementing the assay with additional MGDG. This value was thirty times greater than what I determined and even further removed from the values of Yamamoto & Higashi (1978). The MGDG extant on native VDE undoubtedly changed with purification and so most likely altered the enzyme's apparent $K_M$ just as the absence of added MGDG is documented to result in slower de-epoxidation (Yamamoto et al. 1974, Hager & Holocher 1994). The purity of the violaxanthin used to determine $K_M$ also was a factor; the purer the reactant the lower the calculated $K_M$. 
Purification apparently altered the $K_M$ of VDE for ascorbate to a small extent as well. I calculated the VDE $K_M$ for ascorbate to be 8.54 mM, which was in agreement with Neubauer & Yamamoto (1994), who determined this value in intact chloroplasts to be 3.1 mM. The $K_M$'s determined herein were slightly higher than those previously reported and presumably indicative of the true nature of the enzyme rather than components accompanying it in a semi-purified preparation. It has been speculated that an enzyme's $K_M$ value represents the in vivo concentration of (available) components (Segel 1976).

Sielewiesiuk & Gruszecki (1991) presented a model of the xanthophyll cycle's kinetics with the key assumption being that V availability was the rate-limiting step. They further proposed that their model applied in light of intermediate, non-saturating intensity (which further removes the model from situations in situ). Siefermann & Yamamoto (1974, 1975b) first noted how light intensity varied the availability of V, affecting the extent of de-epoxidation. A portion of the violaxanthin pool, ranging from 5 to 20% in different plants, remains inaccessible to de-epoxidation regardless of light intensity (Pfünder & Bilger 1994). Sielewiesiuk & Gruszecki (1991) take this observation much further and describe the de-epoxidation of V "as a simple monomolecular reaction instead of using the Michaelis-Menten kinetics." Such an understanding for modeling purposes may seem plausible given that VDE has a very low $K_M$ for V, so that as soon as V becomes available it associates with VDE. However, the requirement of VDE for ascorbate is another matter. The in vitro assay for VDE activity is a first-order reaction with
ascorbate present in saturating concentrations (30 mM). Consequently the 505 nm increase appears to fit Sielewiesiuk & Gruszecki’s (1991) model since the de-epoxidation rate depends on the concentration of violaxanthin alone (or VDE). The situation in chloroplasts, however, is not so simple. Neubauer & Yamamoto (1994) have demonstrated that the amount of available lumen ascorbate is influenced both by membrane barriers as well as by the Mehler-peroxidase reaction (which uses ascorbate). It is unlikely that the ascorbate concentration is maintained in the chloroplast lumen at a constant saturating level with respect to VDE, in contrast to this monomolecular model. The kinetics of the de-epoxidation reaction in situ depend on the interaction of V, VDE and ascorbate.

The pI of VDE determined from numerous two-dimensional SDS-PAGE gels using an ampholyte range of 4.0 to 6.5 (eg. figures 5 + 6) was 5.42. The pI of VDE nearly coincides with the pH optimum for VDE, measurements of which have ranged from pH 5.0 - 5.3 (Hager 1969, Yamamoto 1979, 1985). VDE at the pH which it is catalytically most active carries little net charge. This lack of charge may enable the enzyme to interact with pigments integrated in a lipid bilayer. On the other hand, Pfündel & Dilley (1993) suggested VDE activity in vivo is effectively altered in the pH 5.8 to 6.3 range. Perhaps the pH range at which VDE is regulated to work in situ is not its optimal catalytic range in vitro. The working conditions for VDE may necessitate the enzyme operating with a small net charge on it. In which case VDE may interact with either the head groups
of polar lipids or with some charged protein component of thylakoids. VDE, in fact, may interact with the minor LHC antenna complexes CP 24, 26, and 29 as V to Z conversion has been reported to take place there (Dainese et al. 1992).

OG solubilized the VDE/MGDG complex and was the least inhibitory of the detergents tested (table 4). Other detergents were tested by Yamamoto et al. (1974) who reported that 0.02% SDS and 0.05% Triton X-100 were inhibitory to VDE. The fact that Triton X-100 inhibited to VDE was interesting in that Triton was used to solubilize active diadinoxanthin de-epoxidase (Schimmer & Krinsky 1969), the enzyme with activity parallel to VDE in other photosynthetic organisms. Of all the detergents tested, CTAB exerted the most potent inhibition on VDE activity. Yet in the reversed micelle procedure of Hilhorst et al. (1983), CTAB was used to recover an active NADH-regenerating system. The choice of which detergent to use in a given application is admittedly most often empirical (Chapman et al. 1991, Weselake & Jain 1992). In the case of VDE, the use of OG was crucial to assess the value of the lipid precipitation step in VDE purification.

OG does have a history of successful use in plant protein purification. Pick & Racker (1979) added octylglucoside to minimize the intermolecular hydrophobic interactions and the aggregation of chloroplast ATPase complexes. OG has also been used as an affinity ligand in reversed micelle protein purification of concanavalin-A (Woll et al. 1987). In their reviews on the purification of thylakoid membrane proteins
specifically or plant proteins in general, both Chapman et al. (1991) and Weselake & Jain (1992) mentioned numerous applications of OG in protein purification, including of the oxygen evolving PS II complex, cytochrome b6f complex, CFo-CF1 complex ATPase, and cytidine 5'-triphosphate:choline phosphate cytidyltransferase.

On the basis of a glycan immunoassay, I found that VDE was not glycosylated. Whereas glycosylation increases protein solubility, it is not reversible so as to account for VDE's reversible solubility with thylakoids (Yamamoto & Higashi 1978, Hager & Holocher 1994). Glycosylation additionally increases protein stability but VDE may not require further stability. No studies of VDE turnover have been undertaken, however, the amino acid residue at the N-terminus for mature VDE is valine (table 2) which belongs to the class of "most stabilizing residues" as defined by the "N-end rule" (Bachmair et al. 1986, Varshavsky 1992). Strikingly different half-lives were found, from less than 3 minutes to more than 20 hours, in chimeric proteins expressed in yeast (Saccharomyces cerevisiae) which differed only in their N-terminal amino acid. The "N-end rule" is an interesting study on one level of control for protein turnover, nevertheless, it is still a far extrapolation to speculate on the stability of VDE.

When V was added to a VDE preparation, neither the mobility of the VDE band on a non-denaturing gel shifted, nor did any spectral properties from 300 to 600 nm alter. This latter result was in contrast to Hager & Perz (1970). They reported the appearance of a new $A_{max}$ at 380 nm when V bound to VDE even without reacting.
However, special manipulation of the components was necessary to see this 380 nm peak. They mixed V in methanol with a VDE sample, precipitated the mixture with ammonium sulfate and then resuspended the pellet in phosphate buffer. Such a solution differed in 320 to 520 nm absorbance from solutions of either protein alone or V in methanol which underwent a similar procedure. I assume the noted spectral differences reflect the degree to which VDE solubilized V. In my experiments V remained soluble throughout the experiment either in organic solution or in MGDG aggregates or in a VDE complex. Perhaps absorbance of pigment alone swamped any spectral differences with pigment-protein associations, in any case, there were no spectral changes when V and VDE were simply mixed together without precipitation and subsequent resuspension of the protein.

The presence of 2% (w/v) CaCl₂ had been noted to considerably inhibit zeaxanthin synthesis and growth in *Flavobacterium*, whereas concentrations below 0.5% (w/v) CaCl₂ had no effect (McDermott et al. 1973). Neither the addition of EDTA nor of CaCl₂ shifted the position of the VDE band in SDS-PAGE. This indicates VDE lacks a Ca²⁺ binding site. The inhibition of Z synthesis is therefore not acting on VDE or its *Flavobacterium* homolog. EDTA may somehow be involved in the stimulation or preservation of VDE activity (as noted below), but had no discernable effect on the Rf of VDE at least in a mini-gel system.
2. Characteristics of VDE polyclonal antibodies

For all the work that went into the generation and characterization of antibodies, the antibodies are of limited utility. Of the four attempts to generate VDE-specific Abs, only the fourth yielded a measure of success. These purified polyclonals inhibited VDE activity more than the non-specific inhibition of denatured Abs (figure 17) and more than purified pre-immune sera at concentrations less than 35 μg/ml. However, the difference in inhibition of VDE activity between the denatured and intact Ab solutions was relatively small. For one Ab concentration the denatured Ab yielded only one and a half times more VDE activity than the intact. Possibly the inhibition of VDE by the denatured polyclonals was due to some unidentified component of rabbit sera which was not removed in the purification. In contrast, antibodies have been successfully used to selectively precipitate other carotenoid biosynthetic activities (phytoene and phytofluene desaturase; Hugueney et al. 1992).

Another limitation on the use this polyclonal antibody was that there was a fair deal of cross-reactivity in a western blot. This was observed even after the concentrations of the first and second antibody were optimized and after a second detergent rinse subsequent to the 2nd antibody conjugation was added. The polyclonal Ab reacted with other polypeptides present in a Sephadex preparation of VDE, albeit not as strongly as with VDE (figure 18). At best I may conclude that the principal antigenic reaction of the Ab’s was with VDE. Cross-reactivity in polyclonals has been noted before and useful
applications have nevertheless been found for them (e.g. - Henrysson et al. 1989 with the minor chlorophyll a/b-binding complex, CP 29).
A. Introduction

Violaxanthin de-epoxidase (VDE) is the enzyme of the xanthophyll cycle catalyzing the conversion of violaxanthin (V) to zeaxanthin (Z). In chloroplast thylakoids the reaction requires the presence of ascorbate and an acidic lumen. VDE activity occurs in all surveyed Angiospermae, Gymnospermae, Pterophyta (ferns), Bryophyta, Chlorophyta (green algae), and Phaeophyta (brown algae), as well as in individual species of the Rhodophyta (red), Chrysophyta (golden-brown), and the Xanthophyta (yellow-green) algae (reviewed in Hager 1975, and Demmig-Adams & Adams 1993). Parallel enzymatic activity, that which removes the epoxide group from the xanthophyll pigment diadinoxanthin, exists in diatoms, other Chrysophyta, Xanthophyta, Chloromonadophyta, Pyrrophyta (dinoflagellates), and Euglenophyta (euglenoids). The only known oxygen evolving photosynthetic organisms without xanthophyll de-epoxidizing activity are the remaining Rhodophyta (red) and Cryptophyta algae as well as Cyanobacteriae (the blue-green bacteria). Despite a lack of a xanthophyll de-epoxidase, this last group of organisms may nevertheless contain Z.
Advances in the study of VDE have proceeded mainly from in vitro studies of higher plants. Yamamoto et al. (1974) and Yamamoto & Higashi (1978) found that VDE of lettuce contained monogalactosyldiacylglyceride (MGDG) and that VDE's catalytic rate depended on the ratio of pigment to MGDG. Gauged by VDE activity using various epoxy carotenoids as reactants, Yamamoto & Higashi (1978) concluded that VDE was specific for 3-hydroxy-5,6-epoxy carotenoids in the 3S, 5R, 6S configuration and which were all trans with respect to the polyene chain as well as the epoxide and hydroxyl groups in the end rings. VDE is localized within the thylakoid lumen and reversibly associates with the thylakoid membrane based on pH, being membrane-bound at pH 5 and soluble at neutral pH (Yamamoto & Higashi 1978, Hager & Holocher 1994).

In isolated chloroplasts, VDE activity was found to be inhibited by DTT (Yamamoto & Kamite 1972). Pfündel et al. (1992) found a pretreatment of 17.6 μE m\(^{-2}\)\((7000 \text{ J m}^{-2})\) of UV-B light inhibited half the VDE activity in isolated chloroplasts, although a similar level of inhibition in whole leaves required a light dose two orders of magnitude higher. In Bramley's (1993) review on inhibitors of carotenoid biosynthesis he concluded, "Until it is shown that a particular inhibitor binds to the pure enzyme, and thus inhibits catalytic activity, it can still be argued that the effects shown in crude preps in vitro are influenced by other reactions in the cell extract." VDE had been partially purified from spinach (Hager & Perz 1970) and from romaine lettuce (Yamamoto & Higashi 1978). In the preceding chapters I report the purification of VDE from lettuce a further 17 fold
beyond what both the previous works achieved and have characterized some of VDE's biochemistry.

Neutral red and 9-aminoacridine are widely used as *in situ* indicators of organelle pH (Siefermann-Harms 1978, Noctor *et al.* 1993). The local anesthetic, dibucaine, may be similarly used (Vanderkooi 1984), as well as used to probe the control of photophosphorylation and energy-dependent nonphotochemical quenching of chlorophyll fluorescence (Laasch & Weiss 1988). It had been noted in our lab that the use of these chemicals in chloroplast or thylakoid preparations affected the amount of Z formed. Consequently I carried out inhibition studies using purified VDE and these *in situ* pH indicators to determine whether they directly affected VDE activity.

### B. Materials & Methods

The plant material used, as well procedures used to isolate purified VDE, concentrate samples, and conduct VDE activity assay were as described in chapter II. Inhibitor studies were conducted where a given inhibitor was incubated with the VDE sample for 7' with stirring before initiating the VDE reaction with ascorbate.

### C. Results

Various chemicals were tested for their ability to preserve or inhibit VDE activity. As a reference point, VDE purified through to the Sephadex stage and kept at room temperature (RT) retained 64.4% of its activity after 6 hours and 36.7% after 32.5 hours.
A VDE sample stored five days at room temperature lost all activity, but for one stored at 2°C, VDE activity decreased only 12.7% during that same period.

VDE stability during storage depended on the container’s being glass as well as the enzyme not being concentrated beyond 10 μg/ml. Glass-stored VDE (purified through to the Mono Q stage) had nearly four times the activity at both 4°C and 25°C compared to VDE stored in polypropylene microfuge tubes over a time-course of 5 to 6 hours.

Furthermore, it was repeatedly noted that concentrated samples (concentrated for the purpose of either additional chromatography or SDS-PAGE) were much less stable with regards to VDE activity. Instead of the relative stability of VDE activity noted above, samples concentrated by Centricon 30 would lose >50% of their activity in the course of 24 hours even at 2°C (results not shown). However, this result might also be attributable to the sample coming into contact with the polypropylene retentate cups during the course of concentration.

The addition of MGDG to a VDE sample at RT caused the VDE to lose nearly 90% of its activity in 6 hours (vs. 35.6% without) and to be completely inactive after 30 hours. The presence of the antioxidants BHT or BHA either alone or together made no difference in preserving VDE activity at RT. Quite a different result occurred when a VDE solution contained BSA. The addition of 1% BSA increased 30 hour RT-stored VDE activity nearly 31% over initial VDE activity without BSA. An even larger difference over control VDE activity (59%) resulted when VDE was immediately assayed upon addition of
a smaller concentration of BSA in 1x Denhardt’s solution (0.02% Ficoll + 0.02%
polyvinylpyrrolidone + 0.02% BSA) and 2 mM EDTA.

DTT immediately inhibits the conversion of V to Z in the purified enzyme system
as determined by the $\Delta A_{505-540}$ (data not shown). DTT has also been routinely used to stop
VDE activity in systems ranging from intact leaves to thylakoids. I determined that neutral
red (NR) was another inhibitor to VDE activity, and Figure 19 contains the $I_{50\%}$ and Dixon
plots for NR's effect on VDE activity. Panel A shows that 7.2 ± 2.8 μM NR inhibits 50%
of the starting VDE activity. In panel B the Dixon plots ([NR] vs. 1/v) for different
ascorbate substrate concentrations intercept above the x-axis indicative of competitive
inhibition between neutral red and ascorbate (Segel 1976).

Figure 20 contains the $I_{50\%}$ and Dixon plots for 9-Aminoacridine (9-AA). Panel A
shows the $I_{50\%}$ of 9-AA for VDE to be 57 ± 15 μM. In figure 20B the Dixon plots at
different ascorbate concentrations yield ambiguous results as to the type of inhibition 9-
AA has on VDE activity. The plots cross at different points near to the x-axis with the
confidence intervals for the intersection including both the x-axis and an area above it.
This most likely indicates a mixed-type of inhibition is involved (competitive and non-
competitive) between 9-AA and VDE.

Figure 21 contains the $I_{50\%}$ and the Dixon plots for dibucaine. From panel A the
$I_{50\%}$ for dibucaine on VDE activity can be seen to be 125 ± 25 μM. In panel B the Dixon
plot shows that, as for 9-AA, dibucaine has an ambiguous, mixed-type of inhibition on
Figure 19A - VDE activity plotted against NR concentration to determine $I_{50w}$. The different plots vary in their level of reactants. Figure 19B contains Dixon plots at different ascorbate concentration of $v$ (U ml$^{-1}$VDE activity) vs NR concentration. 95% confidence intervals are indicated by the dotted lines. The area of intersection is indicated by shading. The $r^2$ values for these plots were 0.999 for 4 mM ascorbate, 0.872 for 15 mM, and 0.942 for 30 mM. The VDE sample was purified through the Mono Q stage and had an activity of 3.865 U ml$^{-1}$. 35 µl of VDE sample was used per assay. The citrate buffer, water, and NR were premixed on ice for at least 30 minutes before use in an assay. A 3 mM NR stock in methanol was prepared fresh.
Figure 19B - Dixon plot of NR on VDE activity.
The different plots vary in their level of ascorbate. Figure 20B contains Dixon plots at different ascorbate concentrations of $v^{-1}\text{(U ml}^{-1} \text{VDE activity)}$ vs 9-AA concentration. The linear regression correlation coefficients on these plots all were significant at $r^2 > 0.95$.

The VDE sample was partially purified through the Mono Q stage and had an activity of 2.830 U ml$^{-1}$. 40 µl of VDE sample was used per assay. A 6 mM 9-AA stock in methanol was prepared fresh the day of the kinetics assays.
Figure 20B - Dixon plot of 9-AA on VDE activity.
The different plots represent a variety of reactant concentrations. Figure 21B contains Dixon plots at different ascorbate levels of $v^1$ of VDE activity vs dibucaine concentration. Figure 21C contains Dixon plots of different violaxanthin concentrations of $v^1$ of VDE activity vs dibucaine concentration. Linear regression on all Dixon plots in figures 21B and 21C had $r^2 \geq 0.95$. The 95% confidence intervals are indicated by dotted lines. The VDE sample was partially purified through the Mono Q stage and had an activity of 4.159 U ml$^{-1}$. 40 μl of this VDE sample was used per assay. A 30 mM dibucaine stock in methanol was prepared fresh the day of the kinetics assays. The citrate buffer, water, and dibucaine were premixed on ice for each inhibitor level.
Figure 21B - Dixon plot of dibucaine on VDE activity with respect to ascorbate.

Figure 21C - Dixon plot of dibucaine on VDE activity with respect to violaxanthin.
VDE with respect to ascorbate. Panel C is also a Dixon plot for dibucaine but with respect to the reactant violaxanthin. The plots converge above the x-axis, indicative of a competitive inhibition, dibucaine for violaxanthin. However, the concentration at which dibucaine needed to be present in order to be an effective competitor was nearly four hundred times greater than that of V ($I_{50\%} = 125 \mu M$ when $[V] = 0.33 \mu M$).

**D. Discussion**

VDE was surprisingly stable at RT, maintaining nearly two-thirds of its original activity after 6 hours at RT and still more than a third of its activity after 32.5 hours. These were the results from VDE stored in glass. Polypropylene was found to adversely effect VDE activity, reducing it nearly four-fold compared to glass-stored VDE in less than 6 hours. As expected, colder temperatures better preserved VDE activity: a VDE sample stored 5 days at 2°C had 87.3% of the activity initially determined. In marked contrast, Hager & Holocher's (1994) freeze/thaw preparation of VDE was totally inactive after 5 days storage at -20°C. Cycles of freezing and thawing denature protein and their isolation procedure may have destabilized VDE (Deutscher 1990).

VDE has been classed as a lipoprotein, as it contains MGDG essential for enzymatic activity (Yamamoto & Higashi 1978). Typically when fatty acids are present in a solution, they render the labile components more susceptible to oxidative inactivation. However, the antioxidants BHT or BHA (alone or in conjunction) had no measurable effect in preserving VDE activity at RT. On the other hand, the addition of MGDG (270
119

μM) made VDE more prone to lose its activity when stored at RT: nearly 90% of VDE activity was lost in 6 hours. In the above test VDE was unstable in the presence of an abundance of MGDG, but the same instability may result from a lack of lipids. It has been suggested that for membrane-associated enzymes, loss of membrane lipid was critical for enzyme stability and/or activity (Chapman et al. 1991). Delipidation of the sarcoplasmic reticulum Ca^{2+}-ATPase correlated with its decreased enzymatic activity (de Foresta et al. 1989). Such may also be the case for VDE, a reversibly membrane-associated protein, loss of thylakoid lipids from VDE may have reduced its stability and/or activity, which partially offset the gains in specific activity by purification.

Zeaxanthin epoxidase, presumably also a membrane or membrane-associated protein, had its activity stimulated by the addition of BSA. It was concluded that BSA protected the epoxidase system (Siefermann & Yamamoto 1975a, Siefermann-Harms 1977). BSA is used in chloroplast preparations to protect the activities of other chloroplast components from the deleterious effects of fatty acids, among other things (Siefermann & Yamamoto 1975a, Walker 1987). As with Z epoxidase, VDE activity was similarly stimulated and stabilized during storage by the addition of BSA. This result held whether BSA alone was added or when BSA complemented with the other components of Denhardt’s solution plus 2 mM EDTA were added to a VDE sample.

With regard to DTT inhibiting the formation of Z, the effect of DTT on purified VDE was similar to that on whole leaf and isolated chloroplast systems. DTT inhibited
purified VDE activity (measured as $\Delta A_{505-540}$) the same as in chloroplasts (Yamamoto & Kamite 1972) and intact leaves (Bilger & Björkman 1994, Choudhury et al. 1993, Demmig-Adams et al. 1990). At the same time, DTT does not impact quantum efficiency, oxygen evolution, adenylate energy charge or NPQ after Z is formed (reviewed in Pfundel & Bilger 1994). Olaizola et al. (1994) have recently reported that DTT completely blocked the light-induced increase of the de-epoxidized diatoxanthin in the marine diatom *Phaedactylum triacorumutum*. The diadinoxanthin cycle in this organism parallels the xanthophyll cycle in higher plants, and consequently DTT is probably inhibiting an enzyme related to VDE. Presumably DTT inhibits these de-epoxidizing enzymes by reducing an essential disulfide bridge in the protein. It was suggested that UV-B light inhibited VDE activity by similarly breaking an essential cystine crosslinkage (Pfundel et al. 1992).

The effects of NR, 9-AA, and dibucaine on VDE were examined (figures 19-21). These compounds are used to determine pH in situ within chloroplasts (Pick & Avron 1976, Siefermann-Harms 1978, Vanderkooi 1984, Laasch & Weis 1988, Noctor et al. 1993) yet their presence also affects de-epoxidation (Yamamoto, Gilmore and Mohanty personal communication, Günther et al. 1994). Consequently, their use can complicate the interpretation of fluorescence quenching results. Although these chemicals do give information as to the state of membrane energization, an independent variable in explaining fluorescence quenching, they can also impact quenching by affecting Z. I found that NR, 9-AA, and dibucaine each inhibited the activity of purified VDE.
The concentration at which NR inhibited half the VDE activity ($I_{50\%}$) was $7.2 \pm 2.8$ μM; for 9-AA this value was $57 \pm 15$ μM, and for dibucaine the $I_{50\%}$ equaled $125 \pm 25$ μM. Measuring the inhibitory effect of NR in chloroplasts (pea or lettuce) Gilmore (personal communication) found the $I_{50\%}$ of Z formation to be 2 μM. NR’s inhibition of VDE in chloroplasts was similar to what I measured for the purified enzyme, thus the inhibition of NR on VDE is most likely a direct one within the chloroplast. The $I_{50\%}$ of NR on VDE is also nearly identical to the $I_{50\%}$ of NR on photophosphorylation (~10 μM, Pick & Avron 1976). Kinetic evidence indicates that NR apparently competes with ascorbate to inhibit VDE (figure 19B) despite the heterocyclic NR having no obvious structural similarity to the sugar acid ascorbate.

Günther et al. (1994) found the presence of 5 μM 9-AA "largely inhibited" VDE activity in osmotically shocked spinach chloroplasts. Gilmore measured the $I_{50\%}$ of 9-AA on Z formation to equal 0.6 μM. In contrast to NR’s effect being similar on VDE in chloroplasts or purified VDE, the $I_{50\%}$ of 9-AA for VDE in chloroplasts is 100 times smaller than for purified VDE (figure 20A). The inhibition by 9-AA on inhibition of VDE was in a manner which could not be resolved by Dixon (figure 20B) or Lineweaver-Burk plots (latter not shown). Clearly, the increased sensitivity of Z formation in chloroplasts to the presence of 9-AA shows that the inhibition in chloroplasts is an indirect, though potent, effect.
Laasch & Weiss (1988) concluded that dibucaine "selectively uncoupled" the bulk pool of protons responsible for photophosphorylation but left intact other pH-dependent processes. Dibucaine acts by reversibly associating with the thylakoid in the light (Günther & Laasch 1991). Indeed, Barghouthi et al. (1993) found that dibucaine's electrostatic interactions were key in determining both its location being at least partially buried in the lipid bilayer and the extent of its binding to the thylakoid. Laasch et al. (1991) and Luo et al. (1994) also found evidence indicating that dibucaine accumulated either at the aqueous lipid boundary layer or actually immersed in the lipid membrane. NR and 9-AA are also membrane-associated, since cationic 9-AA binds to the negatively charged thylakoid and NR partitions to the membrane phase (Renganathan et al. 1993). Consequently NR, 9-AA, and dibucaine are all ideally placed in the chloroplast to interfere with VDE catalytic activity and/or with whatever processes by which the product of VDE subsequently dissipates excess light energy.

Mohanty and Yamamoto (1995) found dibucaine had an $I_{50\%}$ of 85 $\mu$M for VDE in thylakoids (activity induced at pH 5 with ascorbate), though dibucaine's effect on the non-photochemical quenching of chlorophyll fluorescence (NPQ) was greater (in dark-adapted thylakoids the $I_{50\%} = 6.2$ $\mu$M dibucaine and was 98% inhibited with 20 $\mu$M). The concentrations of dibucaine necessary to exert a similar level of inhibition of VDE in chloroplasts was at least 10x that of NR or 9-AA, and using purified VDE compounded the amount of dibucaine necessary to inhibit 50% of the VDE activity compared to the
VDE in thylakoids (125 μM vs 85 μM). The greater sensitivity of NPQ to dibucaine means
dibucaine must first exert its inhibition on ΔpH and/or another site before directly
inhibiting VDE. The Dixon plots (figure 21B+C) indicated that dibucaine competed with V
for the catalytic site on VDE. V is structured with a central alkene chain and with epoxide
groups residing on both end-rings (figure 5), while dibucaine contains an alkyl chain
connected to a quinoline ring via a carbonyl carbon. Thus, a rough measure of homology
exists between the two and perhaps at sufficiently high concentrations, dibucaine would
disrupt V binding to the enzyme. However, dibucaine’s inhibition of VDE more likely lies
in its partitioning to the lipid phase and simply disrupting V solubility and/or V interaction
with VDE. Dibucaaine’s anesthetic action has been hypothesized to be by way of a specific
binding site rather than a general conformational mechanism (Chyan et al. 1994). If
dibucaine has a specific component it binds to in disrupting NPQ, the component is
assuredly other than VDE as the inhibition of NPQ is more sensitive to dibucaine than
VDE, and even the direct effect on VDE by dibucaine may not be direct.

Because NR, 9-AA, and dibucaine directly inhibited purified VDE (in addition to
indirectly affecting Z-formation within the chloroplast) it must be realized that their use in
chloroplast or thylakoid systems both directly and adversely effects the normal functioning
of VDE. Noctor et al. (1993) used both 9-AA (2 μM) and dibucaine (1-200 μM) in
concentrations which significantly inhibit VDE in both the purified state and in situ.
Perhaps by so doing they thereby gave undue prominance to factors other than the
xanthophyll cycle in dissipating excess light energy. Therefore, when determinations of quantum yield, Z-formation, or photoprotection are made, the use of NR, 9-AA, or dibucaine should either be avoided or used only in parallel experiments.
CHAPTER V

CONCLUSION

The most significant result of this work documented in the preceding chapters was the purification of VDE. To achieve the final purification a novel lipid affinity precipitation step (using MGDG) was developed, the results from which could not be duplicated either by various reversed micelle protein purification techniques or by the substitution of MGDG by at least 8 other membrane lipids. Indeed, lipid affinity precipitation may prove to be a useful procedure in other attempts to purify membrane or membrane-associated proteins.

VDE was determined to be a smaller polypeptide than originally thought - 43 kD rather than in the 54 to 60 kD range. Additional characterizations of VDE made included determining its isoelectric point to be 5.4 and $K_m$ values for ascorbate and violaxanthin to be 8.54 mM and 0.352 $\mu$M respectively.

Studies on the inhibitors of VDE activity were also made. This too was important as inhibition of a purified protein rules out the possibility of an inhibitor effect in vivo being just the indirect consequence of changes in accompanying cell processes. DTT directly inhibits purified VDE activity. Dibucaine, NR, and 9-AA inhibit the activity of purified VDE in different manners. NR apparently competed with ascorbate for VDE ($IC_{50}$ = 7.2 ± 2.8 $\mu$M), dibucaine competed with V ($IC_{50}$ = 125 ± 25 $\mu$M), and 9-AA seems to
have acted as a mixed-type of inhibitor, both competitively and non-competitively ($I_{50\%} = 57 \pm 15 \mu M$). BSA and Denhardt’s solution stimulated VDE activity and protected it during storage. VDE stored in polypropylene was enzymatically much less active compared to VDE stored in glass.

The two largest components of VDE’s amino acid composition were found to be the two acidic residues. VDE was partially sequenced and the sequences found to be unique in the GeneBank data base. Polyclonal antibodies to VDE have been generated. This amino acid sequence information has subsequently been used to fish out a cDNA encoding functional VDE (Bugos & Yamamoto, personal communication).

The possession of both antibodies to VDE and the cloning of its gene opens further avenues of VDE-related study. These tools may be used to explore the site of VDE activity in situ, its developmental pattern of expression, and expression in response to various stimuli and stresses. The level of xanthophyll cycle activity may be manipulated by antisense and overexpression to confirm (or deny) its role in the plant’s handling of excess light energy. The Abs may be used for a greater understanding of transit peptides as VDE is just the fourth lumenal protein to be cloned. VDE is a nuclear-encoded protein, so its transit peptide must direct it across three membranes to get to its site of activity. Protein and nucleotide probes for VDE may also be used to study the evolutionary history of VDE, potentially a key enzyme necessary for the establishment of land plants and even establishment in the upper strata of the water column where light may also be in excess to
an organism's photosynthetic capacity. Consequently VDE probes may be used as a measure of relatedness among all photosynthetic organisms. Using comparisons with other known proteins as well as site-directed mutagenesis, an understanding of VDE's different domains is possible - the catalytic site and the thylakoid association domain for example. The purification of VDE documented here has set the stage for the crystallization and exploration of VDE's three-dimensional structure by x-ray diffraction. Gene regulation studies of VDE are also now possible, and eventually the possible engineering of crops with a greater tolerance for excess light. Such would expand the regions that could sustain a given crop plant.
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