Violaxanthin De-Epoxidase

Purification of a 43-Kilodalton Lumenal Protein from Lettuce by Lipid-Affinity Precipitation with Monogalactosyldiacylglyceride

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Violaxanthin de-epoxidase catalyzes the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin in the xanthophyll cycle. Its activity is optimal at approximately pH 5.2 and requires ascorbate. In conjunction with the transthylakoid pH gradient, the formation of antheraxanthin and zeaxanthin reduces the photochemical efficiency of photosystem II by increasing the nonradiative (heat) dissipation of energy in the antennae. Previously, violaxanthin de-epoxidase had been partially purified. Here we report its purification from lettuce (Lactuca sativa var Romaine) to one major polypeptide fraction, detectable by two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using anion-exchange chromatography on Mono Q and a novel lipid-affinity precipitation step with monogalactosyldiacylglyceride. The association of violaxanthin de-epoxidase and monogalactosyldiacylglyceride at pH 5.2 is apparently specific, since little enzyme was precipitated by eight other lipids tested. Violaxanthin de-epoxidase has an isoelectric point of 5.4 and an apparent molecular mass of 43 kD. Partial amino acid sequences of the N terminus and tryptic fragments are reported. The peptide sequences are unique in the GenBank data base and suggest that violaxanthin de-epoxidase is nuclear encoded, similar to other chloroplast proteins localized in the lumen.

VDE is an enzyme of the xanthophyll cycle that is localized in the chloroplast lumen and catalyzes the conversion of V to A and Z in the presence of an acidic lumen and ascorbate. In vivo, de-epoxidation is induced when a high transthylakoid pH gradient develops in plants exposed to high light intensities. The accumulation of V, A, and Z along with the transthylakoid pH gradient, mediates nonradiative dissipation of light energy in the antennae (reviewed by Björkman and Demmig-Adams, 1993). This nonradiative dissipation of light energy is an alternative energy path that diverts energy from PSII, effectively down-regulating PSII’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, the mechanism is debated (Horton et al., 1994; Pfundel and Bilger, 1994; Yamamoto and Bassi, 1995).

The components of the xanthophyll cycle make up from 10 to 40% of the total carotenoids in leaves, depending on species and growth conditions. Growth in high light results in a larger pool of xanthophyll-cycle pigments (Thayer and Björkman, 1990). The cycle is also termed the V cycle to distinguish it from another xanthophyll cycle in diatoms involving diadinoxanthin and diatoxanthin (Hager, 1975). Study of the cycle itself has a long history. Sapozhnikov et al. (1957) first observed that the V concentration in leaves was reversibly decreased by light-dark treatments. Yamamoto et al. (1962) demonstrated that the changes were due to the stoichiometric and cyclical conversions among V, A, and Z.

Light induces the forward de-epoxidase reaction by establishing the necessary acidic lumen through the proton pump. The required acidity for de-epoxidase activity can also be generated by ATP hydrolysis or supplied by buffer (Hager, 1969; Yamamoto et al., 1972). In isolated chloroplasts, the addition of ascorbate, which is presumably lost during isolation, is also required as an essential and specific reductant for de-epoxidase activity. Ascorbate also supports a pseudocyclic electron transport mediated by the Mehler peroxidase reaction that can generate sufficient lumen acidity for de-epoxidation (Neubauer and Yamamoto, 1992).

Previously, VDE was partially purified from spinach (Hager and Perz, 1970) and lettuce (Lactuca sativa) (Yamamoto and Higashi, 1978). Although far from pure, these preparations allowed the characterization of several properties of the de-epoxidase. It was found that purified VDE itself requires a specific reductant for de-epoxidase activity. Ascorbate also supports a pseudocyclic electron transport mediated by the Mehler peroxidase reaction that can generate sufficient lumen acidity for de-epoxidation (Neubauer and Yamamoto, 1992).

Previously, VDE was partially purified from spinach (Hager and Perz, 1970) and lettuce (Lactuca sativa) (Yamamoto and Higashi, 1978). Although far from pure, these preparations allowed the characterization of several properties of the de-epoxidase. It was found that purified V was a poor substrate for VDE unless suspended with MGDG, the major lipid of chloroplast thylakoids (Yamamoto et al., 1974). Moreover, VDE itself requires a small amount of absorbed MGDG for activity (Yamamoto and Higashi, 1978).

Abbreviations: A, antheraxanthin; DGDG, digalactosyldiacylglyceride; Hex-II, hexagonal two-phase; MGDG, monogalactosyldiacylglyceride; PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyl-D-N-glycerol; PI, phosphatidylinositol; PS, phosphatidyl-L-serine; V, violaxanthin; VDE, violaxanthin de-epoxidase; Z, zeaxanthin.

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The de-epoxidase is stereospecific for xanthophylls that have the 3-hydroxy-5,6-epoxide group in a 3S, 5R, 6S configuration. Also, the polyene chain of the carotenoid must be all-trans. Thus, neoxanthin, which is 9-cis, is an inactive substrate and becomes active when isomerized to the all-trans form (Yamamoto and Higashi, 1978). The partially purified de-epoxidase was estimated to be 54 kD (Hager et al., 1990). A preliminary report of this work has appeared (Rockholm and Yamamoto, 1993).

DTT has been used extensively as a tool for examining the effects of de-epoxidation on nonradiative energy dissipation in both isolated chloroplasts and whole leaves. It is interesting that in thylakoids partial inactivation of VDE activity with DTT indicates that it has at least one critical disulfide linkage (Yamamoto and Kamite, 1972). It is interesting that in thylakoids partial inactivation of VDE activity with DTT results in A accumulation (Gilmore and Yamamoto, 1993). DTT has been used extensively as a tool for examining the effects of de-epoxidation on nonradiative energy dissipation in both isolated chloroplasts and whole leaves.

Herein we report the purification of VDE to a major component of 43 kD, as detected by two-dimensional IEF/SDS-PAGE. The critical final step in purification depends on the apparent specific association of VDE with MGDG into a precipitable complex. Because of this specificity, the treatment is named "lipid-affinity precipitation." We also report the partial amino acid sequences of the N terminus and several internal peptides obtained by trypic digestion. A preliminary report of this work has appeared (Rockholm and Yamamoto, 1993).

MATERIALS AND METHODS

VDE Activity Assay

VDE activity was assayed spectrophotometrically (DW2000; SLM Instruments, Urbana, IL) as A_{500} minus A_{540} in a model reaction system described previously, with slight modifications (Yamamoto, 1985). The reaction mixture was prepared fresh for each assay in a cuvette by mixing 100 μL of 270 μM MGDG in methanol, 100 μL of 10 μM V in acetone, 1.5 mL of 200 mM citrate buffer (pH 5.20), and variable amounts of sample and deionized water to 2.97 mL total volume. The reaction mixture was stirred continuously and after a stable absorbance baseline was established, 30 μL of 0.1 M sodium ascorbate were added to initiate the reaction. Activity was calculated from the initial rate of absorbance change using the difference extinction coefficient of 63 mM\(^{-1}\) cm\(^{-1}\). A unit of VDE activity is defined as 1 μmol of V de-epoxidized per minute (Yamamoto, 1985).

Purification of VDE

Market Romaine lettuce (Lactuca sativa var Romaine) was washed, de-ribbed, and chilled at 2°C. Typically 700 to 1000 g of leaf material were obtained from 5 kg of lettuce heads. The initial purification steps were carried out as described by Yamamoto and Higashi (1978) with some modifications. Lettuce leaves were homogenized at 1 g mL\(^{-1}\) grinding medium (50 mM Mops, pH 7.20, 400 mM sorbitol, 10 mM NaCl, and 16 mM sodium ascorbate) and sonicated through a 36-μm-mesh nylon cloth. The sonicate was centrifuged at 83,000g for 2.5 h at 3°C. The resulting pellets were resolubilized in 62.5 μL of 0.5 M sodium ascorbate and assayed for enzyme activity or examined by SDS-PAGE. Other lipids tested for their ability to precipitate VDE were cardiolipin (C), DGDG, PA, PC, PE, PG, PI, and PS, all obtained from Sigma.

Protein Determination, SDS-PAGE, and Electroblotting of Proteins

Protein concentration was determined using a modified Lowry method that is suitable for samples containing Tris-HCl, sodium citrate, or detergent (Bensadoun and Weinstein, 1976). BSA was used as the protein standard.

Separation by one-dimensional SDS-PAGE was carried out on 10% polyacrylamide minigels (102 × 83 × 0.5 mm) using a slight modification of the method of Laemmli (1970). Two-dimensional IEF/SDS-PAGE was also run using minigels according to the method of Moisaydi and Harrington (1989) with an ampholyte range between pH 4.5 and 6.5. Gels were stained with Coomassie brilliant blue R-250. Alternatively, proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore, 0.45 μM, Millipore) according to the method of LeGendre and Matsudaire (1989). The blotting unit was chilled, and the
proteins were transferred at a 100-mA constant current for 70 min. The membrane was briefly stained, and the VDE band was excised for amino acid analyses. The Biotechnology-Molecular Biology Instrumentation Facility (University of Hawaii, Honolulu) analyzed the samples for amino acid composition and N terminus sequence, and the Beckman Center (Stanford University Medical Center, Stanford, CA) determined the sequences of the N terminus and tryptic digests.

**RESULTS**

**Purification of VDE**

The procedure of Yamamoto and Higashi (1978) yields a VDE fraction that elutes from Sephadex G100 in a trough of the A$_{280}$ trace. Although VDE is purified severalfold at this stage (Table I), the fraction is still highly impure, as evidenced by the numerous proteins that are detectable in a two-dimensional IEF/SDS-PAGE (Fig. 1). Anion-exchange chromatography of Sephadex fractions on Mono Q showed VDE activity corresponding with the appearance of an A$_{280}$ peak (Fig. 2A). Analyses of successive fractions eluting from Mono Q by SDS-PAGE indicated a significant correlation ($r^2 = 0.953$) between the band of approximately 43 kD and VDE enzymatic activity (Fig. 2, B and C). No other protein band correlated similarly with activity. Rechromatography on Mono Q or chromatography on various hydrophobic interaction, cation-exchange, and size-exclusion columns did not achieve significant further purification. Precipitation at pH 5.20 of VDE with MGDG, however, proved highly successful. The precipitate had high specific activity and showed only one major polypeptide of about 43 kD by two-dimensional IEF/SDS-PAGE (Fig. 3). This purification was achieved at the expense of yield by collecting the most active fractions at each stage starting with the Sephadex G100 eluate. Thus, according to Table I, the yield was about 0.3% of the activity in chloroplasts. This is a conservative estimate, since the VDE in chloroplasts is probably overestimated relative to the assay of extracts. The former is mostly an assay of VDE in situ. Similarly, a firm conclusion cannot be drawn about the relative abundance of VDE in chloroplasts based on the purification factor of 15,000. Nevertheless, it can be concluded that VDE is a minor chloroplast protein. The purification relative to the pH 7.2 extract was 79-fold and about 17-fold higher than previously achieved (Sephadex G100 stage).

**Table 1. Purification table for VDE**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein (µg)</th>
<th>Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast</td>
<td>1.01 x 10$^7$</td>
<td>626</td>
<td>6.2 x 10$^{-2}$</td>
<td>1</td>
</tr>
<tr>
<td>pH 7.2 extract</td>
<td>1.20 x 10$^4$</td>
<td>140</td>
<td>11.7</td>
<td>189</td>
</tr>
<tr>
<td>Sephadex G100</td>
<td>1.71 x 10$^3$</td>
<td>91.2</td>
<td>53.3</td>
<td>860</td>
</tr>
<tr>
<td>Mono Q</td>
<td>14.6</td>
<td>11.1</td>
<td>760</td>
<td>1.2 x 10$^4$</td>
</tr>
<tr>
<td>Lipid-affinity</td>
<td>2.02</td>
<td>1.85</td>
<td>916</td>
<td>1.5 x 10$^4$</td>
</tr>
<tr>
<td>pellet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Two-dimensional IEF/SDS-PAGE of the peak activity fraction from a Sephadex G100 column. The fraction (6.8 ml) was concentrated to 100 µl and about one-half of the sample was used. The ampholyte was an equal mixture of pH 3.0 to 10.0 and pH 5.0 to 8.0. The masses of the molecular markers are indicated on the left in kD. The slab gel was 12.5% acrylamide and was stained with Coomassie brilliant blue R-250.

**Characteristics of the Lipid-Affinity Precipitation**

VDE did not precipitate when centrifuged in pH 5.2 buffer alone. With MGDG present, the precipitation of VDE was pH dependent, occurring at pH 5.2 but not at neutral or alkaline pHs (data not shown). The order of the nine tested lipid's ability to precipitate VDE from high to low was MGDG > DGDG > PC > PE > PG > PS > C > PI > PA (Fig. 4A). MGDG precipitated from about 4 to 38 times more VDE than the other eight lipids. The apparent specific association of VDE with MGDG suggests the term "lipid-affinity precipitation." The amount of VDE precipitated was also influenced by the MGDG concentration. MGDG at 2.9 µM was optimal; increasing concentrations to 29 µM precipitating less VDE. The presence of V (0.033 µM with 14.4 µM MGDG) slightly increased the amount of VDE precipitated, whereas the presence of an equal amount of Z significantly reduced the VDE precipitated, by more than 60% relative to the situation where V was present (Fig. 4B).

**Properties of Purified VDE**

Lipid-affinity-precipitated VDE was solubilized, run on SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane for partial amino acid sequence determination. Table II shows the sequences of the N terminus of VDE as well as of various tryptic fragments, the latter numbered according to their elution from capillary electrophoresis chromatography. The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A). The identical N-terminal sequence was also determined for the 43-kD polypeptide band eluting from the Mono Q column and resolved by SDS-PAGE (Fig. 2A).
Figure 3. Two-dimensional IEF/SDS-PAGE of the lipid-affinity precipitate (1 unit of VDE) resolubilized in 0.5% n-octyl-β-D-glucopyranoside and stained with Coomassie blue. The gel was 10% acrylamide, and the tube gel had equal amounts of pH 3.0 to 10.0 and 4.5 to 6.5 ampholyte. The masses (in kD) of molecular markers are indicated on the left.

Figure 2. Chromatography of VDE on Mono Q. A, The A₂₈₀ elution as a percentage of 0.02 full scale (heavy line), salt gradient (thin line), and VDE activity (stippled line). B, SDS-PAGE (10% acrylamide) of Mono Q fractions. Fraction numbers and molecular mass markers (in kD) are shown above and to the left of the gel, respectively. C, Bar graph of the densitometric measurements of the 43- and 58-kD bands in the indicated fraction. The line graph overlay is of the VDE activity in each fraction.

Figure 4. VDE activity in resolubilized pellets after lipid precipitation (n = 4). The error bars denote 95% confidence intervals. A, Various lipids (2.9 μM) were mixed with partially purified VDE (0.277 unit [U] activity) and 200 mM sodium citrate buffer (pH 5.2, 11.4 mL) and centrifuged. B, Partially purified VDE (3.487 unit) was mixed with MGDG (14.7 μM) and citrate buffer alone or additionally with either V or Z (0.33 μM). Different VDE preparations were used for the experiments in A and B.

The purification of VDE to one major component was achieved by the introduction of two additional steps to a previously published procedure (Yamamoto and Higashi, 1978). Anion-exchange chromatography on Mono Q gave a major improvement in specific activity (Table I). Lipid-affinity precipitation of VDE as a complex with MGDG, although improving specific activity “only” 120% over the previous Mono Q step, removed nearly all traces of contaminating protein (Fig. 3). Lipid-affinity precipitation using MGDG appears to be a novel approach (Fig. 4A).

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determined by two-dimensional IEF/SDS-PAGE using an ampholyte range of 4.0 to 6.5 (Fig. 3). The Kₘₜ of purified VDE for V and ascorbate were calculated by Lineweaver-Burk plots to be 0.352 μM for V and 4.4 mM for ascorbate (plots not shown).
MGDG is a Hex-II lipid and does not form bilayers when hydrated at physiological temperatures. Natural MGDG contains mostly unsaturated fatty acids in the cis configuration, giving MGDG a triangular shape unsuitable for bilayer formation (Gounaris and Barber, 1983). This shape is thought to promote the formation of tubular structures with the hydrophobic fatty acid tails facing outward that tend to aggregate in large arrays (Cullis et al., 1983). Hydrophobic interaction between VDE and MGDG is very likely the mechanism underlying the lipid-affinity precipitation, although possibly not the exclusive one. PE also can organize as a Hex-II lipid depending on pH, degree of the unsaturation in its lipid, calcium ion concentration, and temperature (Cullis et al., 1983). Here PE was no more effective for precipitating VDE than the other lipids under the conditions used. It is not excluded, however, that PE may precipitate VDE under conditions that assure formation of the Hex-II structure. Higher concentrations of the lipids increased the difference in recovery of VDE relative to MGDG (data not shown). The MGDG concentration used for precipitating VDE is about one-half that for saturating de-epoxidase activity (Yamamoto et al., 1974). Although the critical concentration for MGDG to precipitate VDE was not determined, higher concentrations of MGDG precipitated less VDE.

VDE associates with MGDG near the pI of VDE but not at either neutral or alkaline pH. Binding of VDE to thylakoids at the pH optimum for activity is the principle behind the differential pH extraction of VDE (Yamamoto and Higashi, 1978). Hager and Holocher (1994) recently confirmed the pH-dependent reversible association of VDE with the thylakoid membrane. Thus hydrophobic interaction is used twice in the current purification procedure, once for the differential extraction from thylakoids and again in the precipitation with MGDG. This binding of VDE to MGDG at acidic pH is probably also reflective of the binding that probably occurs in situ when the lumen of the thylakoid becomes acidified by the light-driven proton pump.

MGDG’s uniqueness is not limited to its ability to precipitate VDE. MGDG is the most abundant naturally occurring polar lipid by virtue of its being the dominant lipid in the world’s most extensive membrane system, the thylakoid (Allen et al., 1966). However, its role in thylakoid structure has not been completely explained. MGDG was significantly more effective in restoring photosynthetic electron transport in Triton-solubilized thylakoids compared to DGDG, PG, PE, or PC (Siefermann-Harns et al., 1982, 1987). Furthermore, the maximal extent of V de-epoxidation occurred in the presence of MGDG (five other lipids were tested) using a partially purified VDE sample (Yamamoto et al., 1974).

VDE precipitation by MGDG appears to be specific, although a low level of activity was recovered through the use of other lipids. DGDG is the second most abundant thylakoid lipid and is structurally similar to MGDG, and yet recoveries of VDE activity using it are similarly low and thus apparently nonspecific. PA, the most polar of the lipids examined, resulted in a much lower recovery of VDE activity. Such specificity suggests that the interaction between MGDG and VDE is not simply a hydrophobic one. Accordingly, the presence of V and Z also affects recovery (Fig. 4B), suggesting that the interaction may relate to some aspect of enzyme-substrate binding.

Precipitation of VDE by MGDG was optimal at a relatively low MGDG concentration (2.9 μM), lower than the 9 μM present in the assay of VDE in the model assay mixture (Yamamoto, 1985). In the assay, pure V is suspended at pH 5.20 with MGDG, presumably in the Hex-II form, and the de-epoxidase reaction is initiated by the addition of ascorbate. The decreasing recovery of VDE by lipid-affinity precipitation with increasing MGDG concentration may be due to hydrophobic interaction of MGDG with itself becoming greater than that of MGDG with VDE.

In vitro, V is a suitable substrate for VDE when suspended with MGDG. It is possible that de-epoxidation in vivo occurs through a similar lipid interface. Fractionation of pigment-protein complexes show V and the products of de-epoxidation associated mainly with the minor PSII fraction.

### Table II. Partial amino acid sequences for VDE

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
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<tr>
<td>N terminus</td>
<td>VLQVRKTCACCLKE</td>
</tr>
<tr>
<td>Tryptic fragment No. 9</td>
<td>LNDGFFTY</td>
</tr>
<tr>
<td>Tryptic fragment No. 11</td>
<td>T/E D/L DGSF - T (Q)</td>
</tr>
<tr>
<td>Tryptic fragment No. 15</td>
<td>SPTLPESTIPNFLQ T/(Q)</td>
</tr>
<tr>
<td>Tryptic fragment No. 21</td>
<td>(A) V/Q (T) F V/Q D P (T)</td>
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</table>

### Table III. Amino acid composition of VDE in mole percentages

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mole Percent</th>
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</thead>
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<tr>
<td>Basic</td>
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<tr>
<td>Arg</td>
<td>3.222</td>
</tr>
<tr>
<td>His</td>
<td>0.805</td>
</tr>
<tr>
<td>Lys</td>
<td>4.479</td>
</tr>
<tr>
<td>Uncharged</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>N.D.</td>
</tr>
<tr>
<td>Gly</td>
<td>4.802</td>
</tr>
<tr>
<td>Ser</td>
<td>2.970</td>
</tr>
<tr>
<td>Thr</td>
<td>5.437</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.153</td>
</tr>
<tr>
<td>Acidic and uncharged</td>
<td></td>
</tr>
<tr>
<td>Asx (Asp + Asn)</td>
<td>8.011</td>
</tr>
<tr>
<td>Glx (Glu + Gln)</td>
<td>8.896</td>
</tr>
<tr>
<td>Nonpolar</td>
<td></td>
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<tr>
<td>Ala</td>
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</tr>
<tr>
<td>Ile</td>
<td>3.041</td>
</tr>
<tr>
<td>Leu</td>
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</tr>
<tr>
<td>Met</td>
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</tr>
<tr>
<td>Phe</td>
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</tr>
<tr>
<td>Pro</td>
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</tr>
<tr>
<td>Trp</td>
<td>N.D.</td>
</tr>
<tr>
<td>Val</td>
<td>4.434</td>
</tr>
</tbody>
</table>

* N.D., Not determined.
tions (Peter and Thornber, 1991). However, there is no direct evidence that V in pigment protein complexes is the substrate for VDE. The first-order kinetics of de-epoxidation instead suggest that the de-epoxidase acts on a homogenous but substrate-limiting pool of V that increases with light intensity (Siefermann and Yamamoto, 1974). These observations can be explained by hypothesizing that (a) the pigments in the pigment-protein complexes are in rapid equilibrium with a pool of lipids, probably MGDG, surrounding these complexes; (b) the equilibrium distribution of V between lipid and protein phases is light dependent; and (c) VDE acts on V in the lipid fraction.

The molecular mass of VDE by native-gel electrophoresis and size-exclusion chromatography had been estimated to be 54 kD (Hager, 1975) and 60 kD (Yamamoto and Higashi, 1978), respectively. Recently, Hager and Holocher (1994) correlated VDE to a 58-kD polypeptide obtained from freeze-thaw treatments of thylakoids. Although a 58-kD polypeptide was present in our fractions from Mono Q chromatography, it did not correlate with VDE activity (Fig. 2). Furthermore, the 58-kD polypeptide was scarcely detectable in the lipid-affinity pellet. Thus VDE in Romaine lettuce was identified as a 43-kD protein. Western blots of Romaine lettuce (R. Bugos and H. Y. Yamamoto, personal communication). Therefore, it is unlikely that spinach VDE is significantly larger than the homologous enzyme in Romaine lettuce.

We repeated the determination of the VDE $K_m$ for V and ascorbate, which had been previously calculated from partially purified enzyme preparations. The $K_m$ of VDE for V was 0.352 $\mu M$ and for ascorbate 4.4 $\mu M$ by the Lineweaver-Burk method. Yamamoto and Higashi (1978) calculated a $K_m$ for V using VDE purified through to the Sephadex stage to be as low as 0.049 $\mu M$. At a higher MGDG:VDE ratio, the value is similar to that found here (0.145 $\mu M$). Hager (1975), using an assay that did not include MGDG, reported a considerably higher $K_m$ for V of 10.6 $\mu M$. The $K_m$ can be expected to vary with conditions that affect the formation of the enzyme-substrate complex, including the presence or absence of MGDG. In the absence of MGDG, de-epoxidation is significantly slower (Yamamoto et al., 1974). The $K_m$ of 4.4 $\mu M$ ascorbate is close to the value of 3.1 $\mu M$ ascorbate determined for intact chloroplasts (Neubauer and Yamamoto, 1994).

In light of the apparent hydrophobic interaction in lipid-affinity purification, two established protocols (Dekker, 1993) for reversed-micelle protein purification were tested (data not shown). Although the components differ, both methods use surfactants and buffer mixed in an organic solvent such that reversed micelles form, trapping some of the buffer within the micelles. Manipulating pH (and salt content) so that the surfactant head group and protein were oppositely charged and optimally attracted caused protein transfer to the aqueous core upon mixing of the micelle “with some degree of specificity” (Dwyer, 1993). Protein was recovered from within these reversed micelles by removing the organic layer and mixing in neutral buffer. Such methods, however, may not take into account lipid-specific interactions. The reversed micelle system of Wolbert et al. (1989) extracted 11 of 19 proteins tested. In our hands, the Wolbert et al. (1989) and Hilhorst et al. (1983) procedures successfully isolated a number of control proteins. In fact, each procedure isolated a different spectrum of thylakoid proteins; however, neither procedure recovered VDE activity or a detectable 43-kD polypeptide band on SDS-PAGE. This appears to further support the view that a specific lipid-affinity interaction occurs between MGDG and VDE.

We also examined MGDG precipitation at pH 5.2 from the initial extract at pH 7.20. This treatment did not precipitate VDE exclusively but did precipitate a limited number of proteins. Consequently, MGDG precipitation cannot be applied as a single-step purification of VDE, but the principle of lipid precipitation may be applicable for the purification of other membrane-associated proteins, especially in the final stages.

Each of the five partial amino acid sequences determined from VDE shown in Table II is unique in the GenBank data base. Furthermore, since GenBank contains the entire chloroplast genome from a number of different species, VDE appears not to be coded in the chloroplast because the partial sequences match neither previously designated proteins nor the product of any open reading frame. We conclude that the 43-kD polypeptide is VDE and is a newly described protein. However, isolation of a cDNA encoding the partial amino acid sequences and expression of activity will be required for definitive proof of the 43-kD protein’s identity.

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