Xanthophyll Cycle Enzymes Are Members of the Lipocalin Family, the First Identified from Plants*

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Violaxanthin de-epoxidase and zeaxanthin epoxidase catalyze the addition and removal of epoxide groups in carotenoids of the xanthophyll cycle in plants. The xanthophyll cycle is implicated in protecting the photosynthetic apparatus from excessive light. Two new sequences for violaxanthin de-epoxidase from tobacco and Arabidopsis are described. Although the mature proteins are well conserved, the transit peptides of these proteins are divergent, in contrast to transit peptides from other proteins targeted to the thylakoid lumen. Sequence analyses of both violaxanthin de-epoxidase and zeaxanthin epoxidase establish the xanthophyll cycle enzymes as members of the lipocalin family of proteins. The lipocalin family is a diverse group of proteins that bind small hydrophobic (lipophilic) molecules and share a conserved tertiary structure of eight β-strands forming a barrel configuration. This is the first reported identification of lipocalin proteins in plants.

The xanthophyll cycle is comprised of de-epoxidation and epoxidation interconversions of three xanthophylls (violaxanthin, antheraxanthin, and zeaxanthin), catalyzed by two enzymes that are localized on opposite sides of the thylakoid membrane. Violaxanthin de-epoxidase (VDE) is localized in the lumen of thylakoids and catalyzes de-epoxidation of violaxanthin in the presence of ascorbate and an acidic lumen, the latter formed by the light-induced proton pump (1–5). Zeaxanthin epoxidase is localized on the stroma side of the thylakoid membrane and catalyzes the epoxidation of zeaxanthin in the dark or under low light intensities (6, 7). The reaction is optimal near pH 7.5, and the enzyme utilizes oxygen, ferredoxin, and FAD as co-substrates (6–10). A role for zeaxanthin was therefore postulated (11) to protect the photosynthetic apparatus against the adverse effects of excessive light. Recent evidence demonstrates that accumulation of both antheraxanthin and zeaxanthin, along with the transthylakoid pH gradient, mediates the non-radiative dissipation of excess energy as heat (12–17). The xanthophyll cycle is thought to have evolved early in the development of higher plants as it is present in all plants examined thus far (18).

Pervaiz and Brew (19, 20) first identified a group of proteins, based on sequence homology, that have a common role in binding and transport of small hydrophobic molecules. These proteins, designated the lipocalins, represent a diverse group of proteins from the animal kingdom (for review see Ref. 21) and recently from a prokaryote (22). These lipocalin proteins have a common tertiary structure of an eight-stranded anti-parallel β-barrel, and only one protein to date displays catalytic activity. We report that violaxanthin de-epoxidase and zeaxanthin epoxidase are members of the lipocalin family. To our knowledge, they are the first lipocalins described from plants and only the second reported to demonstrate enzymatic activity. The deduced polypeptide sequences of three VDE proteins are compared, and the transit peptides are analyzed against other thylakoid lumen proteins.

EXPERIMENTAL PROCEDURES

cDNA Library Construction and Screening—A cDNA library was constructed from poly(A)+ RNA isolated from a pooled sample of young tobacco (Nicotiana tabacum cv. Xanthi) leaves using the Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech) and ligated into λ ZapII (Stratagene). The Arabidopsis cDNA library (designated as λ PRL2) was obtained from the Arabidopsis Biological Resource Center at Ohio State University. The library was derived from pooled mRNA from various tissues of the Columbia wild type of Arabidopsis thaliana (L.) Heynh. The cDNA libraries were screened using a random primed 32P-labeled probe prepared from the coding region of the lettuce violaxanthin de-epoxidase cDNA (23). Hybridization was performed according to Church and Gilbert (24) using a hybridization and washing temperature of 55 °C. Strongly hybridizing cDNAs were excised according to the manufacturer’s instructions.

Sequencing—Both strands of cDNA were sequenced completely using an Applied Biosystems model 373A automated sequencer.

RESULTS AND DISCUSSION

Violaxanthin de-epoxidase cDNAs were isolated and sequenced from Arabidopsis and tobacco. Analysis of the deduced polypeptide sequences of mature VDE proteins indicates high conservation among dicotyledonous plants (Fig. 1). Tobacco VDE shares 91.6 and 89.8% similarity and 82.6 and 82.0% identity with the Arabidopsis and lettuce VDE proteins, respectively. In the mature proteins only nine amino acid positions have different amino acids in all three proteins. All three proteins have a cysteine-rich domain, a lipocalin signature, and a highly charged domain as described previously for the lettuce violaxanthin de-epoxidase (23). The 13 cysteine residues that were reported for the lettuce sequence are invariant in all three proteins. Cysteines are functionally important because dithiothreitol is an inhibitor of VDE (25). Partial inhibition of VDE activity with low dithiothreitol concentrations results in an accumulation of antheraxanthin (13) suggesting that there is more than one disulfide linkage related to activity. One difference between the amino acid sequences is that the lettuce and Arabidopsis VDE have a highly charged peptide repeat (Glu-Val-Glu-Lys) whereas the tobacco sequence does not (underlined sequences in Fig. 1). The significance of this duplication is unknown because it does not appear to affect enzyme activity.

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§ The abbreviation used is: VDE, violaxanthin de-epoxidase.

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The transit peptides for the three proteins are boxed. Identical amino acid residues are indicated with an asterisk (cated with a sequence analysis software). Identical amino acid sequences was carried out with the program PILEUP (GCG)

\[\text{VDE proteins reveals no strong overall homology in primary structure except for the N- and C-terminal ends (Fig. 1). The VDE transit peptides are significantly longer than the transit peptides from the other lumenal proteins compared above.}

Two very short regions of local homology are observed in the VDE transit peptides at both ends of the peptide (Fig. 1). At the N terminus all three VDE transit peptides start with a Met-Ala dipeptide. Earlier analysis of 26 transit peptides of stromal proteins revealed that 85% had this same N-terminal dipeptide (30). Moreover, 18 thylakoid lumen proteins from various dicot plants including plastocyanin and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II show this same N-terminal Met-Ala dipeptide. This dipeptide probably signals the removal of the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can follow the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can

![Image](http://www.jbc.org/jbc/275/25/15322/fig2.jpg)

**FIG. 1.** Amino acid sequence comparisons of VDE proteins. The multiple alignment of the tobacco, Arabidopsis, and lettuce deduced amino acid sequences was carried out with the program PILEUP (GCG sequence analysis software). Identical amino acid residues are indicated with an asterisk. Dots indicate regions where gaps were inserted to maximize alignment. Asterisks mark the 13 invariant cysteine residues. The transit peptides for the three proteins are boxed.

Earlier analysis demonstrated that the lettuce VDE transit peptide is a typical bipartite type with an N-terminal portion representing a transit peptide for targeting to the chloroplastic stroma followed by a short hydrophobic thylakoid targeting signal peptide-like domain at the C-terminal end of the peptide (23, 26). Also, the cleavage site for the lettuce VDE was determined from the N-terminal sequence of purified VDE from lettuce (27). A comparison of the transit peptides for the three VDE proteins reveals no strong overall homology in primary structure except for the N- and C-terminal ends (Fig. 1). The transit peptides have a mean (± S.D.) percent similarity and identity of 49.3 ± 4.1 and 24.8 ± 2.2, respectively. We performed sequence comparisons on transit peptides from six plastocyanin, six 23-kDa polypeptides, and five 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II; all are proteins targeted to the thylakoid lumen. Mean percent similarities of 73.4 ± 7.3, 70.4 ± 8.7, and 80.4 ± 4.4 and mean percent identities of 60.6 ± 7.7, 60.4 ± 8.7, and 63.4 ± 5.8 were calculated for plastocyanin and 23- and 33-kDa polypeptides of the oxygen-evolving complex, respectively. In addition, the transit peptides for three zeaxanthin epoxidases, a protein targeted to the chloroplast stroma, have a mean percent similarity and identity of 77.5 ± 12.4 and 70.1 ± 15.4, respectively. The values for similarity and identity among the VDE transit peptides are significantly lower than for the other transit peptides discussed, indicating that the VDE transit peptides are more divergent between plant species. This divergence in the primary structure, the hydrophobic profiles for the transit peptides are quite similar (Fig. 2). The transit peptides are mostly hydrophilic except for the C-terminal end, which has a characteristic short hydrophobic region. Another difference is the variable lengths of VDE transit peptides. It was reported earlier that similar proteins in different organisms have comparable transit peptide lengths (29). For example, six plastocyanin sequences, five 33-kDa polypeptides, and six 23-kDa polypeptides of the oxygen-evolving complex associated with photosystem II, all lumenal proteins, have transit peptide lengths of 66–72, 79–85, and 73–82 residues, respectively. However, the three transit peptides for VDE have a range of 113–134 amino acids. Not only are the VDE transit peptides more variable in length, but they are overall significantly longer than the transit peptides from the other lumenal proteins compared above.

Two very short regions of local homology are observed in the VDE transit peptides at both ends of the peptide (Fig. 1). At the N terminus all three VDE transit peptides start with a Met-Ala dipeptide. Earlier analysis of 26 transit peptides of stromal proteins revealed that 85% had this same N-terminal dipeptide (30). Moreover, 18 thylakoid lumen proteins from various dicot plants including plastocyanin and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II show this same N-terminal Met-Ala dipeptide. This dipeptide probably signals the removal of the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can then be modified by acetylation (31, 32). At the cleavage site for the three VDE transit peptides, all exhibit a short homologous region having a consensus sequence of (Ala/Val)-(Asp/Glu) as VDE but also has a similar N terminus of the mature protein starting with a hydrophobic-(Asp/Glu) residue. Earlier analysis of 26 transit peptides of stromal proteins revealed that 85% had this same N-terminal dipeptide (30). Moreover, 18 thylakoid lumen proteins from various dicot plants including plastocyanin and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II show this same N-terminal Met-Ala dipeptide. This dipeptide probably signals the removal of the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can then be modified by acetylation (31, 32). At the cleavage site for the three VDE transit peptides, all exhibit a short homologous region having a consensus sequence of (Ala/Val)-(Asp/Glu) as VDE but also has a similar N terminus of the mature protein starting with a hydrophobic-(Asp/Glu) residue. Earlier analysis of 26 transit peptides of stromal proteins revealed that 85% had this same N-terminal dipeptide (30). Moreover, 18 thylakoid lumen proteins from various dicot plants including plastocyanin and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II show this same N-terminal Met-Ala dipeptide. This dipeptide probably signals the removal of the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can then be modified by acetylation (31, 32). At the cleavage site for the three VDE transit peptides, all exhibit a short homologous region having a consensus sequence of (Ala/Val)-(Asp/Glu) as VDE but also has a similar N terminus of the mature protein starting with a hydrophobic-(Asp/Glu) residue. Earlier analysis of 26 transit peptides of stromal proteins revealed that 85% had this same N-terminal dipeptide (30). Moreover, 18 thylakoid lumen proteins from various dicot plants including plastocyanin and the 16-, 23-, and 33-kDa polypeptides of the oxygen-evolving complex associated with photosystem II show this same N-terminal Met-Ala dipeptide. This dipeptide probably signals the removal of the N-terminal methionine (30–32). Most eukaryotic proteins lose their N-terminal methionine co- or post-translationally by a methionine aminopeptidase, especially when the methionine is followed by an alanine. The resulting N-terminal alanine can then be modified by acetylation (31, 32). At the cleavage site for the three VDE transit peptides, all exhibit a short homologous region having a consensus sequence of (Ala/Val)-(Asp/Glu) as VDE but also has a similar N terminus of the mature protein starting with a hydrophobic-(Asp/Glu) residue.
Because the xanthophyll cycle enzymes have a common substrate (antheraxanthin), it is reasonable to consider that zeaxanthin epoxidase may have a similar tertiary structure to VDE. The zeaxanthin epoxidase gene was recently identified from an abscisic acid-deficient mutant of *Nicotiana plumbaginifolia* that was impaired in zeaxanthin epoxidation (40). The resulting zeaxanthin epoxidase cDNA was also used to isolate cDNAs from pepper (10) and tomato (41). A motif search using the GCG sequence analysis software was performed with the three deduced polypeptide sequences for zeaxanthin epoxidase, but no lipocalin signature motif was detected. However, after closer examination of the sequences, a lipocalin signature motif can be identified in all three sequences. The lipocalin motif in the zeaxanthin epoxidase sequences has some slight differences that are not accounted for by the consensus sequence for the lipocalin signature motif.

The lipocalins are classified as kernel or outlier based on homology of three motifs from three structurally conserved regions of the lipocalin fold (21, 35, 42). The three structurally conserved regions are localized in: (a) the first β-strand and a short 310-helix preceding this β-strand; (b) portions of the sixth and seventh β-strands including the connecting loop; and (c) a portion of the eighth β-strand including the loop and part of the C-terminal α-helical structure (Fig. 3). In the folded protein these three regions are in close proximity to one another and are localized on one side of the barrel (21, 42). The first and largest group is known as the kernel lipocalins and shares homology in all three motifs whereas the other group is known as the outlier lipocalins and is more divergent because significant homology is only observed in motif I (the lipocalin signature). Outlier lipocalins exhibit weaker homology in motifs II and III in contrast to the kernel lipocalins. Sequence alignments of the motifs in a number of lipocalins from diverse species including mammalian, crustacean, insect, and prokaryote illustrate this homology (Fig. 3). Motif I is well conserved in all sequences with the key features of an invariant Gly followed by a positively charged residue in most cases and an invariant Trp followed by a residue with a ring structure. Included in these comparisons are two outlier proteins (human α,α-acid glycoprotein and rat von Ebner’s gland protein) exhibiting homology in motif I but somewhat less homology in motifs II and III. Also included in this motif comparison is the enzyme prostaglandin D synthase, the only known lipocalin to date to have catalytic activity. Here we introduce violaxanthin de-epoxidase and zeaxanthin epoxidase to the lipocalin family. They are the first lipocalins identified from plants and are unique in that they also have catalytic activity. These enzymes of the xanthophyll cycle share homology in the structurally conserved regions especially in motif I with the invariant Gly and Trp present. Homology in motifs II and III is much weaker than in kernel lipocalins, suggesting that these enzymes would fall in the class of outlier lipocalins.

To further analyze whether the xanthophyll cycle enzymes fit the overall structure of the lipocalin model, the distances between the three motifs were compared for the various lipocalin proteins. Both kernel and outlier lipocalins have the same characteristics with respect to the spacing of the motifs. Typically, the distances between motifs I and II range from 65 to 73 amino acids whereas the range between motifs II and III is 10–17 amino acids (Fig. 4). The tobacco VDE fits the pattern of other lipocalins with distances of 72 and 15 amino acids between motifs I and II and motifs II and III, respectively. The main difference between VDE and the other lipocalins is the long overall structure of the lipocalin model, the distances between the motifs in a number of lipocalins from diverse species including mammalian, crustacean, insect, and prokaryote illustrate this homology (Fig. 3). Motif I is well conserved in all sequences with the key features of an invariant Gly followed by a positively charged residue in most cases and an invariant Trp followed by a residue with a ring structure. Included in these comparisons are two outlier proteins (human α,α-acid glycoprotein and rat von Ebner’s gland protein) exhibiting homology in motif I but somewhat less homology in motifs II and III. Also included in this motif comparison is the enzyme prostaglandin D synthase, the only known lipocalin to date to have catalytic activity. Here we introduce violaxanthin de-epoxidase and zeaxanthin epoxidase to the lipocalin family. They are the first lipocalins identified from plants and are unique in that they also have catalytic activity. These enzymes of the xanthophyll cycle share homology in the structurally conserved regions especially in motif I with the invariant Gly and Trp present. Homology in motifs II and III is much weaker than in kernel lipocalins, suggesting that these enzymes would fall in the class of outlier lipocalins.

![Fig. 3. Model of the lipocalin fold and motif comparisons. The main structure consists of eight β-strands (arrows labeled 1–8) forming the anti-parallel β-sheet along with an N-terminal helical turn and a C-terminal α-helix. This structure folds into a barrel, and the N-terminal polypeptide chain passes along the bottom of the barrel to form the closed end. There are three main structurally conserved regions (I–III) in the model, and sequences of these regions (motifs I–III) are compared. Identical regions are shaded with a black background whereas regions displaying high homology are shaded gray. The proteins (with accession numbers) are as follows: HMGC, human α1-microglobulin (P02760); RBP, human plasma retinol-binding protein (P02753); PDS, human prostaglandin D synthase (M61900); AGP, human α1-acid glycoprotein-1 (P02763); NGL, human neutrophil gelatinase-associated lipocalin (P80188); VGP, rat von Ebner’s gland protein 1 (P20289); MRP, rat α2-microglobulin-related protein (P30152); CRU, lobster crustacyanin A2 subunit (P80007); BBP, cabbage white butterfly bilin-binding protein (P09464); INS, tobacco hornworm insecticytin (Q80650); VCL, Vibrio cholerae vlpA lipoprotein (AF025663); LVDE, lettuce violaxanthin de-epoxidase (U31462); AVDE, Arabidopsis violaxanthin de-epoxidase (U41433); TVDE, tobacco violaxanthin de-epoxidase (U34817); LZEP, tomato zeaxanthin epoxidase (Z83835); PZEP, pepper zeaxanthin epoxidase (X91491); and TZEP, tobacco zeaxanthin epoxidase (X95732).](http://www.jbc.org/doi/10.1074/jbc.15323)
shows that the binding pocket of insecticyanin is decidedly hydrophobic (38), and the binding pocket for retinol-binding protein is composed of both hydrophobic and uncharged residues (36). Analysis of the xanthophyll enzymes to determine the amino acid residues important in binding and catalytic activity to help further our understanding of the role of these enzymes in photoprotection in plants is now warranted.

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