Mutation Analysis of Violaxanthin De-epoxidase Identifies Substrate-binding Sites and Residues Involved in Catalysis*

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Plants are able to deal with variable environmental conditions; when exposed to strong illumination, they safely dissipate excess energy as heat and increase their capacity for scavenging reacting oxygen species. Both these protection mechanisms involve activation of the xanthophyll cycle, in which the carotenoid violaxanthin is converted to zeaxanthin by violaxanthin de-epoxidase, using ascorbate as the source of reducing power.

In this work, following determination of the three-dimensional structure of the violaxanthin de-epoxidase catalytic domain, we identified the putative binding sites for violaxanthin and ascorbate by in silico docking. Amino acid residues lying in close contact with the two substrates were analyzed for their involvement in the catalytic mechanism. Experimental results supported the proposal of substrate-binding sites and point to two residues, Asp-177 and Tyr-198, which are suggested to participate in the catalytic mechanism, based on complete loss of activity in mutant proteins. The role of other residues and the mechanistic similarity to aspartic proteases and epoxide hydrolases are discussed.

In natural environments, light intensity is variable and often exceeds the saturation limit of photosynthesis (1, 2). As a consequence, excitation energy in excess may lead to production of reactive oxygen species and to oxidative stress, in a process called photoinhibition (2, 3). Photosynthetic organisms have evolved several mechanisms to dissipate excess energy safely and to increase the capacity for scavenging reactive oxygen species. Major role is played by the xanthophyll cycle (4, 5) in which the diepoxide xanthophyll violaxanthin is converted into the epoxide-free zeaxanthin. Zeaxanthin is a key molecule for plant photoprotection, being involved in singlet oxygen scavenging as well as singlet chlorophyll quenching (6–10).

Violaxanthin to zeaxanthin conversion is catalyzed by a luminal enzyme, called violaxanthin de-epoxidase (VDE). The reducing power for the reaction is provided by ascorbate (11), probably in its protonated form (12). VDE is activated when light-driven proton translocation across the thylakoid membrane exceeds the dissipation rate of the proton gradient by ATPase, leading to a decrease in pH in the thylakoid lumen. Inactive VDE is a soluble protein, but upon activation, it associates with the thylakoid membrane (13) where its substrate violaxanthin is located (14). When light intensity decreases, the stromal enzyme zeaxanthin epoxidase converts zeaxanthin back to violaxanthin (15, 16).

Both VDE and zeaxanthin epoxidase have been suggested to belong to lipocalins, a multigenic protein family characterized by a conserved structural organization with an 8-strand β-barrel (15). VDE and zeaxanthin epoxidase are classified among outlier lipocalins because they do not present all three conserved regions typical of this multigenic family. Because of their rather low similarity with other lipocalins, their true membership of the lipocalin family has been challenged (17). In addition, VDE and zeaxanthin epoxidase are the only lipocalins, together with prostaglandin D synthase, that have enzyme activity, although most family members are involved in molecular transport (15, 18, 19). Beside the protein domain sharing similarity with lipocalins, which represents approximately half of the protein, VDE has two additional domains, with no clear homology to any other known protein, called the cysteine-rich and glutamate-rich domains (20, 21).

The structure of the VDE putative lipocalin domain (VDEcd) was resolved by x-ray crystallography, showing that VDE is indeed a lipocalin with the typical conserved three-dimensional organization of an 8-strand β-barrel (22). That work also showed a pH-dependent conformational change associated with protein activation and dimeric organization at pH 5.0, which allows both violaxanthin rings to react at the same time (22).

In this work, we investigated the VDE structure in more detail, with the aim of identifying the key residues involved in its catalytic activity. We first used in silico docking analysis to identify putative binding sites for its substrates violaxanthin and ascorbate. The importance of residues located in close proximity to the substrates was then accessed by mutational analysis. On these bases, we propose that two residues, Asp-177 and Tyr-198, are fundamental for the catalytic mechanism and we discuss similarities with other enzyme catalytic sites.
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**EXPERIMENTAL PROCEDURES**

**Docking**—The ligands, violaxanthin and ascorbic acid, were docked into the crystal structure of the active form of the VDE lipocalin domain (VDEcd) obtained at pH 5 (Protein Data Bank code 3CQR (22)), using AUTODOCK 4.0 (23). The parameters for the molecules were calculated by the AUTODOCK standard parameterization procedure. The Lamarckian Genetic Algorithm and 25 million energy evaluations per run were applied as a search method for the various docking results (for further details, see supplemental material). Before performing the docking procedure, we calculated the residue protonation state, i.e. the pKa values of the lipocalin domain (VDEcd), using MCCE version 2.4 (24) and DELPHI version 4 (25, 26). MCCE default parameters were used, and at least three independent runs were performed. The protonation state at pH 5 was thus calculated for all residues of VDE according to their calculated pKa value. An in-depth analysis of these results will be presented elsewhere.  

The following various combinations were considered for docking: (a) violaxanthin and dimeric VDE; (b) ascorbic acid and the VDE-violaxanthin complex; and (c) ascorbic acid and dimeric VDE. The potential grid map for each atom type was calculated by means of a cubic box centered in the putative binding cavity, with a distance of 0.375 Å between grid points. For each complex, 200 docking runs were performed, giving a total of 600 calculations. Ligand locations were then hypothesized by choosing the lowest energy conformations within the most densely populated clusters. In the case of the ascorbic acid docking experiments, the two most densely populated clusters were considered. Cluster analysis was based on the root mean square deviation distance among the ligands on each run (root mean square deviation cutoff, 2 Å). The minimal distances between violaxanthin, ascorbate, and the protein were calculated by choosing the lowest energy conformations within the total of 600 calculations. Ligand locations were then hypothesized as described above, was incubated with thylakoids as described previously (33). Briefly, 40 µl of partially purified VDE was added to 120 µg of chlorophyll of thylakoids and 40 µl of buffers with variable pH values (MES, HEPES, or Tris). Samples were incubated in the dark at 4 °C with mild agitation for 2 h and centrifuged at 13,000 × g for 10 min to precipitate thylakoids. The presence of VDE in supernatants (and pellets) was accessed by an antibody against His tag (from Sigma) or with homemade antibody raised against A. thaliana VDE (32).

**Membrane Binding Assays**—VDE, overexpressed and purified as described above, was incubated with thylakoids as described previously (33). Briefly, 40 µl of partially purified VDE was added to 120 µg of chlorophyll of thylakoids and 40 µl of buffers with variable pH values (MES, HEPES, or Tris). Samples were incubated in the dark at 4 °C with mild agitation for 2 h and centrifuged at 13,000 × g for 10 min to precipitate thylakoids. The presence of VDE in supernatants (and pellets) was accessed by an antibody against the His tag, which thus specifically binds the recombinant protein.

**RESULTS**

**Docking of Violaxanthin in VDEcd Structure at pH 5**—After VDEcd crystals at pH 5 had been obtained, one main objective was to reveal the structure of the enzyme-substrate complex. Unfortunately, all trials to obtain crystals of such a complex failed, because the substrate either inhibited crystal growth or did not bind to the protein previously crystallized. Analysis of protein packing within the crystals of the VDE lipocalin domain (VDEcd) showed that the barrel cavity, where the carotenoid violaxanthin is expected to bind, is rendered inaccessible by the interference of neighboring molecules, suggesting a reason for these failures. We therefore attempted the alternative approach of structural modeling to gain information on the violaxanthin-binding site and to characterize the structural determinants of the protein-active site. The violaxanthin molecule was docked in silico into the VDEcd structure at pH 5 by AutoDock 4.0. The docking calculations provided us with several clusters of conformations. The most densely populated (160 of 200 decoys), also corresponding to the conformations with lower energy values, allowed us to choose a preferred conformation. The latter was then funneled into a procedure of local optimization by the use of flexible side chains within the AutoDock 4.0 software (see supplemental material). The lowest energy ligand-protein conformation is shown in Fig. 1; one violaxanthin molecule is bound to a VDE dimer, through a connection between the cavities of each monomer, a model consistent with a previous proposal (22). We also performed the same docking experiments.
using antheraxanthin, which also binds to the VDE dimer like violaxanthin (data not shown).

**Identification of Amino Acids Potentially Involved in Catalysis**—To assess experimentally the validity of the *in silico* model, we tentatively identified residues potentially involved in enzyme activity by selecting those located within a distance of 6 Å from the ring of the modeled violaxanthin molecule. Fig. 1C shows the residues identified; they are also listed in Table 1, together with their minimal distances from the violaxanthin ring.

To complement structural information, we also analyzed VDE sequences from various algal and plant species. Among the former group, we considered in particular VDE protein sequences from diatoms, because they do not belong to Viridiplantae and are evolutionarily distant from vascular plants (34). They have a diadinoxanthin-diatoxanthin cycle but are also able to convert violaxanthin into zeaxanthin (35). Analysis of the diatom genome revealed that VDE from plants and diatoms shares a common evolutionary origin with one well conserved protein (34). In this case, we can assume that the key residues for catalysis are conserved. Fig. 2 shows a sequence alignment with the VDE lipocalin domain from various plant and diatom species; the protein sequences show a remarkable similarity, and 50–55% of residues were completely conserved in plants and diatoms.

From the alignment shown in Fig. 2 it can be inferred that conservation is even higher for residues identified as close to violaxanthin; 11 of 13 (85%) are identical in all species, which points to strong evolutionary pressure for their conservation and therefore an important role for enzyme activity or structural integrity.

**Mutational Analysis of Putative Residues Involved in Enzyme Activity**—If the docking model is correct, we expect residues identified as the closest to the violaxanthin head group to play an important role in the de-epoxidation reaction. To verify this hypothesis, all 11 conserved residues were subjected to site-directed mutagenesis. The rationale for amino acid substitution was to alter chemical properties (charge, polarity or aromatic nature) without major changes in their size. Table 1 lists all mutations performed.

All mutant proteins were expressed in *E. coli* Origami cells and purified by affinity chromatography. The purified enzymes

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**TABLE 1**

Amino acid residues identified as closest to violaxanthin and their role in enzyme activity

| Residue | Distance from violaxanthin | Mutation to | Activity (% WT) |
|---------|---------------------------|-------------|----------------|
| His-121 | 4.0                       | Ala         | 5 ± 1          |
| Phe-123 | 4.3                       | Ala         | 34 ± 12        |
| Asn-134 | 5.7                       |             |                |
| Ile-135 | 3.4                       |             |                |
| Gln-153 | 2.4                       | Ala         | 49 ± 10        |
| Glu-153 | 5.7                       | Leu         | 56 ± 22        |
| Tyr-175 | 5.5                       | Phe         | 42 ± 11        |
| Asp-177 | 3.0                       | Ala         | ND             |
| Asp-178 | 5.9                       | Ala         | ND             |
| Trp-179 | 3.0                       | Ala         | ND <2%          |
| Tyr-198 | 5.5                       | Phe         | ND <2%          |
| Tyr-214 | 3.5                       | Phe         | ND <2%          |

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**FIGURE 1.** Model of violaxanthin docking in VDEcd at pH 5. A, VDEcd structure at pH 5 (Protein Data Bank 3CQR) is shown with docked violaxanthin molecule; Surface of amino acids is shown for protein, whereas violaxanthin is shown as *orange spheres*. One monomer is shown in *gray* and the other in *light blue*. B, same structure after 90° rotation; amino acid volume is 80% transparent, and chains are shown as *blue ribbons*. C, detail of structural model. Residues identified as close to violaxanthin ring (under 6 Å threshold) are shown as *sticks* and labeled according to numbering from *A. thaliana* mature protein.
were quantified by Western blotting to verify expression levels, which were found to be similar for WT and all mutants. Because a fraction of expressed VDE is normally found insoluble after cell lysis, a reduction in purification yield was expected if the mutations caused strong alterations in protein folding. In our case, instead, the invariance of protein expression suggests that none of the mutations significantly affected protein folding.

The activities of mutant enzymes were measured, and the results are listed in Table 1. With the only exception of N167A, all mutations produced a significant effect on VDE activity. This supports the idea that the identified residues have indeed been highly conserved through evolution, because of their importance for enzyme activity. Nevertheless, not all mutations altered enzyme activity equally; in the case of mutations on Asp-177 and Tyr-198, we did not detect any residual activity, whereas strongly reduced, mutants at sites Trp-179 and Gln-119 strongly reduced activity, mutants at sites Trp-179 and Gln-119 strongly reduced activity, mutants at sites Trp-179 and Gln-119 strongly reduced activity. In addition, although strongly reduced, mutants at sites Trp-179 and Gln-119 strongly reduced activity, mutants at sites Trp-179 and Gln-119 strongly reduced activity. The activity of the Tyr-214 still retained the ability to yield small amounts of zeaxanthin, which was consistent with spectrophotometric assays. This was especially important in the case of mutations on the tyrosyl OH group, ascorbate does not bind in the proper orientation and probably cannot provide reducing power to the reaction. Conversely, T245A showed activity equivalent to that of WT, implying that this residue is not involved in the enzymatic mechanism. These results thus support the hypothesis that the identified residues have indeed been highly conserved through evolution, because of their importance for enzyme activity.

Ascorbate Docking into VDEcd Structure—The VDE catalytic reaction requires ascorbate as the source of reducing power. For more insights on its binding site, we extended docking analyses to this second substrate. According to the literature, VDE should bind protonated ascorbic acid rather than ascorbate (12); however, docking experiments with both forms did not yield significantly different results.

Because there is no information on the order of binding of the two substrates, we performed ascorbate docking experiments with both the apo-VDE structure and the VDE-violaxanthin complex. Invariance of results indicates that ascorbate can access its binding site independently of the presence or absence of violaxanthin. We also verified that the inverse is true, i.e. violaxanthin docking is not significantly affected by the presence of ascorbate.

Docking calculations with ascorbate yielded two densely populated clusters of 60/200 and 100/200 conformations, the former having slightly more favorable binding energies (Fig. 3 and supplemental Table 1). Because in silico data were not conclusive, we considered both clusters as putative ascorbate-binding sites (Fig. 3, A and B). It should be noted that in both cases ascorbate binds in a very similar position, although with different orientations. Analysis of interactions with the polypeptide chain showed that, in both clusters, ascorbate interacts with Thr-112, Asp-114, and Gln-119. Instead, interactions with Tyr-198 and Thr-245 are specific for only one of the binding conformations. All residues except Thr-112 are conserved in VDE sequences from various species, as shown in the alignment in Fig. 2, supporting their significance for protein activity.

To validate the in silico calculations experimentally, all the conserved residues were subjected to site-directed mutagenesis. The corresponding measured activities are listed in Table 2. Thr-112 was not mutated, not only due to its variability through the family of VDEs but also because its interaction with ascorbate involved the protein backbone and was likely to be poorly affected by any mutation. Strong reduction in activity was observed for the residues that were proposed to interact with ascorbate in both models, Asp-114 and Gln-119, the mutation of the former leading to complete inactivation of the enzyme. However, Asp-114 has previously been suggested to be important for VDE dimer stability (22), which prevented attributing loss activity only to ascorbate binding. Conversely, Gln-119 is located far from the violaxanthin epoxy group, is not engaged in inter-monomer interactions, and is probably not involved in protein conformational changes, suggesting that the mutant phenotype is indeed due to altered ascorbate binding.

These data support the results on the ascorbate binding region obtained in silico. The remaining mutants are useful for distinguishing between the two possible binding conformations. Y198F showed complete loss of activity, suggesting its great importance for ascorbate association; in the absence of the tyrosyl OH group, ascorbate does not bind in the proper orientation and probably cannot provide reducing power to the reaction. Conversely, T245A showed activity equivalent to that of WT, implying that this residue is not involved in the enzymatic mechanism. These results thus support the hypothesis that...
that ascorbate binds as shown in the model in Fig. 3A. Fig. 3, C and D, shows the structural model of the VDE-ascorbate complex in more detail, which allows recognizing the presence of a small binding pocket where the ascorbate is located. It is worth noting that the identified ascorbate-binding site is located close to the violaxanthin ring, in a very good position to provide reducing power for the catalytic reaction.

Assessment of Membrane Binding Capacity of Mutants—Violaxanthin is a hydrophobic molecule found dissolved in the thylakoid membrane. Thus, VDE, a soluble protein at neutral pH, needs to bind to the membrane to perform catalysis (16, 36). Therefore, it is possible that the observed reduction in mutant activity is a secondary effect, due to the inability of the enzyme to bind to the membrane, rather than an alteration in its catalytic activity. To exclude this possibility in any of the mutants analyzed above, we verified the ability of each mutant to associate with the membrane by incubating the protein at various pH values in the presence of a thylakoid membrane preparation. At acidic pH values, VDE binds to the membrane and can be precipitated by brief centrifugation, whereas unbound VDE remains in the supernatant (21, 33), where it can be detected by Western blotting (Fig. 4). In the case of WT, it is clear that VDE at pH 5 or below is hardly detectable in the supernatant, showing that it precipitates together with thylakoids. Fig. 4 shows the same test for the mutant proteins, revealing the greatest reduction in their activity; in all cases, membrane association occurs very similarly to that of WT, and at a pH below 5, the protein is bound to the membrane. These results clearly show that the strong decrease in activity was not due to impairment of the membrane association. The same test was performed for all mutants analyzed in this work, and in no case did we observe alteration of binding to the membrane (data not shown). These results confirm that the reduction in activity we observed was indeed due to effects on the catalysis and/or binding of substrates. In addition, these experiments provide support to the fact that all analyzed mutant proteins are correctly folded, i.e., if they had been misfolded, they would not have been able to bind to the membrane in a pH-dependent manner, a capacity that requires a specific conformational change (37). For further confirmation, we also measured the CD spectra of inactive mutants, which showed that their folding was very similar to that of WT (supplemental Fig. 1).

**DISCUSSION**

**Identification of VDE Active Site**—In this work, we docked in silico violaxanthin and ascorbate to the VDE structure at pH 5 to build a model of the enzyme-substrate complex. This model was thereafter experimentally supported by site-directed mutagenesis, which confirmed positive identification of binding sites for both VDE substrates, i.e., violaxanthin and ascorbate.

In silico and mutational analyses also allowed us to identify, among the residues lying close to the binding cavity, those directly involved in enzyme activity. Two residues were found to be essential for activity, Asp-177 and Tyr-198, and any change in charge/polarity caused complete loss of activity. In these mutants, no traces of antheraxanthin or zeaxanthin were detectable upon activity tests, even when prolonged well beyond standard reaction times (data not shown). It is worth recalling here that the effect of these mutations cannot be
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attributed to disrupted protein folding, because protein expression and purification yields were substantially unaffected in the mutants with respect to WT, and membrane binding activity tests were unaltered (Fig. 4). It is highly unlikely that an unfolded protein can bind to thylakoid membranes in a pH-dependent manner, as binding requires a conformational change (37). Finally, inactive mutants also have CD spectra indistinguishable from WT (supplemental Fig. 1).

It has previously been shown that peptatin A, an inhibitor of Asp proteases, also affects VDE activity. This observation suggested that the reaction centers of VDE and aspartic protease have a common structural organization (37) and supported the first idea that an active aspartate is involved in the VDE catalytic mechanism (38).

More importantly, other enzymes active in the degradation of epoxy groups, such as epoxide hydrolases, also rely on aspartate residues for epoxy cycle opening (39). In these hydrolases, the first reaction step involves a nucleophilic attack on the epoxy group which, in an acidic environment, is known to be sensitive to such an attack. In the case of VDE, we may hypothesize that the reaction starts with protonation of epoxide oxygen, followed by a nucleophilic attack on the carbon.

All previous considerations point to the identification of Asp-177 as responsible for the first reaction in VDE catalysis. It is worth noting that calculation of $pK_a$ values in the structure yielded a value of 5.8 for Asp-177, which is therefore mostly protonated at pH 5.4. Thus, Asp-177 is the best candidate to act as a proton donor to the violaxanthin epoxy group. This hypothesis also matches the violaxanthin binding model, which locates Asp-177 within 3 Å of the epoxy group (Fig. 5A).

The other residue fundamental for activity is Tyr-198, which is located relatively far (7.6 Å) from the epoxy group. Other residues, like Trp-179, are found closer but are not as important for enzyme activity. However, the primary importance of Tyr-198 for VDE activity is easily understood by analysis of the docking experiments; when ascorbate is bound in its pocket with the proper orientation, it forms a hydrogen bond with Tyr-198 and thus allows efficient electron donation to violaxanthin when the epoxy ring opens (Fig. 5A).

Other Residues with an Important Role in VDE Activity—In addition to Asp-177 and Tyr-198, other residues play an important role in VDE activity, as their mutation greatly reduces enzyme activity, although small amounts of zeaxanthin are still produced. Among these residues is Trp-179, which contributes to violaxanthin binding by hydrophobic interaction, as explained by the fact that overpopulation of aromatic residues interacting with carotenoid molecules has been observed in many other carotenoid-binding proteins. A tryptophan molecule interacting with the head ring of a xanthophyll has been found in the LHCII structure (40), and interactions between carotenoids and aromatic residues have also been observed in photosynthetic reaction centers, bacterial light-harvesting proteins (41), and the orange carotenoid-binding protein (42). All these pigment protein complexes bind carotenoids, but they do not share any evolutionary relationship with each other, indicating that aromatic residues are particularly suitable for carotenoid association. We speculate that the protein-carotenoid substrate is a multicomponent interaction, and in the absence of Trp-179, violaxanthin is still bound but probably with reduced affinity and/or altered orientation, which explains the strong mutant phenotype.

Tyr-214 is another aromatic residue with a major effect on VDE activity. However, in this case, the phenotype is not due to its aromatic nature but to the presence of an OH group; its mutation into phenylalanine maintains its aromatic nature but still induces a drastic reduction in protein activity. One explanation for this phenotype is the fact that Tyr-214 is hydrogen-bound to His-121 in the structure at pH 7, suggesting that it may be involved in the pH-induced conformational change, like His-121 (22).

The next closest residues to violaxanthin in the structural model...
are Phe-123 (4.3 Å), Gln-153 (2.4 Å), and Phe-155 (2.9 Å). They are probably involved in enzyme-substrate complex stabilization, as their mutation induces only partial inactivation, and significant amounts of zeaxanthin are still produced. The case of Gln-153 is interesting because, in the structural model, the residue is involved in a putative hydrogen bond with violaxanthin, which may help to bind the carotenoid in its correct orientation. Consistent with the hypothesis that polar interactions with the substrate are significant, the strongest effect is observed when Gln is substituted with Leu. The consequences with the substrate are significant, the strongest effect is thin, which may help to bind the carotenoid in its correct orientation.

A Dimeric Model for VDE—Structural data on VDE suggest that, in its active conformation, the protein is a dimer. This organization is unlikely to be a crystallization artifact, because the monomer-monomer interfaces are larger for VDE than for other lipocalins well known to be dimers (22, 43, 44). Our docking experiments support the identification of the violaxanthin-binding site in a VDE dimer, and mutational analysis is consistent with in silico calculations.

In this respect, it is interesting to compare the structural model of violaxanthin binding presented here with experimental data on VDE activity in various carotenoid species. VDE has been shown to be active with carotenoids in all-trans configurations. Violeoxanthin, which is identical to violaxanthin except for the presence of a double bond in cis configuration, is not de-epoxidized by VDE, even on a single end of the molecule (29). Because the rest of the molecule is identical, this difference can only be due to the molecular shape of the carotenoid, which cannot fit into the VDE cavity. This result led to the estimation of 30 Å for the length of the violaxanthin-binding site (29). This is consistent with the dimeric model which, in the active form, contains a cavity having the size of a violaxanthin molecule, and the distances between the active sites are around 30 Å (Fig. 5B). Instead, in the case of a monomeric VDE, the cavity would be around 15 Å long, and violeoxanthin would be bound as well as violaxanthin.

Several inter-chain interactions further support the dimeric model, and particular significance must be attributed to that between Asp-114 and Arg-138 in the companion monomer. Mutations in either of these two residues, impairing salt bridge formation, have been shown to abolish VDE activity completely (22). Our results clearly show that these two residues are neither in close proximity to the substrate nor to any other residue essential for the activity of VDE, and thus the involvement of this salt bridge in dimer stabilization is the most probable hypothesis explaining the strong mutant phenotype.

All these considerations support the idea that the active VDE form is a dimer. However, we might still ask why this oligomeric state had never been observed before. The most probable answer is that VDE requires lipids for its activity (29, 45), whereas dimerization and lipid association are closely connected. This is consistent with the observation that we never succeeded in maintaining VDE in solution at low pH without lipids. However, unfortunately, the presence of large lipid particles impairs determination of native molecular weight and thus of oligomeric state.

Finally, the dimeric model raises the question as to how antheraxanthin forms. One explanation is that we identified two ascorbate-binding sites for one dimer. If one of these sites is empty then, upon violaxanthin binding, we would expect antheraxanthin to form instead of zeaxanthin. This fits two further considerations. First, it has been shown in isolated chloroplasts that ascorbate can be limiting for zeaxanthin formation, and in this case, we expect antheraxanthin to form (46). Second, the $K_m$ value for ascorbate is much larger than that for violaxanthin (1 mm versus 5 μM (12, 47)), so that one ascorbate-binding site might remain empty in a fraction of active sites.

An alternative/additional explanation why the dimeric VDE fails to produce zeaxanthin alone is found in the above-mentioned in silico calculation, which indicates that Asp-177 has a $pK_a$ of 5.8 in the dimeric form. If protonation of this amino acid is essential for protein activity, we would expect that about 10% of the active centers not to be able to perform the reaction, with the consequent formation of antheraxanthin instead of zeaxanthin. However, antheraxanthin, which eventually forms, can still rebind to VDE and be converted into zeaxanthin.

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**REFERENCES**

1. Kühlheim, C., Agren, J., and Jansson, S. (2002) Science 297, 91–93
2. Li, Z., Wakao, S., Fischer, B. B., and Niyogi, K. K. (2009) Annu. Rev. Plant Biol. 60, 239–260
3. Barber, J., and Andersson, B. (1992) Trends Biochem. Sci. 17, 61–66
4. Hager, A., and Holocher, K. (1994) Biochim. Biophys. Acta 1273, 15321–15324
5. Bugos, R. C., and Yamamoto, H. Y. (1998) Photosynth. Res. 52, 218–224
6. Bugos, R. C., and Yamamoto, H. Y. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6520–6525
Identification of Violaxanthin De-epoxidase Active Site

21. Hieber, A. D., Bugos, R. C., Verhoeven, A. S., and Yamamoto, H. Y. (2002) *Plant Cell* **21**, 2036–2044

22. Arnoux, P., Morosinotto, T., Saga, G., Bassi, R., and Pignol, D. (2009) *Plant Cell* **21**, 2036–2044

23. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) *J. Comput. Chem.* **19**, 1639–1662

24. Song, Y., Mao, J., and Gunner, M. R. (2009) *J. Comput. Chem.* **30**, 2231–2247

25. Rocchia, W., Sridharan, S., Nicholls, A., Alexov, E., Chiabrera, A., and Honig, B. (2002) *J. Comput. Chem.* **23**, 128–137

26. Rocchia, W., Alexov, E., and Honig, B. (2001) *J. Phys. Chem. B* **105**, 6507–6514

27. Fufezan, C., and Specht, M. (2009) *BMC Bioinformatics* **10**, 258

28. Prinz, W. A., Aslund, F., Holmgren, A., and Beckwith, J. (1997) *J. Biol. Chem.* **272**, 13661–13667

29. Yamamoto, H. Y., and Higashi, R. M. (1978) *Arch. Biochem. Biophys.* **190**, 514–522

30. Gilmore, A. M., and Yamamoto, H. Y. (1991) *Plant Physiol.* **96**, 635–643

31. Laemmli, U. K. (1970) *Nature* **227**, 680–685

32. Ballottari, M., Dall’Osto, L., Morosinotto, T., and Bassi, R. (2007) *J. Biol. Chem.* **282**, 8947–8958

33. Gisselsson, A., Szilagyi, A., and Akerlund, H. E. (2004) *Physiol. Plant.* **122**, 337–343

34. Coesel, S., Obornik, M., Varela, J., Falciatore, A., and Bowler, C. (2008) *PLoS ONE* **3**, e2896

35. Lohr, M., and Wilhelm, C. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 8784–8789

36. Rockholm, D. C., and Yamamoto, H. Y. (1996) *Plant Physiol.* **110**, 697–703

37. Kuwabara, T., Hasegawa, M., Kawano, M., and Takaichi, S. (1999) *Plant Cell Physiol.* **40**, 1119–1126

38. Kawano, M., and Kuwabara, T. (2000) *FEBS Lett.* **481**, 101–104

39. Arand, M., Cronin, A., Oesch, F., Mowbray, S. L., and Jones, T. A. (2003) *Drug Metab. Rev.* **35**, 365–383

40. Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X., and Chang, W. (2004) *Nature* **428**, 287–292

41. García-Martín, A., Pazur, A., Wilhelm, B., Silber, M., Robert, B., and Braun, P. (2008) *J. Mol. Biol.* **382**, 154–166

42. Kerfeld, C. A., Sawaya, M. R., Brahmandam, V., Cascio, D., Ho, K. K., Trevithick-Sutton, C. C., Krogmann, D. W., and Yeates, T. O. (2003) *Structure* **11**, 55–65

43. Campanacci, V., Bishop, R. E., Blangy, S., Tegoni, M., and Cambillau, C. (2006) *FEBS Lett.* **580**, 4877–4883

44. Lascombe, M. B., Grégoire, C., Poncet, P., Tavares, G. A., Rosinski-Chupin, I., Rabillon, J., Goubran-Botros, H., Mazié, J. C., David, B., and Alzari, P. M. (2000) *J. Biol. Chem.* **275**, 21572–21577

45. Latowski, D., Akerlund, H. E., and Strzałka, K. (2004) *Biochemistry* **43**, 4417–4420

46. Neubauer, C., and Yamamoto, H. Y. (1994) *Photosynth. Res.* **39**, 137–147

47. Havir, E. A., Tausta, S. L., and Peterson, R. B. (1997) *Plant Sci.* **123**, 57–66

48. DeLano, W. L. (2008) *The PyMOL Molecular Graphics System*, DeLano Scientific LLC, Palo Alto, CA