De-epoxidation of Violaxanthin in Light-harvesting Complex I Proteins*

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The conversion of violaxanthin (Vx) to zeaxanthin (Zx) in the de-epoxidation reaction of the xanthophyll cycle plays an important role in the protection of chloroplasts against photodynamic damage. Vx is bound to the antenna proteins of both photosystems. In photosystem II, the formation of Zx is essential for the pH-dependent dissipation of excess light energy as heat. The function of Zx in photosystem I is still unclear. In this work we investigated the de-epoxidation characteristics of light-harvesting complex proteins of photosystem I (LHCI) under in vivo and in vitro conditions. Recombinant LHCI (Lhca1-4) proteins were reconstituted with Vx and lutein, and the convertibility of Vx was studied in an in vitro assay using partially purified Vx de-epoxidase isolated from spinach thylakoids. All four LHCI proteins exhibited unique de-epoxidation characteristics. An almost complete Vx conversion to Zx was observed only in Lhca3, whereas Zx formation in the other LHCI proteins decreased in the order Lhca4 > Lhca1 > Lhca2. Most likely, these differences in Vx de-epoxidation were related to the different accessibility of the respective carotenoid binding sites in the distinct antenna proteins. The results indicate that Vx bound to site V1 and N1 is easily accessible for de-epoxidation, whereas Vx bound to L2 is only partially and/or with the slower kinetics convertible to Zx. The de-epoxidation properties determined for the monomeric recombinant proteins were consistent with those obtained for isolated native LHCI-730 and LHCI-680 in the same in vitro assay and the de-epoxidation state found under in vivo conditions in native LHCIs.

Photosynthesis of higher plants is driven by the light-induced electron transport from photosystem II (PSII)¹ to photosystem I (PSI). In both photosystems, light-harvesting chlorophyll (Chl) a/b-binding (LHC) proteins collect the light energy for the primary light reactions in the reaction centers to achieve the efficient capture of solar energy (1). In addition to this basic light-harvesting function, antenna proteins play a key role in the dissipation of excess light energy and, thus, in the protection of the photosynthetic apparatus against photodynamic damage (2, 3). This protective role is related to the action of carotenoids, which are essential components of all Chl-binding proteins (4). In higher plants, 10 different antenna proteins (Lhcb1–6 in PSII and Lhca1–4 in PSI) constitute the family of Chl a/b-binding proteins (5). Although the amino acid sequence and, thus, the secondary structure are highly conserved among the different antenna proteins in higher plants (6), the carotenoid composition of each member of the LHC family is unique. Four possible carotenoid binding sites (denoted as L1, L2, N1, and V1) in Chl a/b-binding proteins of both photosystems have been identified in recent studies of the pigment binding properties of the different LHC proteins (summarized in Ref. 7). According to these data, the binding of carotenoids to L1 and L2 is supposed to be obligatory so that at least two carotenoids per monomer are bound to all LHC proteins. Although the binding of lutein (Lut) to L1 appears to be conserved in all antenna proteins, the L2 site can also be occupied by other carotenoids (8–10). The occupancy of the remaining binding sites, N1 and V1, is variable (for further details, see Ref. 7). Violaxanthin (Vx), which is reversibly convertible to zeaxanthin (Zx) in the so-called xanthophyll cycle (11), is bound to each of the LHC proteins in different stoichiometries and at variable binding sites, either L2, N1, or V1 (7).

In PSII, the formation of Zx in the de-epoxidation reactions of the xanthophyll cycle is essential for the pH-regulated dissipation of absorbed light energy as heat (12). This photoprotective mechanism is frequently measured as the qE, which is the pH-dependent component of the non-photochemical quenching of Chl fluorescence (see Ref. 13 for a recent review). According to current understanding, qE is based on a synergistic action of the luminal pH, xanthophyll binding, and conformational changes in antenna proteins of PSII, giving rise to the generation of quenching centers (14–16). There is further evidence that only one or two molecules of Zx per PSII are required for qE (17–19). To date, the function of the remaining major fraction of the xanthophyll cycle pigment pool (VAZ pool), which is composed of >20 Vx molecules per electron transport chain (20), is still unclear. Recently, an additional role of Zx as membrane stabilizer analogous to the function of tocopherols has been proposed (21). Indeed, the detailed analysis of the npq1 mutant from Arabidopsis, which is defective in the gene encoding the Vx de-epoxidase (VxDE) and therefore lacks Zx, underlined this proposed function of Zx (22, 23). It is unclear, however, to what extent this additional function might be related to the conversion of a non-protein-bound pool of Vx molecules in the thylakoid membrane.

It is well known from early work on the xanthophyll cycle that only a fraction of ~60–80% of the VAZ pool is convertible to Zx in higher plants (24). Later studies with antenna-depleted plants implied that this limited conversion of Vx can be

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§ The abbreviations used are: PSI, photosystem I; PSII, photosystem II; LHC, light-harvesting complex; LHCⅠ, LHCⅡ proteins of PSI; Lut, lutein; VAX, violaxanthin-antheraxanthin-zeaxanthin; Vx, violaxanthin; VxDE, Vx de-epoxidase; Zx, zeaxanthin.
attributed to protein-bound xanthophylls, whereas non-protein bound Vx is completely convertible to Zx (20, 25). In vivo analyses of the dynamics of conversion of protein-bound xanthophylls revealed that both the extent and kinetics of Zx formation differ in single antenna subcomplexes (26), indicating different characteristics of the Vx binding sites in distinct antenna proteins.

The mechanism of xanthophyll conversion and its regulation is still under debate. Studies of the characteristics of Vx de-epoxidation from the stroma side of the thylakoid membrane indicate that the VxDE converts Vx in the lipid matrix and not at the protein (27, 28). The comparison of the temperature dependence of Vx de-epoxidation from both sides of the thylakoid membrane led further to the conclusion that the release of Vx from the carotenoid binding sites and/or the diffusion of Vx in the lipid matrix might be the rate-limiting reaction of the de-epoxidation reactions (28). Thus, the conversion of protein-bound Vx to Zx would require the following: (i) the release of Vx into the lipid phase; (ii) the diffusion of Vx to the VxDE; (iii) the de-epoxidation of Vx in the lipid matrix; and (iv) the rebinding of Zx to the protein. Both the release of Vx from the carotenoid binding site into the lipid phase of the membrane and the rebinding of Zx are likely to be controlled by the pH-regulated conformational changes of antenna proteins (7).

The importance of the respective carotenoid binding sites for the convertibility of Vx has been demonstrated under in vitro conditions using recombinant Lhc1 proteins as a model system (29). It was concluded from that work that Vx bound to one of the Lut binding sites, most likely L1, is not convertible to Zx. By contrast, Vx bound to the other Lut binding site, most likely L2, and to the N1 site was found to be fully convertible to Zx, although with different kinetics (29).

The role of Zx formed in the antenna proteins of PSI is still unclear. Four different LHC proteins, Lhca1–4, are associated with the core complex of PSI, and each of them is apparently present in a single copy (30). Under mild detergent treatment, LHCI proteins can be isolated from the PSI holocomplex as two subfractions, LHCI-730 and LHCI-680 (31). LHCI-730 is known to be a heterodimer composed of Lhca1 and Lhca4, whereas LHCI-680 is constituted of Lhca2 and Lhca3 (32–35). The proposed dimeric organization of LHCI proteins was confirmed by the crystal structure of PSI (30). The formation of Zx in PSI-LHCI complexes isolated from preilluminated leaves has been reported for different species in earlier studies (26, 36–41). In all species, generally lower maximal DEPSs were found for LHCI proteins (–0.5–0.45) in comparison with LHII proteins (–0.5–0.9). To date, it is not known whether LHCI subpopulations differ in their Zx binding capacity which may be important for the function of the various LHCI proteins.

To gain insight into the potentially different de-epoxidation properties of Vx attached to the various LHCI proteins, we investigated the de-epoxidation characteristics of monomeric and dimeric LH2 proteins in vivo and in vitro conditions. To obtain additional information about the possible role of different carotenoid binding sites in the convertibility of Vx in PSI, recombinant monomeric LH2 proteins were used for these studies.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth**—Tomato plants (*Lycopersicon esculentum*; brand name “Hellfrucht,” Gartenland, Aschersleben, Germany) were grown in a green house under a 16 h light/8 h dark regime at a light intensity of 120 μmol photons m⁻² s⁻¹ supplied by additional fluorescent tubes (LSW714–860 LumiluxPlus Eco, Osram, München, Germany). Plants were watered once a week with a commercially available fertilizer and harvested after ~4 weeks.

**Isolation of Native LHCI-730 and LHCI-680**—PSI holocomplexes and native LHCI-subcomplexes were isolated from tomato thylakoids as described (10, 35).
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The apoproteins of Lhca1–4 were reconstituted with either total pigment extracts from tomato thylakoids (total) or a mixture of Chl α, Chl b, Vx, and Lut (Lut/Vx) as indicated under “Experimental Procedures.” After reconstitution, LHCs were separated from free pigments by sucrose density gradient centrifugation. For quantification, pigments were extracted with 2-butanol from the respective gradient bands and analyzed by HPLC. Mean values (± S.D.) of 6–10 independent experiments of each overexpressed Lhca protein are shown.

| Protein | Pigment mixture | Pigments per 12 chlorophylls |
|---------|----------------|----------------------------|
|         | Nt | Vx | Lut | ε-Car | Car | Chl a/b |
| Lhca1   | Total | 0.34 ± 0.14 | 0.30 ± 0.05 | 1.71 ± 0.15 | 0.05 ± 0.01 | 2.41 ± 0.30 | 3.48 ± 0.16 |
| Lhca2   | Lut/Vx | 0.12 ± 0.03 | 0.22 ± 0.04 | 1.67 ± 0.05 | 0.05 ± 0.01 | 2.06 ± 0.07 | 2.28 ± 0.05 |
| Lhca3   | Total | 0.38 ± 0.05 | 0.31 ± 0.02 | 2.23 ± 0.13 | 0.20 ± 0.05 | 3.10 ± 0.07 | 6.14 ± 0.07 |
| Lhca4   | Lut/Vx | 0.14 ± 0.04 | 0.20 ± 0.03 | 1.56 ± 0.08 | 0.05 ± 0.02 | 1.96 ± 0.13 | 2.59 ± 0.41 |

**Note:** All data were performed with a pH of 5.2 at 20 °C.

**TABLE I**

**Pigment stoichiometries of reconstituted Lhca1–4**

| Protein | Pigment mixture | Pigments per 12 chlorophylls |
|---------|----------------|----------------------------|
|         | Nt | Vx | Lut | ε-Car | Car | Chl a/b |
| Lhca1   | Total | 0.34 ± 0.14 | 0.30 ± 0.05 | 1.71 ± 0.15 | 0.05 ± 0.01 | 2.41 ± 0.30 | 3.48 ± 0.16 |
| Lhca2   | Lut/Vx | 0.12 ± 0.03 | 0.22 ± 0.04 | 1.67 ± 0.05 | 0.05 ± 0.01 | 2.06 ± 0.07 | 2.28 ± 0.05 |
| Lhca3   | Total | 0.38 ± 0.05 | 0.31 ± 0.02 | 2.23 ± 0.13 | 0.20 ± 0.05 | 3.10 ± 0.07 | 6.14 ± 0.07 |
| Lhca4   | Lut/Vx | 0.14 ± 0.04 | 0.20 ± 0.03 | 1.56 ± 0.08 | 0.05 ± 0.02 | 1.96 ± 0.13 | 2.59 ± 0.41 |

**Fig. 1.** Time course of violaxanthin de-epoxidation in reconstituted LHCI monomers. Recombinant apoproteins were reconstituted in the presence of Chl α, Chl b, Vx, and Lut. Reconstituted LHCIs were purified by sucrose density gradient centrifugation. Prior to de-epoxidation, the reconstituted complexes were mixed with monogalactosyl diacylglycerol. The VxDE used was a partially purified protein extract from spinach thylakoids. In vitro de-epoxidation was performed with a pH of 5.2 at 20 °C. The reaction was started by the addition of ascorbate. Samples were taken at the indicated times, and the pigments were extracted with 2-butanol and analyzed by HPLC. In all experiments the concentrations of Vx and VxDE were in a similar range. Mean values (±S.D.) of three independent experiments are shown.

**Kinetics**

As the next step we investigated the *in vitro* de-epoxidation of native LHCI complexes that are supposed to be organized as hetero and/or homodimers in intact PSI (34). According to the structure of PSI from the pea, the individual Lhca proteins are present in a single copy per PSI (30). Our isolation procedure of native LHCI resulted in the separation of two fractions, namely LHCI-730, consisting of an Lhca1/Lhca4 heterodimer, and LHCI-680, containing monomers of Lhca2 and Lhca3. Vx de-epoxidation in native LHClCs (Fig. 2) was studied under the same conditions as those applied previously to recombinant LHCIs. In LHCI-730, only a small portion of Vx (up to ~15%) was detectable under our experimental conditions in Lhca2. Almost complete and very rapid de-epoxidation of Vx, however, was observed in Lhca3 (Fig. 1D). In contrast to all other complexes, no rapid Vx conversion was inducible in Lhca4 (Fig. 1E). Fitting of the data with a single exponential term (see Ref. 29 for details) indicated that at least a fraction of ~60% of the total Vx was convertible to Zx with kinetics that were slowed down by a factor of ~10 in comparison with non-protein-bound Vx. The possibility cannot be excluded, however, that the complete pool of Vx molecules is available for de-epoxidation in Lhca4 but that the conversion is limited by the loss of VxDE activity during 2 h of incubation at 20 °C. Obviously, the reconstituted LHCI proteins differ from each other with respect to the convertibility of bound Vx. Only a limited portion of Vx was accessible for de-epoxidation in Lhca1, Lhca2, and Lhca4, but most of the Vx in Lhca3 was convertible to Zx, although with different kinetics.
was converted to Zx (Fig. 2A). The turnover kinetics were very similar to those observed for reconstituted Lhca4 (cf. Fig. 1E).

By contrast, a more rapid de-epoxidation of a larger portion of Vx (~30%) was inducible in LHCI-680 (Fig. 2B). The kinetics resembled those found with reconstituted Lhca1–3 proteins. However, ~10% of the bound xanthophyll cycle pigments were already de-epoxidized at the beginning of the experiment. In PSI holocomplexes, up to ~40% of the Vx was convertible to Zx (Fig. 2C).

We also determined the extent to which the formed Zx was rebound by the LHC proteins after 120 min of in vitro de-epoxidation. For this purpose, the complexes were separated from free pigments by sucrose density gradient centrifugation. The pigment composition of the resulting bands was analyzed by HPLC, and the pigment ratios are summarized in Table II. In general, <20% of the total pigments were present in the upper yellow greenish free pigment band, indicating that the pigment-protein complexes were highly stable under our experimental conditions. The only exception was Lhca3, in which about two-thirds of the pigments were found in the free pigment band (Table II). Thus, Lhca3 seems to be less stable than the other monomeric LHCIs. The functionality of the repurified LHCIs was analyzed using 77 K fluorescence emission spectroscopy. All complexes showed similar emission spectra in the epoxidized (i.e. before starting the in vitro de-epoxidation) and de-epoxidized (i.e. after 120 min de-epoxidation and subsequent repurification of the complexes) state (data not shown). In comparison to the band containing the LHCIs, the free pigment band was generally characterized by the following: (i) a very high DEPS; (ii) a lower Chl/Car ratio; and (iii) a lower Lut/VAZ ratio, in most instances (Table II). This finding indicates that destabilization of the pigment-protein complexes is accompanied by the release of carotenoids, predominantly those of Zx.

A comparison of the epoxidized and de-epoxidized samples revealed a non-uniform picture for the different LHCIs (Table II). Hardly any differences were found between the epoxidized and de-epoxidized complexes of Lhca1 and LHCI-730, whereas the lower Chl α/β ratio in the de-epoxidized complexes of Lhca2, Lhca4, and LHCI-680 indicate a loss of some Chl α in these complexes during or after the de-epoxidation reaction. In Lhca3 an equal loss of both chlorophylls was obtained, a finding that again supports the notion that monomeric Lhca3 is significantly less stable in its de-epoxidized state. The reduction of Chl b and carotenoids in the de-epoxidized holocomplexes of PSI may indicate a loss of Lhca proteins, because the core complex does not bind any Chl b. It is also worth noting that in those LHCs that showed the highest DEPS, Lhca3, and Lhca4, the Lut/VAZ ratio was increased by a factor of ~1.5 (Lhca4) and 2.5 (Lhca3) in the de-epoxidized state (Table II). Obviously, de-epoxidation induced a disproportionate release of xanthophyll cycle pigments bound to these monomers. In summary, with the exception of Lhca3, most of the Zx molecules formed

**TABLE II**

| Protein     | Chl α/β | Chl/Car | Lut/Chl | Lut/VAZ | DEPS | Volume |
|-------------|--------|--------|--------|--------|------|--------|
| Lhca1       |        |        |        |        |      |        |
| Epoxidized  | 2.86   | 4.60   | 0.16   | 2.70   | 0    | 90     |
| Free pigment| 2.92   | 3.29   | 0.24   | 3.61   | 0.85 | 10     |
| Lhca2       |        |        |        |        |      |        |
| Epoxidized  | 3.21   | 5.76   | 0.13   | 2.63   | 0    | 90     |
| Free pigment| 3.53   | 2.37   | 0.24   | 1.32   | 0.66 | 10     |
| Lhca3       |        |        |        |        |      |        |
| Epoxidized  | 4.64   | 3.46   | 0.20   | 2.13   | 0    | 35     |
| Free pigment| 2.88   | 1.58   | 0.40   | 0.90   | 0.89 | 65     |
| Lhca4       |        |        |        |        |      |        |
| Epoxidized  | 3.04   | 6.02   | 0.13   | 3.57   | 0.00 | 80     |
| Free pigment| 3.11   | 2.81   | 0.23   | 1.62   | 0.67 | 20     |
| LHCI-730    |        |        |        |        |      |        |
| Epoxidized  | 2.76   | 6.08   | 0.12   | 2.03   | 0.02 | 90     |
| Free pigment| 2.38   | 5.36   | 0.16   | 5.77   | 0.36 | 80     |
| LHCI-680    |        |        |        |        |      |        |
| Epoxidized  | 2.73   | 5.27   | 0.13   | 2.45   | 0.12 | 90     |
| Free pigment| 2.24   | 5.39   | 0.13   | 2.37   | 0.26 | 80     |
| PSI holocomplex |    |        |        |        |      |        |
| Epoxidized  | 7.35   | 17.55  | 0.04   | 2.04   | 0.04 | 90     |
| Free pigment| 6.19   | 3.26   | 0.20   | 1.74   | 0.96 | 10     |

* Repurification of de-epoxidized samples by density gradient centrifugation yielded very faint bands that did not allow precise volume determination. Therefore, the relative distribution of the pigments among the free pigment band and the LHC band is given on the basis of estimated volumes.
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Table III

Comparison of in vitro and in vivo de-epoxidation

| Protein complexes | De-epoxidation state (DEPS) |
|-------------------|----------------------------|
|                   | In vitro | In vivo |
| LHCI-730          | 0.17     | 0.18   |
| LHCI-880          | 0.26     | 0.25   |
| PSI holocomplex    | 0.35     | 0.29   |

during in vitro de-epoxidation were rebound by the LHCs.

We further tested whether the different DEPS in LHCI subcomplexes obtained with the in vitro assay applies also to in vivo conditions. For this purpose, PSI and LHCIs were isolated from leaves that had been preilluminated for 1 h at saturating light intensities to induce maximum DEPS. Pigment analyses from leaves that had been preilluminated for 1 h at saturating conditions were sufficient to induce the maximum DEPS in intact leaves. Mean values of 3–4 experiments are shown; S.D. was not >8% of the respective values.

L. esculentum is in contrast to the proposed binding capacity of three carotenoids per monomer in a recent work with recombinant Lhca apoproteins from Arabidopsis thaliana (45). The binding of two carotenoids per monomer (most likely to L1 and L2) is supposed to be obligatory in all LHCI proteins (7). Although the binding of Lut to L1 is conserved in all antenna proteins, the L2 site can also be occupied by other carotenoids (8, 9), and the occupancy of the other binding sites, N1 and V1, is variable. In Lhca1, the binding of Lut and Vx to N1 has been concluded from a mutational analysis of the recombinant complex from A. thaliana (47). The high degree of sequence identity between Lhca1 in A. thaliana and L. esculentum makes it unlikely that the lower carotenoid binding capacity of Lhca1 found in the tomato protein is related to structural differences.

Therefore, the reduced occupancy of the third carotenoid binding site (most likely N1) in our study is instead due to different reconstitution conditions and could reflect a generally lower binding affinity of Vx and Lut to N1 in comparison with the two others binding sites (supposedly L1 and L2). This interpretation is in agreement with the lower binding affinity of Vx and Lut to N1 in Lhcb1 (8, 9).

In Lhca3, the additional carotenoid binding site accessible in the recombinant protein has been assigned to the more peripheral V1 site instead of to N1 (46). Assuming that the two Lut molecules are bound to the L1 and L2 site (46), V1 seems to bind rather nonspecifically to neoxanthin, Vx, or β-Car.

Reconstitution with Lutein and Violaxanthin as the Only Carotenoids—After reconstitution with Lut and Vx as the only carotenoids, the Chl/Car ratios in all four complexes were similar to those obtained with total pigment extracts (Table I). The Vx content was found to be increased after reconstitution with Lut and Vx, whereas the Lut content was almost unchanged. Obviously, neoxanthin and β-Car, which were bound in reconstitutions with total pigment extracts, were replaced by Vx in reconstitutions with Lut and Vx as the only carotenoids. This finding indicates that there is a low specificity for the respective binding site with regard to different β-carotenoids but that there is a fixed ratio of β-β-carotenoid/β-e-carotenoid binding in all four recombinant LHCCI. Assuming an obligatory occupancy of L1 by Lut, Vx should be bound to the L2 site in Lhca2 and Lhca4 as suggested previously (10). The average binding of 0.5 Vx per Lhca2 and Lhca4 monomer indicates a similar binding affinity of this binding site for Lut and Vx. In Lhca1, Vx is supposed to bind to both the L2 site and the N1 site (45, 47). The non-integer stoichiometry of carotenoid binding to Lhca1 may indicate that the N1 site can also be empty in this protein. In Lhca3, which binds three carotenoids, Vx is most likely bound to V1, whereas L1 and L2 are occupied by Lut as suggested (7, 46).

Violaxanthin De-epoxidation in Recombinant Light-harvesting Complexes—The de-epoxidation experiments revealed clear differences in the extent and kinetics of xanthophyll conversion in the different recombinant monomeric LHCCI (Fig. 1). From former studies with recombinant Lhcb1 it is known that, similarly as with non-protein-bound Vx, the Vx bound to the more peripheral N1 site is easily accessible for in vitro de-epoxidation and is more rapidly convertible to Zx than is Vx bound to L2 (29). It has been further speculated in earlier work with LHCIi that V1 represents a loose binding site, most likely on the periphery of the complex and probably in equilibrium with the lipid phase (48). The proposed localization of the binding sites N1 and V1 has been confirmed by the very recently published crystal structure of LHCIi trimers (49). Based on these structural data, neoxanthin is bound in a hydrophobic cleft formed by side chains from helices C and B as well as by the chlorin rings and phytanyl chains of two Chl b molecules, which...
are also located between helices C and B (49). By contrast, the V1 site was found to be located at the surface of the LHCII monomer and bound in a hydrophobic pocket at the monomer-monomer interface formed by several Chl molecules, hydrophobic residues of the polypeptides, and a phosphatidylglycerol (49). Assuming a similar overall structure of LHCI proteins, the different characteristics of de-epoxidation that have been determined for the various LHCIs in our study could thus be simply explained by differences in the accessibility of the distinct carotenoid binding sites to the VxDE.

In Lhca3, Vx was found to be almost completely convertible to Zx with rapid kinetics. Because it is proposed that the V1 site in Lhca3 is occupied by Vx (46), this result fully corroborates the hypothesis that Vx bound to V1 is easily accessible for de-epoxidation. In reconstituted Lhca4, however, conversion of Vx followed much slower kinetics. The absence of any fast de-epoxidation kinetics in Lhca4 thus supports the assumption that Vx is bound to the L2 site in Lhca4 and is not associated with the N1 or V1 binding site (7).

The biphasic conversion of Vx to Zx in Lhca1 (Fig. 1B) could reflect the different accessibility for de-epoxidation of Vx bound to either L2 and N1 in this protein. Applied to the situation in Lhcb1/Lhcb2, the rapid conversion of ~20% of the protein-associate Vx in Lhca1 should then be related to the Vx bound to N1 and the slow conversion of the remaining fraction to Vx bound to L2. This interpretation would be consistent with the slow kinetics of de-epoxidation observed in Lhca4, which has been attributed to the conversion of Vx bound to L2.

In Lhca2, Vx is supposed to bind to the L2 site; thus, one would expect a slow conversion of Vx in this protein. However, only 20% of the Vx pool was convertible to Zx but with the same fast kinetics as those found for Lhca1. Thus, either the rapid de-epoxidation of this fraction of protein-associated Vx is related to the conversion of an nonspecifically bound portion of Vx, or the rapidly convertible Vx is not bound to the L2 site but to the N1 (as in Lhca1) or V1 (as in Lhca3) site. In either case, however, one has to conclude that Vx bound to L2 is not accessible for de-epoxidation in Lhca2.

Summarizing these interpretations, our data are consistent with the proposed binding sites of Vx in the different LHCl proteins. We further conclude that Vx is only available for rapid de-epoxidation (i.e. within 10–20 min) when bound to the V1 or N1 site. By contrast, the conversion of Vx bound to L2 generally follows slower kinetics (>2 h) but with slightly faster kinetics in Lhca4 than in Lhca1. In Lhca2, however, conversion of Vx bound to L2 seems to not be possible within the time scale of our experiments.

Similar experiments performed with Lhca1 and Lhca4 in a recent study with recombinant proteins from A. thaliana (41) also show a higher de-epoxidation of Lhca4 as compared with Lhca1. However, in that investigation only ~7% (Lhca1) and 12% (Lhca4) of the protein-bound Vx were convertible to Zx within 30 min, although de-epoxidation was performed at 28 °C. These differences in the DEPS are likely to originate from a generally lower activity of the VxDE in that work, because the conversion of Vx bound to Lhcb1 and Lhcb2 also yielded much lower maximum values of Zx (below 5%; Ref. 41) in comparison with our previous data on Lhcb1 (>50%; Ref. 29), which came from the same type of assay that was used in the present study. Preliminary experiments performed at higher temperature (28 °C, data not shown) led to an acceleration of de-epoxidation but not to an increase of the maximum de-epoxidation state in all four recombinant Lhca proteins from L. esculentum.

Violaxanthin De-epoxidation in Native Light-harvesting Complexes—The DEPS that was obtained after in vitro de-
epoxidation of the isolated native LHCl-730 (isolated as dimers of Lhca1+Lhca4) and LHCl-680 (isolated as monomers of Lhca2 and Lhca3) was lower than what could be expected from the sum of the DEPSs found in the respective recombinant monomers. But the exact same DEPS was determined for the native LHClIs under in vitro and in vivo conditions (Table III). It is worth noting that the DEPS in LHCl-680 is clearly higher than that in LHCl-730, which is in accordance with the more pronounced de-epoxidation found for recombinant Lhca3 (a component of LHCl-680) in comparison with recombinant Lhca4 (a component of LHCl-730). Obviously, the accessibility of Vx is generally reduced in the LHCl-730 dimers in comparison with the respective LHCl-680 monomers. The increased Vx convertibility in LHCl-680 appears not to be related to the monomerization of this subcomplex during isolation, because the same DEPSs were found for both types of native LHClIs under in vivo and in vitro conditions (Table III).

It can be speculated that the reduction of nonspecifically bound Vx (particularly in Lhca1 and Lhca2) on the one hand and the structural changes of proteins in the dimeric state on the other hand are responsible for the lower DEPSs in the native complexes in comparison with those in the respective reconstituted monomeric LHClIs. Strikingly, the DEPS was increased (again observable under both in vitro and in vivo conditions) when PSI holocomplexes were used (Table III). This increase can be explained simply by the presence of loosely and/or nonspecifically bound Vx molecules in PSI holocomplexes that are lost during solubilization of the holocorex for the subsequent isolation of the subcomplexes.

Under in vivo conditions, generally lower DEPSs have been reported in the literature for LHCl proteins (~0.3–0.45) in comparison with LHClII proteins (~0.5–0.9) in different species (26, 36–41). Based on our data, we conclude that this reduced availability of Vx for de-epoxidation in LHCl is related to the non-convertible Vx bound to Lhca2 and to the convertible fraction of Vx in Lhca1 that is only slowly convertible. One can speculate that the Vx bound to Lhca2 (and possibly also to Lhca1) in particular serves either structural or light-harvesting functions in the PSI antenna. Any possible photoprotective function of the xanthophyll cycle in PSI should then be related to Zx formation in Lhca3 and Lhca4. This assignment would not only fit in with the assumption that an energy transfer from LHCl to PSI core may occur via either Lhca3 or Lhca4 (10), but it would also be consistent with the proposed similarities in the structural arrangement of Lhca4 and Lhca3 on the one hand and that of Lhca1 and Lhca2 on the other hand on the basis of the crystal structure of PSI (30).

The function of the light-induced formation of Zx in PSI is still unclear. Unlike the situation in PSII, no Zx-dependent induction of non-photochemical quenching of Chl fluorescence has been reported in PSI. Based on the generally lower fluorescence yield in LHCl proteins in comparison with that of LHcb proteins, it has been speculated that the conformation of LHca proteins may represent a constitutive “dissipative” conformation similar to the (pH- and Zx-dependent) “quenched” conformation of LHcb proteins (47). This hypothesis would exclude a direct function of Zx in energy dissipation in PSI. Similar conclusions have been drawn from a study of the P700 oxidation characteristics in intact leaves under high light stress (50). Although the light saturation curve of P700 oxidation was shifted to higher intensities after high light treatment, this effect was related to energy dissipation in the reaction center rather than in the antenna of PSI. Moreover, this effect was shown to be independent of the formation of Zx (50). Photoinhibition of PSI under in vivo conditions has, to date, been reported only at low temperatures and predominantly in cold-
sensitive plants (51, 52). In cold-tolerant barley plants, the photoinhibition of PSI was indiscernible at low temperatures after the treatment of leaves with KCN, an inhibitor of Cu,Zn-superoxide dismutase and ascorbate peroxidase (53). This can be understood as an inactivation of PSI by a increased formation of active oxygen species under these conditions. A more recent study brought further evidence that active oxygen produced at the reducing site of PSI is also damaging to PSI (54). It is thus reasonable to assume that the Zx formed in LHCl proteins is involved in the scavenging of active oxygen rather than energy dissipation.

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