De-epoxidation of Violaxanthin in the Minor Antenna Proteins of Photosystem II, LHCB4, LHCB5, and LHCB6*

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The conversion of violaxanthin to zeaxanthin is essentially required for the pH-regulated dissipation of excess light energy in the antenna of photosystem II. Violaxanthin is bound to each of the antenna proteins of both photosystems. Former studies with recombinant Lhcb1 and different Lhca proteins implied that each antenna protein contributes specifically to violaxanthin conversion related to protein-specific affinities of the different violaxanthin binding sites. We investigated the violaxanthin de-epoxidation in the minor antenna proteins of photosystem II, Lhcb4–6. Recombinant proteins were reconstituted with different xanthophyll mixtures to study the conversion of violaxanthin at different xanthophyll binding sites in these proteins. The extent and kinetics of violaxanthin de-epoxidation were found to be dependent on the respective protein and, for each protein, also on the binding site of violaxanthin. In particular, violaxanthin bound to Lhcb4 was nearly irreversible for de-epoxidation, whereas violaxanthin bound to Lhcb5 was fully convertible but with slow kinetics. Lhcb6 exhibited heterogeneous violaxanthin conversion characteristics, which could be assigned to different populations of reconstituted Lhcb6 complexes with respect to violaxanthin binding sites. The results support the proposed different binding affinities of violaxanthin to the three putative violaxanthin binding sites (V1, N1, and L2) in antenna proteins. Under consideration of former studies with Lhcb1 and Lhca proteins, the data imply that violaxanthin bound to the V1 and N1 binding site of antenna proteins is easily accessible for de-epoxidation in all antenna proteins, whereas violaxanthin bound to L2 is either only slowly or not convertible to zeaxanthin, depending on the respective protein.

Light-harvesting chlorophyll (Chl)2 a/b-binding (LHC) proteins serve as an efficient antenna system that captures solar energy for the primary light reactions in plant photosynthesis (1). In higher plants, the LHC family is composed of at least 11 different antenna proteins of both photosystems, Lhca1–5 in photosystem I (PSI) and Lhcb1–6 in photosystem II (PSII) (2–4). Related to their high sequence similarity, a similar structure of the members of the LHC family has been supposed (5). Despite this high structural similarity, however, antenna proteins differ from each other with respect to their pigment binding properties. The number of coordinated Chl molecules was found to vary between 8 (in Lhcb4) and 14 (in Lhcb1/2) per monomer, and the number of xanthophylls was found to vary between two (in most of the antenna proteins) and up to four (in trimeric LHCI) (6). Recent analysis of the x-ray structure of trimeric LHCI from spinach (7) and pea (8), which consists of Lhcb1–3 proteins, identified the four different xanthophyll binding sites in these proteins. Two luteins (Luts) are bound to the central L1 and L2 sites associated with the two central transmembrane helices A and B, and neoxanthin (Nx) is bound to the more peripheral helix B and protrudes into the lipid phase, whereas violaxanthin (Vx) is located at the monomer interface (8). Only binding of Lut to the L1 site is a common feature of all LHC proteins, whereas the occupancy of the other binding sites is variable and differs in each of the antenna subcomplexes (6, 9–11).

Xanthophylls serve three basic functions in antenna proteins: (i) the stabilization of the proteins, (ii) the absorption of light energy, and (iii) the photoprotection of the photosynthetic apparatus (12–14). Vx, which is reversibly convertible to zeaxanthin (Zx) in the de-epoxidation reactions of the so-called xanthophyll cycle (15–17), is bound to each of the LHC proteins in different stoichiometries and at variable binding sites, either L2, N1, or V1 (6). Zx has at least two different photoprotective functions; it is essentially required for thermal dissipation of excess light energy (so-called qE-quenching) in the antenna of PSII (18–20) and additionally acts as an antioxidant in the lipid phase of the thylakoid membrane (21, 22). The role of Zx in thermal energy dissipation has been related to the binding of two molecules of Zx to the PsbS protein of PSII (23–25). Based on spectroscopic analysis, a direct involvement of Zx in the quenching process due to the presence of a Chl–Zx heterodimer and the formation of a carotenoid (Car) cation has been proposed as the molecular mechanism of energy quenching in PSII (26, 27). However, the requirement of additional antenna proteins for qE has been shown in different studies (28–33). It is, thus, likely that energy dissipation is related to different mechanisms including different quenching sites and that each single antenna protein contributes to the concerted response of the organized thylakoid macrostructure (20).

Binding of xanthophylls to antenna proteins has been supposed to limit the conversion of Vx to Zx in the xanthophyll
cycle (34, 35). In vivo analysis of the dynamics of xanthophyll conversion demonstrated that the extent and kinetics of Xz formation differ in single antenna subcomplexes (36), most likely reflecting different affinities of Vx binding sites in different antenna proteins. It is reasonable to assume that these characteristics of Vx conversion are related to different functions of the single antenna proteins in the dissipation of excess light energy. To understand the contribution of each of the single antenna proteins and/or specific xanthophyll binding sites to the formation of Zx (and, thus, to energy dissipation), the reversibility of Vx to Zx has been investigated in recombinant antenna proteins. Recent studies on recombinant Lhcb1 (37) and Lhca1–4 (38) brought evidence that Vx bound to V1 and N1 is, like non protein-bound Vx, easily accessible for de-epoxidation, whereas Vx bound to L2 is only partially, and/or with slower kinetics, convertible to Zx. These characteristics are likely to reflect the different affinities of Vx to the respective xanthophyll binding sites in the distinct antenna proteins.

In this work we investigated the de-epoxidation characteristics of the three minor PSII antenna proteins, Lhcb4–6, which are supposed to bind Vx at the L2 site under in vivo conditions (6). Recombinant proteins were reconstituted with different xanthophyll mixtures to gain information about the characteristics of Vx conversion at the different Car binding sites in these proteins. Clearly different de-epoxidation characteristics were found for each of the minor PSI antenna proteins, confirming the importance of protein-specific properties for the regulation of Vx conversion to Zx. Together with former studies on the other LHC proteins our data allow us to relate the multiphasic kinetics of Vx de-epoxidation found under in vivo conditions and isolated thylakoids to different xanthophyll binding properties of the single antenna proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation and Cloning of lhcb4, lhcb5, and lhcb6 Genes**—The cDNA fragments of a tobacco cDNA library (Nicotiana tabacum) were cloned into the EcoRI site of the Lambda ZAP II vector system (Stratagene). For screening of the cDNA library for the minor antenna proteins, heterologous sequence tag primers sets were used: Lhcb6, 5’-ATAATTGATCCGGCGGACCGACTGTCCCTCCAAG-3’ and 5’-GGCCCGAGCTTCA-ACCAAGAGCTCAAAGGCTAT-3’, Lhcb5, 5’-ATAATTGATCCCAAAGGGTGTCGTGCTGCCCCT-3’ and 5’-GCGCAAGGTCTTCAAAAGTGGGCGTCCTTCA-3’; Lhcb4, 5’-GAGAGGGATCCCAAGGGTAT-3’ and 5’-GGCGGCGAGGCTTATAGAGAAGACCGAATGGTGT-3’.

**Cloning of the Overexpression Plasmids**—The coding sequences of the respective mature antenna proteins of Lhcb6, Lhcb5, and Lhcb4 were cloned into the overexpression vector pET21a (Novagen) using the BamHI and HindIII restriction sites. For DNA amplification, the following primer sets were used: Lhcb6, 5’-ATAATTGATCCGGCGGACCGACTGTCCCTCCAAG-3’ and 5’-GGCCCGAGCTTCA-ACCAAGAGCTCAAAGGCTAT-3’, Lhcb5, 5’-ATAATTGATCCCAAAGGGTGTCGTGCTGCCCCT-3’ and 5’-GCGCAAGGTCTTCAAAAGTGGGCGTCCTTCA-3’; Lhcb4, 5’-GAGAGGGATCCCAAGGGTAT-3’ and 5’-GGCGGCGAGGCTTATAGAGAAGACCGAATGGTGT-3’.

**In Vitro De-epoxidation**—In vitro de-epoxidation was carried out with VxDE extracts from spinach chloroplasts as described before (37). For repurification of the de-epoxidized complexes, samples were concentrated with Centricon YM-50 tubes after 2 h of de-epoxidation. The concentrated sample was loaded on a sucrose gradient (0.1–0.8 M sucrose, 5 mM Tricine, pH 7.6, and 0.06% β-dodecyl maltoside) and centrifuged for 18 h at 300,000 × g and 4 °C. The resulting bands were harvested with a syringe, and the pigment content was analyzed by high performance liquid chromatography (HPLC).

**Pigment Analysis**—Pigments were extracted from all samples with 2-butanol (42) and analyzed by reversed-phase HPLC as described earlier (36).

**Spectroscopy**—Fluorescence emission spectra (PerkinElmer Life Sciences luminescence spectrometer LS 55) were recorded at room temperature. The samples were diluted to a Chl concentration of 1 μg/ml in 10 mM HEPES, pH 7.6, 0.06% β-dodecyl maltoside, 20% glycerol, 200 μg/ml glucose oxidase, and 40 μg/ml katalase.

**RESULTS**

Apoproteins of Lhcb4–6 were overexpressed in E. coli and reconstituted with pigment extracts isolated from spinach thylakoids. The xanthophyll mixture used for reconstitution was varied to yield either complexes with the native xanthophyll composition (Lhcb4 and Lhcb5, Lut + Vx + Nx; Lhcb6, Lut + Vx) or complexes, in which either Lut or Nx were replaced by Vx. The pigment composition of the different complexes is summarized in Table 1. The pigment stoi-

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TABLE 1
Pigments stoichiometries of reconstituted Lhcb4-6

| Protein         | Nx     | Vx     | Lut | Σ Car | Chl a/b |
|-----------------|--------|--------|-----|-------|---------|
| Lhcb4-LVN       | 0.53 ± 0.03 | 0.51 ± 0.05 | 0.86 ± 0.13 | 1.90 ± 0.11 | 3.37 ± 0.42 |
| Lhcb4-LV        | 0      | 0.70 ± 0.04 | 0.81 ± 0.08 | 1.50 ± 0.05 | 2.56 ± 0.23 |
| Lhcb4-NV        | 0.56 ± 0.02 | 1.06 ± 0.06 | 0    | 1.62 ± 0.08 | 3.47 ± 0.18 |
| Lhcb5-LVN       | 0.73 ± 0.04 | 0.23 ± 0.04 | 1.28 ± 0.05 | 2.25 ± 0.05 | 2.51 ± 0.27 |
| Lhcb5-LV        | 0      | 0.37 ± 0.06 | 1.53 ± 0.08 | 1.90 ± 0.09 | 1.98 ± 0.15 |
| Lhcb5-NV        | 0.99 ± 0.10 | 1.31 ± 0.02 | 0    | 2.30 ± 0.12 | 1.75 ± 0.12 |
| Lhcb6-LV        | 0      | 0.84 ± 0.09 | 0.81 ± 0.05 | 1.64 ± 0.05 | 1.09 ± 0.04 |
| Lhcb6-V         | 0      | 11.52 ± 0.02 | 0    | 1.52 ± 0.02 | 1.09 ± 0.03 |

The functionality of the recombinant proteins was tested by recording the fluorescence emission spectra at excitation wavelengths that preferentially excite Chl a (440 nm) or Chl b (475 nm) or 475 nm (Zea mays), lutein; V, violaxanthin; N, neoxanthin.

FIGURE 1. Chl fluorescence emission spectra of recombinant Lhcb4–6 proteins. Recombinant apoproteins were reconstituted in the presence of Chl a, Chl b, and different xanthophyll mixtures as indicated. Reconstituted complexes were purified by density gradient centrifugation. The purified complexes were diluted to a Chl concentration of 1 μg/ml, and fluorescence emission was recorded in the range from 600 to 775 nm after excitation at either 440 nm (solid line) or 475 nm (dashed line). The resulting spectra were normalized to 100 in the maximum at about 680 nm. L, lutein; V, violaxanthin; N, neoxanthin.

### References

- [440 nm) or 475 nm (Zea mays), lutein; V, violaxanthin; N, neoxanthin.
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**De-epoxidation of Violaxanthin in Lhcb4–6 Proteins**

Apoproteins of Lhcb4–6 were reconstituted with different pigment mixtures as described under “Experimental Procedures.” After reconstitution, complexes were concentrated and 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. Data are normalized to yield the stoichiometries per monomer assuming that 8, 9, and 10 Chls (a + b) are coordinated per monomer of Lhcb4, -5, and -6, respectively. Mean values (± S.D.) of 6–10 independent experiments are shown. Σ Car, sum of all carotenoids (Nx + Vx + Lut); L, lutein; V, violaxanthin; N, neoxanthin.

| Protein         | Pigments per monomer |
|-----------------|----------------------|
| Lhcb4-LVN       | Σ Chls reported in other studies (45, 46). Thus, either a fraction of recombinant Lhcb6 binds only one xanthophyll per monomer under our reconstitution conditions, or the number of coordinated Chl molecules is lower than 10. The latter possibility is supported by the fact that Lhcb6 is known to be rather unstable and tends to loose Chl molecules in its isolated form (45). If the data are normalized to 8 Chl per monomer, a stoichiometry of two xanthophylls per monomer would also be given in Lhcb6.

Reconstitution of Lhcb6 in absence of Lut (Lhcb6-V) was possible without changes of the Chl a/b ratio or the Car/Chl stoichiometry. It can, thus, be assumed that Lut was stoichiometrically replaced by Vx in Lhcb6-V. Reconstitution of Lhcb5 in the absence of Nx (Lhcb5-LV) or Lut (Lhcb5-NV), however, resulted in a reduction of the Chl a/b ratio, reflecting the variability of this complex in binding either Chl a or Chl b without affecting the xanthophyll binding properties (44). In Lhcb5, it was therefore possible to replace both Nx and Lut by Vx. Only chiorometry of recombinant Lhcb6 binds only one xanthophyll per monomer under our reconstitution conditions, or the number of coordinated Chl molecules is lower than 10. The latter possibility is supported by the fact that Lhcb6 is known to be rather unstable and tends to loose Chl molecules in its isolated form (45). If the data are normalized to 8 Chl per monomer, a stoichiometry of two xanthophylls per monomer would also be given in Lhcb6.

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TABLE 2
Kinetics of Vx conversion

| Sample                        | Amplitude | Rate constant | Amplitude | Rate constant |
|-------------------------------|-----------|---------------|-----------|---------------|
|                               | A<sub>1</sub> | k<sub>1</sub>  | A<sub>2</sub> | k<sub>2</sub>  |
| Non protein-bound Vx          | 85 ± 4    | 310 ± 30      | 15 ± 4    | 10 ± 6        |
| Lhcb4-LVN                     | 15 ± 1    | 270 ± 40      | 31 ± 2    | 290 ± 60      |
| Lhcb4-LV                      | 17 ± 1    | 230 ± 40      | 10 ± 1    | 290 ± 80      |
| Lhcb5-LVN                     | 10 ± 1    | 300 ± 40      | 90 ± 1    | 3 ± 0.2       |
| Lhcb5-LV                      | 76 ± 4    | 250 ± 20      | 24 ± 3    | 10 ± 5        |
| Lhcb5-NV                      | 27 ± 2    | 460 ± 10      | 73 ± 1    | 4 ± 0.3       |
| Lhcb6-LV                      | 36 ± 1    | 90 ± 6        | 8 ± 1     | 3 ± 0         |
| Lhcb6-V                       | 38 ± 1    | 30 ± 3        | 1 ± 1     | 4 ± 0.6       |

Under assumption of an irreversible first order reaction, rate constants were determined for the Vx conversion shown in Figs. 1–3. For simplification, analyses were restricted to the first step of de-epoxidation (Vx → Ax), and for all Lhcb5 complexes (Fig. 3) and non-protein-bound Vx (Fig. 2A), data points were fitted with two exponentials, in all other cases with a single exponential (k<sub>1</sub>). Amplitudes (A<sub>1</sub> and A<sub>2</sub>) and rate constants (k<sub>1</sub> and k<sub>2</sub>) represent mean values ± S.D. of three independent experiments.

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FIGURE 2. Time course of violaxanthin de-epoxidation in recombinant Lhcb6 complexes. Reconstitution was performed in presence of Chl<sub>a</sub>, Chl<sub>b</sub>, and either Lut and Vx (Lhcb6-LV) or with Vx as only xanthophyll (Lhcb6-V). Isolated Vx (A) or purified complexes (B and C) were mixed with monogalactosyl diacylglycerol and a VxDE-enriched extract isolated from spinach chloroplasts as described (37). De-epoxidation was carried out at pH 5.2 and 20 °C. The reaction was started by the addition of 30 mM ascorbate. At the indicated time, aliquots were mixed with 2-butanol for pigment extraction, and the pigment content was analyzed by HPLC. Mean values (±S.D.) of three independent experiments are shown.

nm) (Fig. 1). Independent of the excitation wavelength, fluorescence emission was found to peak at about 680 nm in Lhcb4, supporting the functional coupling of the chlorophylls in these complexes (Fig. 1, A–C). Only in Lhcb4-LV complexes (Fig. 1B), a very faint shoulder at about 660 nm was visible upon excitation of Chl<sub>b</sub> at 475 nm, indicating that a small fraction of the excitation energy is not completely transferred from Chl<sub>b</sub> to Chl<sub>a</sub>. A similar picture was found for Lhcb5 complexes reconstituted with the full complement of xanthophylls (Lhcb5-LVN, Fig. 1D). Reconstitution in the absence of Nx (Lhcb5-LV) or Lut (Lhcb5-NV) resulted in a more pronounced shoulder of the emission spectra at 660 nm (Fig. 1, E and F), indicating again a less efficient energy transfer from Chl<sub>b</sub> to Chl<sub>a</sub> in a small fraction of complexes. It should be noted that the appearance of the shoulder at 660 nm in Lhcb4-LV, Lhcb5-LV, and Lhcb5-NV correlates with the reduction of the Chl<sub>a</sub>/Chl<sub>b</sub> ratio in comparison with the complexes with the native xanthophyll composition, Lhcb4-LVN, and Lhcb5-LVN, respectively (see Table 1). The markedly increased peak at 660 nm upon excitation at 475 nm in Lhcb6 under both reconstitution conditions (Fig. 1, G and H) is in agreement with former studies on recombinant Lhcb6 from maize (45). Thus, even in Lhcb6 complexes with the native xanthophyll composition (Lhcb6-LV), a pronounced fraction of the excitation energy is not efficiently transferred from Chl<sub>b</sub> to Chl<sub>a</sub>. This property of recombinant Lhcb6 might be related to the fraction of complexes that possibly holds only one xanthophyll per monomer (see above).

The enzymatic conversion of Vx to Zx in the reconstituted LHCs was studied during 2 h of incubation at 20 °C with a VxDE extract isolated from spinach thylakoids. Non-protein-bound Vx, which was used as the control substrate to test the VxDE activity in each assay was nearly completely de-epoxidized within 10 min (Fig. 2A). In recombinant Lhcb6 complexes with the native xanthophyll composition (Lhcb6-LV, Fig. 2B), ~35% of the Vx was convertible to Zx with somewhat slower kinetics as non-protein bound Vx, whereas the remaining fraction of Vx was not accessible for de-epoxidation (Table 2). We ensured that the activity of the enzyme did not limit the reaction by testing the conversion of freshly added Vx at the end of the experiment (not shown). Thus, two different populations of Lhcb6-LV complexes must exist that differ in the accessibility of Vx for de-epoxidation. After replacement of Lut by Vx (Lhcb6-V, Fig. 2C), about 40% of the Vx was converted to Zx with even more retarded kinetics than before (Table 2). Obviously, the replacement of Lut by Vx induced only a partial increase of the Vx convertibility. This indicates that Vx is not accessible for de-epoxidation at the (former) Lut binding sites in Lhcb6.

In Lhcb5, the de-epoxidation characteristics were different (Fig. 3). In all complexes a biphasic conversion was found...
The majority of complexes with the native xanthophyll composition (Lhcb5-LVN, Fig. 3B) showed slower conversion kinetics than the corresponding complexes of Lhcb6, and the reaction was not completed within the time frame of the experiment. Fitting of the data indicated that all of the Vx bound to Lhcb-LVN was convertible to Zx but with strongly retarded kinetics in comparison with non-protein-bound Vx (Table 2). It can, thus, be assumed that Vx is generally accessible for de-epoxidation in Lhcb5, but Vx conversion occurs only with very slow kinetics. After reconstitution of Lhcb5 in the absence of Nx (Lhcb5-LV, Fig. 3B), the Vx convertibility was dramatically accelerated (Table 2). The rapid and complete de-epoxidation of most of the Vx (about 75%) under these conditions let us conclude that the presence of Nx is responsible for the restriction of the Vx accessibility in Lhcb5-LVN complexes. On the other hand, when Lut was omitted from the reconstitution assay (in Lhcb5-NV, Fig. 3D), the fraction of rapidly convertible Vx was increased in comparison with Lhcb5-LVN complexes. About 30% of the Vx pool was convertible with fast kinetics (like the majority of Lhcb5-LV complexes), and about 70% was convertible with slow kinetics (like the majority of Lhcb5-LVN complexes) (Table 2). According to the increased fraction of rapidly convertible Vx in Lhcb5-LV complexes, we assume that this portion of Vx is related to complexes that did not bind Nx.

Vx de-epoxidation in recombinant Lhcb4 proteins (Fig. 4) was much more limited under all reconstitution conditions in comparison with Lhcb5 and Lhcb6. In complexes with the
native xanthophyll composition (Lhcb4-LVN, Fig. 4A) and after reconstitution in the absence of Lut (Lhcb4-VN, Fig. 4C) only a small fraction of about 15% of the total Vx was convertible to Zx but with fast kinetics as found for non-protein bound Vx (Table 2). The fraction of convertible Vx was increased to about 30% when Nx was omitted from the reconstitution mixture (Lhcb4-LV, Fig. 4C and Table 2). Again, the conversion occurred with fast kinetics. These characteristics indicate that only a small fraction of Vx is easily accessible for de-epoxidation in Lhcb4, whereas the major portion is not convertible to Zx.

The observed differences in the extent and kinetics of Vx de-epoxidation are likely to reflect different affinities to the respective xanthophyll binding sites. All non-convertible fractions of Vx can, thus, be assigned to tight xanthophyll binding sites. The convertible fraction of Vx, however, must not necessarily display specific characteristics of the respective binding site but could also be due to the unspecific release of Vx during the experiment. In particular, a possible destabilization of the antenna proteins upon 2 h of incubation at 20 °C can be assumed to induce the unspecific release of Vx from the respective binding site, resulting in a rapid conversion into Zx as has been found for non-protein-bound Vx (Fig. 2A).

A critical parameter for a specific interaction of the Vx/DE with its substrate under the various conditions is the rebinding of the formed Zx by the protein. To discriminate between specific and unspecific Vx conversion during the experiment, we (i) determined the pigment composition of all complexes at the end of each experiment and (ii) analyzed to what extent the formed Zx was bound by the proteins. To separate protein-bound and free pigments, samples were re-concentrated after 2 h of de-epoxidation and separated by sucrose density gradient centrifugation. The pigment composition and the de-epoxidation state (DEPS), defined by the quotient (Zx + 0.5 Ax)/(Zx + Ax + Vx), of the two bands in comparison with the epoxidized samples before the experiment is summarized in Table 3. The relative amount of free pigments is an indicator of the loss of pigments and, thus, of the stability of the different complexes throughout the de-epoxidation treatment. Large differences were obtained for the different proteins. Nearly no free pigments were found in the experiments with recombinant Lhcb4 irrespective of the xanthophyll composition of the complexes, underlining the high stability of Lhcb4 (see Table 3). For Lhcb5, however, only the complexes with the xanthophyll composition of the native protein (Lhcb5-LVN) were stable, whereas from complexes reconstituted in the absence of Lut (Lhcb5-NV) or Nx (Lhcb5-LV) at least 50% of the pigments were released from the proteins during the experiment. In the experiments with Lhcb6, on the other hand, the free pigment band made up about 30% of the total pigments, even in complexes with the native xanthophyll composition (Table 3). It should be noted, however, that the fraction of unstable recombinant Lhcb6 was not influenced by the exchange of Lut by Vx and, thus, rather represents an intrinsic property of this protein. It is likely also that the reduced xanthophyll binding capacity (Table 1) and the insufficient energy transfer to Chl a (Fig. 1C) is related to this instable fraction of recombinant Lhcb6.

Comparison of the DEPS of the protein and free pigment bands supplies further information as to whether the destabilization of the proteins (and, thus, the release of pigments) is related to the de-epoxidation of Vx during the experiment or to the purification procedure after the de-epoxidation experiment. The loss of Vx during the experiment should result in a very high DEPS in the free pigment band, since non-protein-bound Vx is very efficiently and rapidly convertible to Zx. Indeed, a very high DEPS in the free pigment band, which is based on estimated volumes because repurification of the de-epoxidized samples yielded only faint bands that did not allow the precise determination of the volume.

| Protein      | Xanthophylls per monomer | Chl a/b | Chl/Car | DEPS | Volume |
|--------------|--------------------------|---------|---------|------|--------|
|              | Lut         | Nx | VAZ |       |       |        | %     |
| Lhcb4-LVN    | 0.86        | 0.53| 0.51 | 3.37 | 4.22  | 0      | 100   |
| Epoxidized   | 1.07        | 0.66| 0.73 | 3.40 | 3.26  | 0.15   | 70    |
| Free pigment |             |    |     |      |       |        |       |
| Lhcb4-LV     | 0.81        | 0   | 0.70 | 2.56 | 5.33  | 0      | 90    |
| Epoxidized   | 0.87        | 0.67| 0.24 | 5.28 | 0.29  | 0.90   | 10    |
| Free pigment | 1.38        | 0.97| 3.13 | 3.40 | 0.80  | 0.50   | 10    |
| Lhcb4-NV     | 0           | 0.56| 1.06 | 3.47 | 4.95  | 0      | 100   |
| Epoxidized   | 0           | 0.54| 1.02 | 3.00 | 5.17  | 0.13   | 10    |
| Free pigment |             |    |     |      |       |        |       |
| Lhcb5-LVN    | 1.28        | 0.73| 0.23 | 2.51 | 4.01  | 0      | 100   |
| Epoxidized   | 1.57        | 0.81| 0.20 | 1.74 | 3.54  | 0.51   | 10    |
| Free pigment |             |    |     |      |       |        |       |
| Lhcb5-LV     | 1.53        | 0   | 3.7 | 1.98 | 4.74  | 0      | 10    |
| Epoxidized   | 1.85        | 0   | 0.40| 1.45 | 3.98  | 0.86   | 50    |
| Free pigment | 2.52        | 0   | 0.70| 1.00 | 2.64  | 0.90   | 50    |
| Lhcb5-NV     | 0           | 0.99| 1.31| 1.75 | 3.92  | 0      | 10    |
| Epoxidized   | 0           | 1.08| 1.06| 1.27 | 4.21  | 0.47   | 30    |
| Free pigment | 0           | 0.95| 0.66| 0.17 | 5.62  | 0.80   | 70    |
| Lhcb6-LV     | 0.81        | 0   | 0.83| 1.09 | 6.08  | 0      | 10    |
| Epoxidized   | 0.76        | 0   | 0.84| 1.04 | 6.24  | 0.17   | 70    |
| Free pigment | 0.92        | 0   | 0.95| 1.73 | 5.31  | 0.43   | 30    |
| Lhcb6-V      | 0           | 0   | 1.52| 1.09 | 6.58  | 0      | 10    |
| Epoxidized   | 0           | 0   | 1.17| 1.10 | 8.53  | 0.06   | 70    |
| Free pigment | 0           | 0   | 1.23| 1.48 | 8.14  | 0.13   | 30    |

* The relative distribution of the pigments among the free pigment band and the protein band is based on estimated volumes because repurification of the de-epoxidized samples yielded only faint bands that did not allow the precise determination of the volume.
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the exception of the two experiments with recombinant Lhcb6), the DEPS of the reisolated protein bands in the de-epoxidized state (Table 3) matched the maximum DEPS obtained at the end of the different de-epoxidation experiments (t = 120 in Figs. 2–4). We can, therefore, conclude that most of the Zx molecules formed during in vitro de-epoxidation were rebound by the LHCs and that the different de-epoxidation characteristics indeed reflect different properties of the xanthophyll binding sites in the respective recombinant proteins.

**DISCUSSION**

The reliable interpretation of the data is dependent on the assumption that the reconstitution procedure yielded stable complexes that are properly folded with respect to pigment binding. The general stability of the complexes was indeed supported by the absence of any pronounced free pigments bands upon concentration of the reconstituted proteins by sucrose density gradient centrifugation and in native gels (data not documented). Differences in the stability among the different samples were found, however, during or after the de-epoxidation treatment (Table 3 and the discussion below). The proper assembly of the pigments into the different binding sites can be assessed only indirectly from the pigment stoichiometries (Table 1), the protein stability (see above), and the fluorescence emission spectra (Fig. 1). Particularly the presence of fluorescence emission from Chl b is a critical parameter. The pronounced Chl b emission in both Lhcb6 complexes (and to a less extent also in Lhcb5-Nv and Lhcb5-LV) correlates with the lower stability of these complexes during de-epoxidation (Table 3). Thus, the experiments with these complexes have to be interpreted more cautiously, although the xanthophyll binding properties must not necessarily be altered in these complexes. The very low Chl b emission in Lhcb4-LV, Lhcb4-NV, and Lhcb5-LVN complexes can be assumed to be uncritical for the de-epoxidation studies since obviously only a very small fraction of complexes might be affected.

A prerequisite for the assignment of the observed de-epoxidation characteristics to distinct binding sites of Vx in the respective proteins is a reasonable prediction of the Vx binding sites under the various conditions. This can be achieved by a careful interpretation of the determined pigment stoichiometry of each complex under consideration of available data from the literature on the occupation of the different xanthophyll binding sites. To make the discussion easier, we will at first examine the three different complexes separately before a more general comparative overview is given at the end.

**De-epoxidation in Recombinant Lhcb4 Proteins**—From biochemical analysis of isolated antenna proteins, it has been derived that two or three xanthophylls per monomer are bound by native Lhcb4 (47–50). All authors agree that, as in each of the (native) LHC proteins, L1 is occupied by Lut in Lhcb4, but the possible binding sites of Vx and Nx in Lhcb4 are controversially discussed. Although Horton and co-workers (49) favor Vx binding to L2 and additionally to N1, the occupation of the third xanthophyll binding site N1 in Lhcb4 has recently been questioned in a study with chimeric Lhcb1 containing the putative N1 binding site of Lhcb4 (50). Because we determined values of about 2 xanthophylls per monomer in recombinant Lhcb4 with the native xanthophyll composition (Lhcb4-LVN) and even less stoichiometries in Lhcb4-LV and Lhcb4-NV complexes, we suppose that Vx is predominantly bound to L2 in the presence of Lut (i.e. in Lhcb4-LVN and Lhcb4-LV) and to L1 in the absence of Lut (Lhcb4-NV). We, thus, have to conclude from our de-epoxidation experiments with recombinant Lhcb4 (Fig. 4) that Vx bound to L1 and L2 is generally not accessible for the conversion to Zx. The small portion of rapidly convertible Vx in all assays with Lhcb4 could then be either explained by an unpecific release of Vx during the experiment due to the destabilization of the protein or, alternatively, by a small fraction of Vx that is bound to the N1 site rather than to L2. We strongly favor the latter possibility, since all recombinant Lhcb4 complexes were found to be very stable under our experimental conditions, as was obvious from the absence of a pronounced free pigment band after the de-epoxidation experiment (Table 3). Furthermore, earlier experiments with recombinant Lhcb1 and Lhca proteins brought evidence that only Vx bound to N1 (and V1) is, in contrast to Vx bound to L2, easily accessible for rapid de-epoxidation like non-protein-bound Vx (37, 38). The remarkable low degree of Vx conversion in recombinant Lhcb4 under all conditions shows, in agreement with other studies on recombinant and native antenna proteins, that Lhcb4 exhibits the lowest DEPS among the LHC proteins (36, 49, 51).

**De-epoxidation in Recombinant Lhcb5 Proteins**—Binding of two (44, 52) or three (49) xanthophylls per monomer has been determined for Lhcb5, but again, different models exist for the occupation of the xanthophyll binding sites. Although binding of Lut to L1 is assumed in all cases, different speculations have been made for the occupancy of the other binding sites L2, N1, and V1. The L2 site of Lhcb5 seems to have no specific affinity for a single xanthophyll species and is supposed to be occupied by either Nx or Vx (53). By contrast, Horton and co-workers (49) concluded from the low binding strength of Vx to Lhcb5 that, rather, this xanthophyll binds to the V1 site, Nx to the N1 site, and that the L2 site may be empty (49). Our data support the existence of three xanthophyll binding sites in Lhcb5. The reduction of the Car/Chl ratio in the absence of Nx (Table 1, Lhcb5-LV complexes) to a value of about 2 in comparison with about 2.3 in presence of Nx (Lhcb5-LVN and Lhcb5-NV) let us suggest that Nx and Vx bind to different sites in Lhcb5. Due to the slow conversion of Vx to Zx in our de-epoxidation experiments with Lhcb5-LVN complexes (Fig. 3A), however, we speculate that Vx is bound to the L2 site rather than to V1. The absence of either Lut (Lhcb5-NV) or Nx (Lhcb5-LV) led to a pronounced destabilization of the protein, as can be derived from the large portion of free pigments after the de-epoxidation treatment (Table 3) and the less efficient energy transfer from Chl b to Chl a (Fig. 1, E and F) in these complexes. The high DEPS of the repurified complexes after de-epoxidation in Lhcb5-LV but not in Lhcb5-NV complexes indicates that the binding affinity of Vx in absence of Nx is strongly reduced. Thus, an important role of Nx for the stability of recombinant Lhcb5 can be derived from our data. This interpretation is in accordance with earlier observations, demonstrating that in the absence of Nx (i) the yield of pigmented recombinant Lhcb5 during reconstitution is reduced (54), and (ii) the denaturation
temperature of the complexes is decreased (44) in comparison with Nx-containing Lhcb5 complexes.

In conclusion, our data show that in contrast to Lhcb4, Vx bound to Lhcb5 is accessible for de-epoxidation. The very slow kinetics of Vx conversion to Zx found in Lhcb5 with the native xanthophyll composition, thus, supports the observation from earlier studies on Lhcb1 (37) and Lhca proteins (38) that Vx bound to the L2 site cannot be converted with fast kinetics.

**De-epoxidation in Recombinant Lhcb6 Proteins**—Recombinant Lhcb6 exhibits some unique features in comparison with the other LHC proteins of PSII. It does not contain a Nx binding site, and it can be reconstituted in the absence of Lut and is rather unstable in isolated form (45, 46). The lower stability of Lhcb6 in comparison with Lhcb4 and Lhcb5 is likely to be responsible for the insufficient energy transfer from Chl b to Chl a (Fig. 1, G and H) and the unspecific release of pigments during repurification of the de-epoxidized complexes (Table 3). It has been proposed that only two xanthophyll binding sites, L1 and L2, can be occupied in Lhcb4 (6). Because Lut is supposed to be obligatorily bound to L1, we therefore assume that Vx is bound to L2 in Lhcb4-LV complexes. The stoichiometric replacement of Lut by Vx in the absence of Lut (without affecting the Chl a/b and Car/Chl ratio) implies that Vx is bound to L1 and L2 in Lhcb6-V complexes.

The observed slow kinetics of Vx conversion in both types of Lhcb6 complexes (Fig. 2 and Table 2) is in agreement with the proposed binding of Vx to L2. The fact that about 60% of the total Vx is not accessible for de-epoxidation in both cases indicates a heterogeneous distribution of Vx among the L2 and L1 site of the respective complexes. In the case of Lhcb6-V this can easily be understood by the proposed binding of Vx to both the L1 and L2 site because the fraction of Vx bound to L1 can be expected to be non-convertible to Zx. In Lhcb6-LV complexes, however, one would expect that the whole pool of Vx is convertible to Zx when binding of Vx to L2 is assumed in these complexes. In fact, the substoichiometric binding of both Lut and Vx to these complexes and the low Car content of about 1.6 per monomer determined in Lhcb6-LV (Table 1) indicates that three different types of complexes must be present; one type binding two xanthophylls per monomer (most likely Lut at L1 and Vx at L2) and two types binding only one xanthophyll, either Lut or Vx, most likely at L1. In accordance with the current understanding of Vx conversion, only the pool of Vx that is bound to L2 should then be convertible to Zx, whereas the portion of Vx bound to L1 would represent the non-convertible pool. It should be noted, however, that also the instability of recombinant Lhcb6 complexes may contribute to the inhomogeneous de-epoxidation behavior.

**Conclusive Remarks**—In summary, our studies on the in vitro de-epoxidation of Vx in recombinant antenna proteins of both photosystems (Refs. 37 and 38 and this study) provide evidence that the conversion of the Vx pool in the thylakoid membrane is controlled by the binding affinity of Vx to the different antenna proteins. Our results indicate that Vx bound to homologous binding sites (L1, L2, N1, and V1) has similar (but not identical) conversion characteristics (and, thus, binding affinities) in the different proteins. The following general rules for the conversion of protein-bound Vx can be derived from our experiments.

1) Vx bound to the N1 and V1 site is rapidly (within 10–20 min) convertible to Zx. 2) Vx bound to the L2 site is either slowly (within hours) or not convertible to Zx. 3) Vx bound to L1 (only in recombinant proteins) is not convertible to Zx. The multiphasic and incomplete conversion of Vx to Zx determined under in vivo conditions in intact leaves or under in vitro conditions in isolated thylakoid membranes could, thus, at least partly be explained by different pools of Vx in the thylakoid membrane. These pools are likely to be defined by the binding affinity to different xanthophyll binding sites in LHC proteins. Based on our data, we derived a simplified model for the explanation of the de-epoxidation characteristics in thylakoid membranes (Fig. 5). Following this model, the rapidly (i.e. within 10–20 min) convertible Vx pool is related to low affinity binding sites provided by trimeric LHCCI (Vx binding at V1), Lhca3 (Vx binding at V1), and a possible pool of non-protein bound Vx, which could be present particularly in high light-grown plants. This rapidly formed Zx can be expected to be involved in rapidly induced (and most likely pH-dependent) energy dissipation processes (e.g. qE-mechanism).

In a second step and upon illumination at saturating light intensities at a longer time scale (up to several hours), de-epoxidation of Vx bound to xanthophyll binding sites with higher affinities (Vx binding at L2 in Lhcb5, Lhcb6, and Lhca4) will lead to a further (but slower) increase of the DEPS. This slowly formed Zx might be involved in more slowly developing energy dissipation processes (e.g. photocinhibitory quenching mechanisms). In fact, such a function has recently been proposed for Lhcb5 (55). Under all conditions, however, a non-convertible pool of Vx with highest binding affinities (Vx binding to L2 in Lhcb4, Lhca1, and Lhca2) is retained in both photosystems. This non-convertible Vx pool could be required to maintain the structural integrity of either single antenna proteins or the suprastructure of the antenna of both photosystems.

The proposed properties of the single LHC proteins with respect to their contribution to the overall de-epoxidation in
De-epoxidation of Violaxanthin in Lhcb4 – 6 Proteins

Intact leaves are indeed reflected by *in vitro* analysis of the DEPS in isolated native antenna proteins after high-light treatment of intact leaves (36, 51, 56) or isolated thylakoids (36). Highest values for the DEPS have always been detected for trimeric LHCII, Lhcb5, and Lhcb6, whereas the lowest values were determined for Lhcb4 and Lhca proteins. However, careful inspection of the earlier *in vivo* data indicates that the situation in intact leaves or thylakoid membranes is obviously much more complex than might appear from the experiments with recombinant antenna proteins. In particular, for the single antenna complexes of PSII, the *in vivo* data do not completely fit with the *in vitro* experiments. Although under *in vivo* conditions LHCII was found to retain some Vx even under prolonged high light, Lhcb4 exhibited always higher DEPS (36, 56) than LHCII was found to retain some Vx even under prolonged high light, Lhcb4 exhibited always higher DEPS (36, 56) than could be expected from the present work. One should have in mind, however, that under *in vivo* conditions a pronounced redistribution of VAZ pigments among the different antenna proteins may occur, particularly under photo-oxidative stress conditions (56). Furthermore, some of the PSII antenna proteins are encoded by several genes (57), so that heterogeneous populations of several antenna proteins will be present *in vivo*. Moreover, dependent on environmental stress, a variable supramolecular organization of the antenna proteins within the membrane can further modulate and regulate the convertibility of Vx more differentially under *in vivo* conditions. Finally, the close packing of the proteins in the grana region of the membranes (where PSII is located) is likely to restrict diffusion of Vx in the lipid phase. Xanthophyll de-epoxidation has been supposed to be rate-limiting for the de-epoxidation reactions *in vivo* (58). Thus, under *in vivo* conditions not only the release of Vx from the carotenoid binding sites (which is determined by the binding affinity of Vx to the respective binding sites in the different antenna proteins) but also the overall organization of the antenna proteins in the membrane will determine the extent and kinetics of de-epoxidation in leaves.

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