Assembly of the Major Light-harvesting Chlorophyll-α/β Complex

THERMODYNAMICS AND KINETICS OF NEOXANTHIN BINDING

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The major light-harvesting chlorophyll-α/β complex in most higher plants contains three carotenoids, lutein, neoxanthin, and violaxanthin. How these pigments are assembled into the complex during its biogenesis is largely unknown. Here we show that neoxanthin but not lutein can dissociate from the fully assembled complex. Its equilibrium binding constant in a detergent system (0.1% n-dodecyl-β-D-maltoside) was determined to be \( \approx 10^6 \text{ M}^{-1} \). Neoxanthin insertion into light-harvesting chlorophyll-α/β complex prefolded from overexpressed apoprotein \((Lhcb1)^2\) from Pisum sativum in the presence of chlorophylls \(a\) and \(b\), and lutein as the sole carotenoid is kinetically controlled by an activation energy barrier of \( \sim 120 \text{ kJ mol}^{-1} \). This is the first thermodynamic and kinetic description of a binding equilibrium between a non-covalently bound pigment of the photosynthetic apparatus and its protein complex. Dissociation of neoxanthin from the major light-harvesting chlorophyll-α/β complex upon temperature increase is discussed in terms of providing a readily available substrate pool for synthesizing abscisic acid as part of a heat and drought stress response.

The photosynthetic apparatus in the thylakoid membrane of green plants contains light-harvesting complexes enhancing its capacity to absorb light quanta that are then converted into chemical potential by the reaction centers. The major light-harvesting complex of photosystem II, LHCIIb, is assembled in its trimeric form, equipped with 14 chlorophylls and 4 xanthophylls per apoprotein (1, 2). The xanthophylls consist of two molecules of lutein (Lu), one neoxanthin (Nx), and one violaxanthin (Vx), bound to the sites L1/L2, N1, and V1, respectively. L1 and L2 are located close to the center of the complex, stabilizing a superhelix of protein helices I and III, whereas N1 and V1 are located more peripherally.

The specificity of xanthophyll binding sites has been addressed both by analyzing xanthophyll biosynthetic mutants (see below) and by employing recombinant in vitro systems. In the latter approach, complexes were formed by having different carotenoids compete for the various binding sites. Lu and Nx were found to bind to the L1/L2 and N1 sites, respectively, with high specificity (3, 4). As long as Lu is provided for binding to L1/L2, the N1 site does not bind any of the all-trans carotenoids Lu or Vx. Zx was shown to be a strong competitor for Lu, whereas Vx competes to a lesser extent. Consistently, when Lu is omitted completely from the refolding experiment, both Zx and Vx support complex formation by binding to L1/L2, although this increasingly compromises the complex stability. Moreover, Zx and Vx in L1/L2 compromise the binding specificity of N1, prompting its partial occupation with Vx. Xanthophyll biosynthetic mutants of Arabidopsis thaliana revealed a flexible response of the aggregational state of LHCIIb as well as of pigmentation of particular binding sites: double mutants lacking epoxycarotenoids as well as Lu exhibit a complete loss of trimeric LHCIIb with resulting monomeric complexes carrying only the remaining xanthophyll Zx (5, 6). The loss of trimer formation and binding flexibility toward “non-native” carotenoids also holds true for single mutants where both the absence of Lu in mutant lut2, which is then replaced by Vx and/or Zx, and the absence of Nx in abai strongly decreases the trimer stability of LHCIIb (5–7).

The specificities of the L1, L2, and N1 binding sites appear to be based mostly on their selectivity for different polyene conformations, followed by a gradual response to different ionon ring types. In the case of L1/L2 a hydroxyl group at C3 on the β-end group turned out to be important (8). In contrast to Lu and Vx, which both exhibit an all-trans configuration of the extended polyene chain, Nx in photosynthetic tissue of chlorophyll b-containing organisms is present in its 9′-cis conformation (9). However, pigment analysis of LHCIIb from the parasitic plant Cuscuta reflexa identified 9′-cis-Vx as the carotenoid in N1 (10), suggesting that this binding site requires a 9′-cis-5,6-epoxy-3-hydroxy carotenoid. This suggestion was confirmed by the x-ray structure of LHCIIb resolved at 2.72 Å (1), which identified a tyrosine residue located in the luminal loop as the hydrogen bond partner for the ionone ring proximal to the 9′-cis conformation of Nx. Furthermore, numerous chlorophylls and amino acid side chains form a hydrophobic cleft that accommodates the bent polyene chain of Nx.

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‡1 This is the first thermodynamic and kinetic study of the binding of neoxanthin in the major light-harvesting complex of photosystem II, LHCIIb.

The abbreviations used are: LHCIIb, major light-harvesting chlorophyll a/b complex of photosystem II; ABA, abscisic acid; β-DM, n-dodecyl-β-D-maltoside; LL-LHCIIb, partially pigmented LHCIIb carrying two molecules of Lu; Lu, lutein; NLL-LHCIIb, fully pigmented LHCIIb carrying two molecules of Lu as well as one Nx; Nx, neoxanthin; Vx, violaxanthin; Zx, zeaxanthin; HPLC, high-performance liquid chromatography.
Whereas both Lu and Nx are generally considered to be tightly bound to LHCIIb, the binding strength of Vx is much weaker. Solubilization of thylakoids usually causes the loss of Vx from LHCIIb unless special care is taken (11). X-ray crystallography localized the Vx binding site at the interface between two monomers in trimERIC LHCIIb (1, 2) where the binding pocket is shaped by several amino acid side chains, chlorophylls, and lipids.

Xanthophylls not only contribute to the structural stability of the pigment-protein complex but fulfill a number of additional tasks (12–17). One of these is the protection against photodamage by quenching either the triplet-excited state of chlorophyll or the singlet state of oxygen. These two quenching processes have been assigned to Lu and Nx, respectively (3). Another function of carotenoids is the dissipation of excess excitation energy in the light-harvesting apparatus involving zeaxanthin, generated by de-epoxidation of Vx in the xanthophyll cycle (12, 13, 18). During this cycle, Vx presumably is extracted from its binding site in light-harvesting complexes to interact with the water-soluble de-epoxidase, which then reinserts its product back into the protein complex (19). This dynamic binding behavior may explain why Vx easily dissociates from LHCIIb. The dynamics of carotenoid binding to LHCIIb is an issue to be considered when the biogenesis of this complex is to be understood. It is unclear how and in which sequence the carotenoids are assembled into the light-harvesting complexes. In an early stage of greening, before the massive accumulation of trimeric LHCIIb sets in, the monomeric complex is transiently found (20). However, no earlier assembly intermediate, containing only a subset of LHCIIb pigments, has ever been observed in greening plants. Of course this does not exclude the existence of such intermediates, because the biogenesis of LHCIIb is not well synchronized in green tissue, and, therefore, such intermediates may appear only in low amounts at any given time point. Time-resolved fluorescence and CD measurements (21, 22) showed that the presence of Lu accelerates complex assembly and protein folding, more so than the presence of any other carotenoids, indicating that the correct occupation of the central xanthophyll binding sites L1 and L2 with Lu possibly precedes the binding of (some) other pigments (23). In fact, LHCIIb containing L2 as the only carotenoid component is stable (4, 24), at least in its monomeric form (5). The present work addresses the question of whether Nx can bind to a potential intermediate of LHCIIb assembly consisting of the folded protein, Lu, and the chlorophylls. We show that in fact Nx binding to LHCIIb is reversible and, by using Nx-specific signals in the CD of LHCIIb, determine the binding constant and activation energy of its binding equilibrium.

**EXPERIMENTAL PROCEDURES**

**Pigment Preparation—**9'-cis-Nx, all-trans-Vx, all-trans-Lu as well as chlorophylls a and b were prepared from pea leaves as described previously (21). 9'-cis-Vx was extracted from papaya fruits (*Carica* sp., local market). The extraction of total pigments from papaya, the hydrolysis step in a KOH-ethanol solution, the extraction of carotenoids, and their fractionation by HPLC followed the procedure for carotenoids isolation from pea leaves (see above). 9'-cis-Vx was identified by the 40 nm hypsochromic shift in the absorption and increased hypodichroicity of its furanoid derivative produced by adding HCl (25) and distinguished from the all-trans isomer by its slightly lower polarity and a 4–5 nm hypsochromic shift of absorbance (26).

**Preparation of Recombinant and Native LHCIIb—**Native LHCIIb was isolated as described before (27). Recombinant LHCIIb was prepared from overexpressed apoprotein carrying a His tag at its C terminus (28). Monomeric samples were reconstituted by the detergent exchange method as described previously (29) but without sucrose in the solubilization buffer to facilitate the subsequent purification step on sucrose gradients (0.1–1 M sucrose, 0.1% β-DM, 5 mM Tricine-NaOH, pH 7.8). Monomeric complexes were harvested with a syringe and kept on ice until use. Trimeric LHCIIb was prepared by detergent exchange reconstitution (29) and affinity chromatography (30) and purified on sucrose gradients as described for monomeric complexes.

**Carotenoid Insertion Reactions**—Insertion reactions were carried out with complexes extracted from sucrose gradients, thus containing ~0.2 M sucrose, 0.1% β-DM, 5 mM Tricine-NaOH, pH 7.8. When unbound pigments were to be extracted upon the insertion reaction, sucrose was depleted from the complex solution. In this case, β-DM was added to give a total detergent concentration of 0.3%. The sample was then diluted 10-fold in 5 mM Tricine-NaOH, pH 7.8, and concentrated again on 30-kDa Centricon devices to the original volume, thereby decreasing the sucrose concentration to ~0.02 M. Concentration of LHCIIb was determined using a molar extinction coefficient at 670 nm of 5.46 × 10^4 (mM × cm)^{-1} (31). Xanthophylls for insertion were first completely solubilized in ethanol (concentration of ~1–2 µg/µl). This solution was diluted 1:10 with 0.1% β-DM, 5 mM Tricine-NaOH, pH 7.8, sonified in a bath sonifier for 1 min, and filtrated through 0.2-µm sterile filters. Pigment concentration in the filtrate was determined by analytical HPLC after butanol extraction (32). Insertion was started by mixing LHCIIb and xanthophyll solutions. The xanthophyll solution never exceeded 5% of the final volume to facilitate the comparison of LHCIIb CD spectra taken before and after the insertion reaction by using a J-810 spectropolarimeter (Jasco, Gross-Umstadt, Germany). The temperature during the insertion reaction was controlled by a thermostatted water bath.

**Determination of Binding Constant K—**According to the law of mass action the binding constant K is defined as in Equation 1.

\[
K = \frac{[NLL\text{-}LHCIIb]}{[Nx] 	imes [LL\text{-}LHCIIb]} \quad (Eq. 1)
\]

The ratio of NLL-LHCIIb/LL-LHCIIb was determined via CD478/492 calibration (see Fig. 9), whereas the portion of unbound Nx in equilibrium [Nx] can be expressed as the difference between total Nx minus Nx concentration bound to LHCIIb. Because of the stoichiometry of 1 Nx per apoprotein this can be calculated as in Equation 2.

\[
[Nx] = [Nx\text{(total)}] - [NLL\text{-}LHCIIb] \quad (Eq. 2)
\]
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Total Nx was quantified by HPLC, whereas [NLL-LHCIIb] can be calculated by taking into account the ratio of NLL-LHCIIb/LL-LHCIIb as determined via CD478/492 calibration and the total concentration of LHCIIb as determined by $A_{670}$ with a molar extinction coefficient of $5.46 \times 10^3 \text{ (m}^{-1} \times \text{cm}^{-1})$ (31) (Equation 3).

\[
[NLL-LHCIIb] = \left( \frac{[LHCIIb(total)]}{[NLL-LHCIIb] + 1} \right) \times \frac{[NLL-LHCIIb]}{[LL-LHCIIb]} \quad \text{(Eq. 3)}
\]

By substituting $[NLL-LHCIIb]$ in Equation 2 with Equation 3 and $[Nx]$ in Equation 1 with Equation 2, $K$ can be determined.

**Determination of Rate Constants**—After obtaining an initial CD spectrum (500–440 nm) of LL-LHCIIb at the respective temperature, Nx was added at a 0.5 m ratio with thorough mixing. During the insertion reaction spectra were taken every 60 s. The temperature was controlled by an implemented Peltier-type thermostat. Amplitudes of negative peaks at 478 nm and 492 nm from every spectrum were extracted, and $[NLL-LHCIIb]$ was determined as described above. Rate constants $k$ were determined by using Equation 4 for a second order reaction,

\[
[Nx]_0 - [Nx] = \frac{[LHCIIb]}{k + [LL-LHCIIb]}(1 - e^{-(k + [LL-LHCIIb])[t]})
\]

with $[P] = [NLL-LHCIIb]$; $[A]_0$ and $[B]_0$ are the concentrations of Nx and LL-LHCIIb, respectively, at the beginning of the reaction, $k$ is the rate constant, and $t = $ time (seconds). An iterative fit procedure was performed by minimizing the sum of square deviations at all time points.

**RESULTS**

**Dissociation of Nx from LHCIIb Depends on Temperature and Protein Concentration**—As a first approach to further understand the dynamics of xanthophyll binding to LHCIIb we tested whether Nx and Lu, like Vx, are reversibly bound, i.e. whether these pigments are able to dissociate from the otherwise intact complex. Native trimeric LHCIIb was first diluted in detergent buffer containing 0.1% β-DM. Afterward potentially dissociating pigments were separated from the remaining complexes by ultracentrifugation at different temperatures to overcome possible activation barriers of carotenoids dissociation.

Fig. 1 depicts the relative pigment compositions of native LHCIIb as a result of both decreasing concentration and increasing temperature during isolation by sucrose density centrifugation. The complex concentrations given in Fig. 1 were measured before ultracentrifugation but are expressed as per gradient volume. The combination of nearly hundredfold dilution before centrifugation with a temperature increase up to 40 °C during preparative ultracentrifugation leads to a specific and complete loss of Nx but not Lu (Fig. 1). The dissociation of Nx from LHCIIb can both be observed with respect to Lu (Fig. 1A) and chlorophyll a plus b (Fig. 1B). Pigment compositions of the remaining complexes after Nx dissociation do not significantly change, as indicated by the fact that the graphs in Fig. 1 (A and B) follow the same profile.

**Nx Rebinds at a Defined Stoichiometry and Correct Orientation**—If this specific loss of Nx is based on an equilibrium reaction we would expect the remaining LL-LHCIIb to rebind Nx either upon reconcentrating the sample or upon adding an excess of unbound Nx. Because samples solubilized in a detergent system are hard to concentrate without changing the concentration of detergent, we chose the second approach. However, because biochemical and spectroscopic analysis of emerging complexes demand higher concentrations than that available after extreme dilution, we chose recombinant LHCIIb refolded in the absence of Nx as the substrate for Nx binding. In the presence of chlorophylls a and b and of Lu as the sole carotenoid, both monomeric and trimeric LHCIIb could be isolated by sucrose density ultracentrifugation (Fig. 2). Although the yield of trimeric complexes decreases when Nx is omitted from the refolding mixture, the composition of chlorophylls and remaining Lu molecules within the resulting monomers and trimers remained largely unchanged (Table 1). These complexes were then incubated with Nx solubilized in detergent and subsequently re-isolated on sucrose gradients. HPLC analysis of pigment composition of emerging complexes shows that both monomeric and trimeric LL-LHCIIb take up Nx in a stoi-
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Rebound Nx Is Functional—It was shown before that LHCIIb exhibits a decreased fluorescence yield compared with fully pigmented NLL-LHCIIb (35). Consistently with this observation, fluorescence rises upon association of Nx to pre-folded LL-LHCIIb as monitored by comparing excitation spectra (Fig. 5). Fluorescence emission before (dashed line) and after (solid line) Nx insertion was measured at 720 nm, beyond the emission maximum, to be able to record an excitation spectrum covering the whole red region of chlorophyll absorption. Close inspection of the Soret region of excitation reveals a contribution of newly inserted Nx at ~492 nm. This shoulder was also present in the spectrum of fully pigmented NLL-LHCIIb obtained by folding the protein in the presence of both Lu and Nx (Fig. 5, dotted line). The insertion reaction was allowed to proceed for 1 h at 12 °C before taking the excitation spectra. The slight difference in the blue region between insertion product and fully pigmented NLL-LHCIIb did not indicate deficient coupling of Nx but was caused by Nx insertion not yet being completed after 1 h under these conditions (see below, Fig. 12).

Taken together, these biochemical and spectroscopic results demonstrate that Nx but not Lu dissociates from LHCIIb,
**Neoxanthin Binding to LHCIIb**

**FIGURE 4.** Nx insertion into LL-LHCIIb restores NLL-LHCIIb-specific CD signals. CD spectra were taken after Nx was inserted into LL-LHCIIb for 14 h at 0 °C at the following molar ratios of Nx/LL-LHCIIb. Squares, 0.32; circles, 0.48; and triangles, 0.62. CD spectra of LL-LHCIIb (dashed line) and NLL-LHCIIb (solid line) are given as references. Spectra were normalized to the isosbestic point (see Fig. 9) at 451 nm.

**FIGURE 5.** Inserted Nx contributes to chlorophyll fluorescence and increases overall quantum yield. Excitation spectra of a LL-LHCIIb sample before (dashed line) and after (solid line) Nx insertion are compared. Insertion was carried out at 12 °C for 60 min. The dotted line represents a reference excitation spectrum of NLL-LHCIIb. This spectrum has been normalized to that of the Nx insertion experiment at their maximum signal in the red spectral domain where Nx does not contribute.

resulting in LL-LHCIIb, and that Nx rebinds to LL-LHCIIb to yield NLL-LHCIIb, which is indistinguishable both from native LHCIIb isolated from thylakoids and from recombinant LHCIIb refolded in the presence of all pigments. Thus the N1 binding site at the same time exhibits a pronounced specificity (3, 4) and a reversible binding mode for Nx.

In Contrast to Lu Bound Nx Can Be Exchanged —To verify the notion of reversible Nx binding by an independent assay, we treated NLL-LHCIIb with excess amounts of other carotenoids competing with either Nx or Lu for binding to their respective binding sites. Earlier experiments had identified Zx as a strong competitor for Lu binding to both sites L1 and L2 during complex formation in vitro (3, 4). The competitor carotenoid for Nx binding to the N1 site was 9′-cis-Vx, the carotenoid actually replacing Nx in LHCIIb from C. reflexa (10). The trans conformer of 9′-cis-Vx, all-trans-Vx, was used as a control. This is the cognate carotenoid of the Vx binding site in LHCIIb. All-trans-Vx competes with Lu for L1 and L2 but much more weakly so than Zx and is very unlikely to exhibit any affinity to the Nx binding site, because it lacks the 9′-cis conformation shared by Nx and 9′-cis-Vx.

Exchange experiments were carried out by incubating NLL-LHCIIb and LL-LHCIIb with different amounts of competitors and re-isolating the complexes on sucrose gradients for subsequent pigment analysis. As shown in Fig. 6, Nx bound to N1 was readily displaced by 9′-cis-Vx, providing further proof that indeed the binding of Nx to LHCIIb follows an equilibrium. After the addition of an equimolar amount of 9′-cis-Vx to NLL-LHCIIb, about half of the Nx was replaced by its competitor demonstrating that the affinity of N1 to 9′-cis-Vx was at least as high as that to Nx. At a larger excess of the competitor the exchange was virtually complete upon 2.5-h incubation, revealing rather fast exchange kinetics. Zeaxanthin, a carotenoid that is produced from Vx by de-epoxidation as part of the xanthophyll cycle, also binds to fully pigmented NLL-LHCIIb when offered in excess, however, binding was clearly substoichiometric and, even more importantly, neither Nx nor Lu stoichiometry was affected by Zx binding (Fig. 7A). When LL-LHCIIb with an empty N1 site was exposed to surplus Zx (Fig. 7B) we observed a minor increase of Zx binding, however, still not affecting Lu stoichiometry (Fig. 7B). Interestingly both NLL-LHCIIb and LL-LHCIIb seem to share a somewhat higher binding affinity for Zx in their trimeric state as compared with the monomeric complexes. These competition assays clearly describe L1 and L2 as non-reversible sites for Lu binding,
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Binding Affinity of N1—The selective dissociation of Nx upon dilution of LHCIIb, its re-association, and its exchange with 9'-cis-Vx all demonstrate that Nx binding to LHCIIb is reversible. The assessment of the binding constant of this equilibrium association requires the determination of the ratio of Nx-containing versus Nx-free LHCIIb (NLL-LHCIIb/LL-LHCIIb). For these measurements, we took advantage of a difference in the CD spectra of these complexes. Fig. 9A shows CD spectra of different mixtures of LL-LHCIIb and NLL-LHCIIb with the solid and dashed spectra representing the respective original samples. The loss of CD amplitude at ≈492 nm with decreasing content of Nx complexes was accompanied by a slight bathochromic shift, whereas the inverse was true for the signal at ≈478 nm. Neglecting this shift we took the local amplitudes at these two wavelengths to set up a calibration curve representing a stepwise dilution of NLL-LHCIIb with LL-LHCIIb as shown in Fig. 9B (closed squares). The data were fitted with an algorithm to extract the ratio between NLL-LHCIIb and LL-LHCIIb from the CD 478/CD 492 signal. The optimum fit (power trendline as determined by Excel) is shown in Fig. 9B and yields the following equation:

\[ y = 0.4497 \times x^{-0.2443} \]

\[ y = NLL-LHCIIb/LL-LHCIIb \]

and yields the follow-

\[ y = CD 478/CD 492 \]

This equation can easily be rearranged to calculate NLL-LHCIIb/LL-LHCIIb from measured CD spectra. Unbound Nx did not contribute significantly to the CD spectra as we know from insertion reactions where Nx was offered in excess and emerging spectra were identical to those after purification (not shown).

Fig. 10 shows the titration of N1 in LL-LHCIIb by performing Nx insertions into LL-LHCIIb at increasing ratios (0.32, 0.48, and 0.62) of offered Nx/LL-LHCIIb. The NLL-LHCIIb/LL-LHCIIb ratio obtained was calculated from the CD spectra by using the calibration shown in Fig. 9B. The curve in Fig. 10 represents the expected yield of NLL-LHCIIb/LL-LHCIIb in the case of complete binding of the offered Nx ([NLL-LHCIIb/LL-LHCIIb]_calc = [Nx/LL-LHCIIb]/(1 - [Nx/LL-LHCIIb])). Because the measured yields coincide with this curve quite well, Nx in fact becomes virtually completely bound to the complex, indicating a high binding affinity. With the overall concentration of LHCIIb (as determined by the absorption at 670 nm, see "Experimental Procedures"), the total amount of Nx added (HPLC quantitation), and the determined ratio of Nx-containing versus Nx-free LHCIIb we are now able to calculate the equilibrium constant \( K \) (Equation 1). The six insertions of Nx as illustrated in Figs. 9 and 10 yield \( K \) values between \( 3 \times 10^8 \text{ M}^{-1} \)

because Lu was not replaced by Zx, although this had proven to be a strong competitor of Lu for binding to L1/L2 in ab initio assembly studies of LHCIIb (3, 4). The ability of Vx to replace Nx was crucially dependent on its cis configuration (Figs. 6 and Fig. 8). When mixed with NLL-LHCIIb, all-trans-Vx did co-isolate with complexes (Fig. 8), similar to the behavior of Zx. However, all-trans-Vx clearly did not displace Lu, and the increasing amount of Vx was not accompanied by a respective loss of Nx, indicating either binding to V1 or unspecific association.

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and 1 \times 10^7 \text{ M}^{-1} \text{ mol}^{-1} \text{ mol}^{-1} (Table 1). In addition to these data, two independent complex preparations, including their calibration curves were analyzed, comprising a total of 27 Nx insertion reactions (not shown). In neither of these data sets did \( K \) fall below 1 \times 10^6 \text{ M}^{-1}. Therefore, we take this as a minimum value for \( K \) at 0 °C. The upper error margin was more difficult to establish because large values for \( K \) correspond to very small amounts of unbound Nx, which cannot be quantified with sufficient precision. The binding affinity was determined with monomeric LHCIIb, because trimer-specific CD signals overlap with Nx-dependent CD changes rendering the spectroscopic calibration of Nx binding much less precise in trimeric than in monomeric complexes. However, Fig. 1 shows that Nx binding is reversible in LHCIIb trimers, too, and the virtually complete binding of Nx to trimeric LL-LHCIIb (Table 1) strongly suggests that the minimum value of the equilibrium constant \( K \) for Nx binding to trimeric LHCIIb is of the same order of magnitude as that measured with monomeric complexes.

**Kinetics of Nx Binding**—The different CD spectra of LHCIIb with and without Nx can also be used to monitor the kinetics of Nx binding to LL-LHCIIb in time-resolved CD measurements. Fig. 11 shows the time course of the CD signals at 478 and 492 nm during an Nx insertion reaction performed at 16 °C. As expected, the CD signal at 478 changes to more positive values and the signal at 492 nm changes to more negative values as Nx binds to the complex. At 16 °C, the process is complete after \( \sim 1 \text{ h} \).

To determine the activation energy of Nx binding to pre-folded LL-LHCIIb lacking Nx, we repeated the time-resolved binding experiment at various temperatures. Fig. 12A shows the time course of NLL-LHCIIb formation, as determined by

![Figure 9](image9.png)

**FIGURE 9. A**, CD spectra of mixtures of Nx-containing and Nx-free LHCIIb. LHCIIb was refolded in the presence or absence of Nx. Resulting monomeric complexes were diluted to equal extinctions at 670 nm and mixed at the ratios given below. **Solid line**, monomeric LL-LHCIIb; **dashed line**, fully pigmented monomeric NLL-LHCIIb. **Dotted spectra** are successive mixtures of these samples with NLL-LHCIIb/LL-LHCIIb ratios as follows: 10, 5, 2, 1.5, 1, 0.5, 0.2, and 0.1.

**TABLE 2**

| Nx/LL-LHCIIb | Binding constant \( K \) (M) |
|--------------|-------------------------------|
| 0.32         | 3.4 \times 10^6               |
| 0.32         | 7.8 \times 10^6               |
| 0.48         | 4.6 \times 10^6               |
| 0.48         | 1.0 \times 10^7               |
| 0.62         | 1.0 \times 10^7               |
| 0.62         | 9.7 \times 10^6               |
| Mean value   | 7.6 \times 10^6               |

and 1 \times 10^7 \text{ M}^{-1} (Table 1). In addition to these data, two independent complex preparations, including their calibration curves were analyzed, comprising a total of 27 Nx insertion reactions (not shown). In neither of these data sets did \( K \) fall below 1 \times 10^6 \text{ M}^{-1}. Therefore, we take this as a minimum value for \( K \) at 0 °C. The upper error margin was more difficult to establish because large values for \( K \) correspond to very small amounts of unbound Nx, which cannot be quantified with sufficient precision. The binding affinity was determined with monomeric LHCIIb, because trimer-specific CD signals overlap with Nx-dependent CD changes rendering the spectroscopic calibration of Nx binding much less precise in trimeric than in monomeric complexes. However, Fig. 1 shows that Nx binding is reversible in LHCIIb trimers, too, and the virtually complete binding of Nx to trimeric LL-LHCIIb (Table 1) strongly suggests that the minimum value of the equilibrium constant \( K \) for Nx binding to trimeric LHCIIb is of the same order of magnitude as that measured with monomeric complexes.

**Kinetics of Nx Binding**—The different CD spectra of LHCIIb with and without Nx can also be used to monitor the kinetics of Nx binding to LL-LHCIIb in time-resolved CD measurements. Fig. 11 shows the time course of the CD signals at 478 and 492 nm during an Nx insertion reaction performed at 16 °C. As expected, the CD signal at 478 changes to more positive values and the signal at 492 nm changes to more negative values as Nx binds to the complex. At 16 °C, the process is complete after \( \sim 1 \text{ h} \).

To determine the activation energy of Nx binding to pre-folded LL-LHCIIb lacking Nx, we repeated the time-resolved binding experiment at various temperatures. Fig. 12A shows the time course of NLL-LHCIIb formation, as determined by
the calibrated CD478/CD492 ratio (see Fig. 9). Due to the applied ratio of reactants (LL/H18528LHCIIb/Nx/H11005 2:1) the kinetics has to be treated as a second order reaction and not a pseudo-first order reaction. The integrated rate law as given under “Experimental Procedures” allows for the determination of the rate constant $k$ using the given concentrations of reactants and the calculated concentrations of newly formed NLL/H18528LHCIIb. The lines in Fig. 12A represent best fits of rate constants $k$, for each temperature, based on the minimum sum of deviation from measured data. These rate constants in dependence on the respective temperatures, plotted according to the Arrhenius algorithm (Fig. 12B), yield an activation energy for the insertion of Nx into LL-LHCIIb of 119 kJ/mol. From the narrow error margins of the data points in the linear regression of Fig. 12B we conclude that the error for the slope is $\pm 6$ kJ/mol.

**DISCUSSION**

Several observations presented here demonstrate that binding of Nx to LHCIIb is a reversible process. Both association (Figs. 11 and 12) and dissociation (Fig. 1) of Nx were shown to occur without noticeably altering the remaining structure consisting of the folded protein, chlorophylls, and Lu. Nx binds to prefolded LL-LHCIIb with a 1:1 stoichiometry, the resulting complex shows the same CD spectrum as recombinant LHCIIb assembled in the presence of Nx (Fig. 4) or as native LHCIIb (not shown), and Nx bound to prefolded LL-LHCIIb engages in energy transfer to chlorophylls (Fig. 5). Finally, Nx is readily displaced by 9'-cis-Vx in fully assembled trimeric LHCIIb (Fig. 6). This structural analog of 9'-cis-Nx normally is not present in photosynthetic membranes but found in fruit such as mango (36), papaya (37), or in petals of *Viola tricolor* (26). Only in the
parasitic angiosperm *C. reflexa*, which is not dependent on photosynthesis, has 9′-cis-Vx been found to replace Nx completely in LHCIIb (10).

In contrast to Nx in N1, neither of the two Lu molecules in L1/L2 shows any indication of reversible binding. Upon dilution and increasing the temperature their stoichiometry relative to chlorophylls *a* and *b* remained constant (Fig. 1). Incubating LHCIIb with an excess of Zx or Vx did not lead to any Lu exchange (Figs. 7 and 8), although we know that Zx is an excellent and Vx a weaker competitor for Lu binding to L1/L2 when these carotenoids are present during complex assembly from the unfolded protein, either *in vitro* (3, 4) or in Arabidopsis mutants unable to synthesize Lu (5). Hence, unlike Nx, Lu cannot be exchanged in the otherwise fully assembled complex. To replace Lu, the protein has to be unfolded and refolded. Taken together, the three carotenoid species bound to LHCIIb under non-stress conditions, Lu, Nx, and Vx, all exhibit different binding modes, strong and irreversible for L1 and L2, strong but reversible for N1, and weak and reversible for Vx. These differences in the binding behavior most likely reflect functional differences between the three carotenoids. In the case of Lu and Vx, the correlation is obvious. The two Lu molecules play a more pronounced structural role than the other two carotenoids, being situated at the very core of LHCIIb, interacting with the intertwining helices 1 and 3 along a hydrophobic cleft and with both stromal and luminal protein loops via their ionon end groups (1, 2). Moreover, Lu is not only involved in light harvesting but also in triplet-chlorophyll (35) and non-photochemical quenching (38, 39) and may therefore be indispensable for the photoprotection of LHCIIb. Vx, on the other hand, has been reported to be of minor importance for light harvesting (40) but fulfills its photoprotective role as the key pigment for the xanthophyll cycle (41). Therefore, Vx must dissociate from LHCIIb to perform its major function.

Why is Nx relatively tightly bound to LHCIIb, similar to Lu, but then, like Vx, can dissociate from the complex? The X-ray structure of LHCIIb revealed a previously unexpected position of this carotenoid in the complex: half of the molecule protrudes from the lateral complex surface into the membrane interior (1, 2) with the second ionon ring structure pointing toward the stromal surface of the thylakoid (2). The other end group is deeply buried inside the complex, interacting with several chlorophylls, amino acid side chains of helix 2, and forming some fast relaxation pathway. The effect is relatively small (6 to 8 quite stable hydrogen bonds, is substantial and will ensure that Nx dissociation from LHCIIb is rather slow at moderate temperatures. However, elevated temperatures above 40°C that are easily encountered by plants in their natural habitat will considerably accelerate dissociation. The kinetic energy localized in LHCIIb due to the dissociation of excess excitation energy (thermo-optic effect) has been suggested to be even higher (43) and would further promote Nx dissociation. Thus, it is principally possible that part of LHCIIb in vivo lacks Nx at least transiently. The fact that LHCIIb isolated from thylakoids invariably contains ~1 Nx per apoprotein does not contradict this notion. Part of the chlorophyll *a/b* complexes inevitably disintegrate during the LHCIIb isolation procedure, and on the basis of the observations reported here one would expect that the Nx thus set free would bind to LL-LHCIIb present in the thylakoid to form NLL-LHCIIb.

One potential benefit of partial dissociation of Nx from LHCIIb at elevated temperatures may be to help to protect the antenna system against photo-oxidation. LL-LHCIIb has a lower fluorescence quantum yield than NLL-LHCIIb, indicating that more of its excitation energy is converted to heat by some fast relaxation pathway. The effect is relatively small (<15% reduction of fluorescence quantum yield as judged by comparing integrals of excitation spectra); however, it is possible that the quenching efficiency is enhanced by some complementing structural change such as protonation of luminal LHCIIb domains at low lumen pH in energized thylakoids. Thus such a regulation of the energy flow in the light-harvesting apparatus by Nx dissociation would set in at elevated temperatures makes sense physiologically. High temperatures often lead to light stress, at least in C3 plants, because stomata will be closed to avoid accelerated transpiration, which in turn will limit the supply of carbon dioxide. Consequently, the consumption of NADPH in the dark reaction of photosynthesis is no longer able to keep pace with its production via electron trans-
port in the thylakoid, leading to overexcitation of PSII and its light-harvesting apparatus.

The second potential advantage of Nx dissociation from LHCIIb is connected with the plant hormone ABA. Synthesis of ABA starts with the cleavage of 9'-cis-epoxycarotenoids. In thylakoids of higher plants, both 9-cis-Vx and 9-cis-Nx are potential substrates for the cleavage dioxygenase producing 2-cis,4-trans-xanthoxin as an intermediate toward ABA synthesis. Because 9-cis-Nx is much more abundant in leaves than 9-cis-Vx (44, 45) and in vitro assays with the recombinant dioxygenase VP14 from maize revealed a lower $K_m$ for 9-cis-Nx as compared with 9-cis-Vx (27 $\mu$M versus 58 $\mu$M, respectively), it was suggested that the primary source for ABA synthesis in leaves of higher plants is 9-cis-Nx (44, 46). In fact, a recent analysis of ABA production in orange fruit and leaves showed that, due to differential expression of two 9-cis-epoxycarotenoid dioxygenases, 9-cis-Vx is the substrate for ABA production in fruit and 9-cis-Nx in leaves (47). Furthermore, because 80–90% of 9-cis-Nx in thylakoids is bound to LHCIIb (3, 48), the major light-harvesting complex of photosystem II is a potential source for fast supply of Nx as an educt for ABA synthesis. Data from the present report show that indeed LHCIIb can release 9-cis-Nx upon a slight increase of temperature, and the 9'-cis-diepoxycarotenoid dioxygenase would then drain Nx from the pool generated in the dissociation equilibrium. The steady-state concentration of ABA in leaves is up to two orders of magnitude lower than that of Nx (49, 50). However, the turnover of ABA is thought to be relatively high, regulating the steady-state level of ABA by tuning the rates of biosynthesis and catabolism (51), so the drain on Nx may be much higher. In fact, in cut off leaves dehydrated for 4 h the content of 9'-cis-Nx dropped to ~50%, whereas the overall carotenoid decrease was only 15% (52). Consequently, the availability of Nx may limit the rate of ABA biosynthesis. That elevated temperatures would accelerate Nx dissociation from LHCIIb and, thus, potentially increase the rate of ABA biogenesis makes sense when considering the fact that one of the stress responses triggered by ABA is the closure of stomata. On the other hand, the drainage of Nx from LHCIIb by epoxycarotenoid dioxygenase may be a key step in the stress-induced reduction of the PSII antenna. When plants are acclimated from low to high light intensity, a process often connected with light stress, part of LHCII is degraded proteolytically (53). A proteolytic activity in the thylakoid likely to be involved in this process has been shown to degrade monomeric but not trimeric LHCIIb (54). Therefore, the increased flow of Nx into ABA biosynthesis under light-stress situations would partially decrease LHCIIb and in turn increase its rate of degradation as a stress response.

In this report we have shown that Nx reversibly binds to LHCIIb and that its dissociation can become rather fast at elevated temperatures. The implications of these findings for ABA-related stress responses in plants remain hypothetical at this point; however, they can be tested experimentally. One prediction would be that in plants with a reduced diepoxycarotenoid dioxygenase the light stress-induced LHCIIb degradation is expected to be slower than in the wild type, and another one that in plants containing LHCIIb with reduced stability, e.g. the lut1 and lut2 mutants of Arabidopsis that are defective in Lu synthesis, should exhibit an increased ABA turnover.

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