Early Steps in the Assembly of Light-harvesting Chlorophyll a/b Complex

TIME-RESOLVED FLUORESCENCE MEASUREMENTS

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The light-harvesting chlorophyll a/b complex (LHCIib) spontaneously assembles from its pigment and protein components in detergent solution. The formation of functional LHCIib can be detected in time-resolved experiments by monitoring the establishment of excitation energy transfer from protein-bound chlorophyll b to chlorophyll a. To detect the possible initial steps of chlorophyll binding that may not yet give rise to chlorophyll b-to-a energy transfer, we have monitored LHCIib assembly by measuring excitation energy transfer from a fluorescent dye, covalently bound to the protein, to the chlorophylls. In order to exclude interference of the dye with protein folding or pigment binding, the experiments were repeated with the dye bound to four different positions in the protein. Initial chlorophyll binding occurs at roughly the same rate as the establishment of chlorophyll b-to-a energy transfer, in the range of 10 s. However, under limiting chlorophyll concentrations, the binding of chlorophyll a clearly precedes that of chlorophyll b. The complex containing the apoprotein, carotenoids, and chlorophyll a but no chlorophyll b is biochemically unstable and therefore cannot be isolated. However, chlorophyll a binding into this weak complex is specific, as it does not occur with a C-terminal deletion mutant of Lhcb1 which still contains most chlorophyll-ligating amino acids but is unable to fold and assemble into functional LHCIib. As a scenario for LHCIib assembly in the thylakoid, we propose the initial formation of a labile Lhcb1-chlorophyll a-carotenoid complex that then becomes stabilized by the binding (or formation in situ) of chlorophyll b.

The major light-harvesting chlorophyll (Chl)1 a/b complex (LHCIib) greatly enhances the efficiency of photosynthesis by enlarging the cross-section of photosystem (PS) II and, under some conditions, also of PS I. The structure of LHCIib has been known in near-atomic detail since 1994 (1) and, more recently, has been further resolved to 2.7 Å (2). Much less detail is known about some steps in the biogenesis of LHCIib.

The apoprotein, Lhcb1–3, is synthesized in its precursor form in the cytoplasm, imported into the plastid, and finally inserted into the thylakoid membrane via the signal recognition particle pathway (3, 4). It is unclear when and where the protein becomes complexed with pigments. The signal recognition particle complex presumably keeps Lhcb1–3 in an unfolded and thus nonpigmented form until it is delivered to the thylakoid; on the other hand, Hoeger and Eggink (5) proposed, based on observations in the green alga Chlamydomonas reinhardtii, that LHCIib assembly takes place in the envelope membrane. We do not know how the Chls are delivered to their apoproteins, whether the last steps in their biogenetic pathway take place in the immediate neighborhood of the site of assembly or whether the pigments are transported there via some carrier (6, 7). All we can safely assume is that Chl molecules do not freely diffuse in the thylakoid membrane because this would presumably give rise to potentially dangerous photodissociative reactions. Furthermore, we know nothing about the mechanism of LHCIib assembly. Is it a spontaneous process or assisted by some molecular machinery? Do the pigments bind all at once, or is there a defined sequence of binding events?

The assembly of LHCIib (and other structurally related Chl a/b proteins) can be achieved in vitro. When the unfolded apoprotein Lhcb1, native or recombinant, is mixed with Chls and carotenoids in detergent solution, the protein spontaneously refolds and binds pigments to yield structurally authentic LHCIib (8–10). We have tried to unravel this process in time-resolved (stopped-flow) experiments, and we found that pigment binding and the completion of protein secondary structure both occur with the same kinetics and thus appear to be closely coupled events (11). These processes occur in two apparent phases in the time ranges of 10 s and several min (12, 13). The rate constants of the two apparent phases are dependent on both Chl and carotenoid concentrations, and therefore, both apparent steps presumably include the binding of Chls as well as carotenoids (14).

As a monitor for Chl binding during LHCIib assembly, the establishment of energy transfer from Chl b to Chl a within the complex was employed. This is a useful parameter, as it reflects the appearance of functional LHCIib if pigment-pigment energy transfer is considered an essential function of light-harvesting complexes. On the other hand, this monitor detects Chl binding only if it includes at least one Chl a and one Chl b that engage in energy transfer. If the first step is the binding of one or several molecules of either Chl a or Chl b, this would evade detection. Therefore, in the work presented here we chose a different monitor for Chl binding, a fluorescent dye attached to the protein that is capable of transferring its excitation energy to either Chl a or Chl b. This approach allowed us to kinetically study the initial steps of Chl assembly into LHCIib.

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1 The abbreviations used are: Chl, chlorophyll; Chlide, chlorophyllide; LHCIib, light-harvesting complex of photosystem II; Lhcb1, light-harvesting chlorophyll a/b-binding protein; ΔLhcb1, C-terminal (49 amino acids) deletion mutant of Lhcb1; CAO, Chlade-a oxidase; Bodipy 507/545 IA, N-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yl)iodoacetamide.
Experimental Procedures

Proteins, Pigments, and Dye—Lhcb1 with single cysteines at amino acid positions 3 (N terminus, S33C), 106 (luminal loop, S106C), 160 (stromal loop, S160C), and 229 (C terminus, S229C), all containing a hexahistidyl tag either at the N terminus (S106C and V229C) or the C terminus (S160C), and recombinant S33C4A (Lhcb1 Δct) were transferred to reconstitution buffer with 2% (w/v) octyl-β-D-glucopyranoside and 0.08% phosphatidyl-DL-glycerol in reconstitution buffer. The pigment solution contained pigments as given previously (9). Chls and carotenoids were isolated from pea thylakoids according to Ref. 12. Chlorophyllide (Chlide) α was prepared as described earlier (15). Bodipy 507/545 IA (N-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yl)iodoacetamide) was prepared as described previously (15). Bodipy 507/545 IA was added to a solution of the complete pigment set, the pigment solution contained 0.048 μM Chlide α, 0.13 μM Chl b, 0.08% (w/v) phosphatidyl-DL-glycerol, and 1.4 M NaCl. Reconstitution buffer (reconstitution buffer) was added to the solution at a molar excess of 3–6-fold over the pigment (3–6-fold molar excess), and the incubation was continued for 2 h at 37 °C. For the purification of labeled ΔLhcb1, the protein was precipitated overnight by adding acetone (2.3-fold volume) and acetic acid (1.0-fold volume). To remove excess dye, the protein pellet (after centrifugation, 45 min, 21,900 × g, 4 °C) was washed with 70% ethanol and centrifuged (5 min, 45,000 × g, room temperature). The air-dried pellet was solubilized at a concentration of 39 μM in 1% (w/v) SDS and reconstitution buffer (100 mM lithium borate, pH 9.0, 12.5% (w/v) sucrose, and 5 mM dithiothreitol). The resulting digestion products were separated by fully denaturing SDS-PAGE (15% polyacrylamide) and visualized by Western blotting and decoration with an anti-Lhcb1 antibody (16).

Time-resolved Fluorescence Measurements—Fluorescence data over a time range of 100 s or 700 s were collected on a FluoroMax-2 spectrometer using a front-face setup. Time-resolved measurements were performed over a time range of 700 s with a data pitch of 0.5 s, integration time of 0.1 s, and over a time range of 100 s with a data pitch of 0.1 s and an integration time of 0.08 s. Silt widths were set to 0.5 and 6 nm for excitation and emission, respectively. Dye fluorescence emission upon excitation at 508 nm was measured at 543 nm. Chl fluorescence emission upon excitation at 470 nm (mostly Chl b excitation) was measured at 660 (Chl b emission) and 680 nm (sensitized Chl a emission). Steady-state spectra upon excitation at 508 nm were recorded from 515 to 750 nm with increments of 0.5 nm and integration times of 0.2 s. Silt widths were set to 1 and 5 nm for excitation and emission, respectively. Fluorescence data over a time range of 50 s were collected on an SX.17MV stopped-flow fluorometer (dead time ~1.4 ms) at 24 °C. The path length for excitation was 2 mm; emission was measured at an angle of 90° with a path length of 10 mm. For measuring dye fluorescence, the excitation was at 508 nm (monochromator with bandwidth 0.8 nm), and emission was at 546 nm (bandpass filter with 8 nm at full width, half-maximum). A thousand data points were collected (0–0.5 s, data pitch of 0.001 s; 0.5–50.5 s, data pitch of 0.1 s). The kinetic traces were fitted to a sum of two exponentials by using the software KaleidaGraph (version 4.1.4.0, SPSS Inc.). Time constants are the reciprocals of the calculated rate constants. The presented data reflect the average of at least three independent measurements. Time-resolved CD measurements were performed as described previously (11).

Results

Pigment Binding Can Be Monitored by the Signal Change of a Fluorescent Dye Monitor—Monomeric light-harvesting complex of photosystem II (LHCIIb) can be refolded in vitro by simply mixing the apoprotein Lhcb1 with Chls and carotenoids in the presence of detergents. Complex formation consists of folding of the protein into an α-helical secondary structure and binding of at least 12 Chls and 3 carotenoid molecules to the protein. In our previous time-resolved measurements of LHCIIb formation, we used the intra-complex energy transfer from Chl b to Chl a as a monitor for complex formation. Upon excitation of Chl b at 470 nm, more and more of the excitation energy is transferred to Chl a as the complex forms, visible as a gradual decrease in Chl b emission (660 nm) and a concomitant increase in Chl a emission (680 nm). In the present work, we also monitored LHCIIb formation by energy transfer measurements, but we introduced an artificial energy donor into the system by site-specifically labeling the protein at singular cysteine positions with the fluorescent dye Bodipy 507/545 IA. This dye, with excitation and emission wavelengths at 508 and 543 nm, efficiently transfers its excitation energy to a Chl (Fig. 1). After reconstituting the dye-labeled Lhcb1 with pigments and illuminating it at 508 nm, the dye emission at 543 nm is virtually completely quenched. At the same time, sensitized Chl a fluorescence appears at 680 nm. In a control measurement (Fig. 1, dashed line), with the dye and chlorophylls at the same concentration as in the labeled LHCIIb solution but not organized in a complex, Chl a emission (due to co-excitation at 543 nm) is much lower. In this control, the emission of unbound Bodipy 507/545 IA is also lower than that of the labeled protein (Fig. 1, gray line), presumably because of an increased fluorescence quantum yield of the protein-bound dye and to an inner filter effect of the chlorophylls present. In Bodipy-labeled LHCIIb, only Chl a emits although the dye can transfer its excitation energy to both Chl b and Chl a (critical Förster distances R0 calculated as 47 and 57 Å, respectively, for a situation with one donor and one acceptor molecule). This is because of rapid energy transfer from Chl b to Chl a within the complex.

The quenching of dye fluorescence in Bodipy 507/545 IA-labeled Lhcb1 during its reconstitution with pigments can be used as a monitor for pigment protein-complex formation (Fig. 2a).
FIG. 1. Energy transfer to Chls from the donor dye (Bodipy 507/545 IA) covalently attached to Lhcb1. Steady-state spectra of fluorescence emission after excitation at 508 nm (dye absorption maxima) of the N-terminally labeled Lhcb1 without the addition of pigments (gray line) and at the end of a refolding experiment yielding functional LHCIIb (black line). The dashed spectrum shows a mixture of dye and pigments in a concentration according to the conditions of the refolding experiment.

FIG. 2. Time-resolved measurements of LHCIIb assembly with two different fluorescence monitors. a, fluorescence emission of Lhcb1-attached dye (N terminus) at 543 nm after rapid mixing in a stopped-flow apparatus under different conditions. Dotted trace, mixing of dye-labeled Lhcb1 with Chls and carotenoids in detergent/lipid solution; black trace, mixing of dye-labeled Lhcb1 with detergent/lipid solution lacking pigments. Gray trace, mixing of dye with Chls and carotenoids in appropriate concentrations. Inset, same experiments as in a but performed on an Applied Photophysics SX.17MV stopped-flow fluorometer with a dead time of about 1.4 ms. Measurements over a time range of 50 s with an Applied Photophysics SX.17MV stopped-flow fluorometer with a dead time of about 1.4 ms revealed an additional apparent step in the decrease of dye fluorescence in the 10-ms range (Fig. 2a, inset, black dots). This decrease also is dependent on the presence of protein and pigments, as it is not seen in the absence of pigments (Fig. 2a, inset, black line) or in the absence of protein, i.e., when dye and pigments are mixed (gray line).

For comparison, the same experiment over the time range of 700 s was performed with excitation of Chl b, monitoring the energy transfer from Chl b to Chl a appearing during complex formation (Fig. 2b). Both the decrease of Chl b emission and the increase of sensitized Chl a emission occur in the same time range as the quenching of Bodipy 507/545 IA fluorescence. All signal changes can be described by two apparent steps with time constants of about 25 and 200 s (see below).

Labeling a protein with a fluorescent dye introduces the risk that the covalently attached label might influence the structure of the protein and thus its folding behavior. Therefore, we attached the label to four different sites in Lhcb1, the N and C termini and the luminal and stromal loop domains, and we compared the kinetics of LHCIIb formation. Fig. 3 shows that the behavior of all labeled proteins as monitored by the dye fluorescence quenching was very similar. The biexponential fit of the four traces revealed the apparent time constants \( \tau_1 \) between 15 and 22 s and \( \tau_2 \) of about 4 min irrespective of the site of dye attachment. The only protein derivative showing slightly different kinetics is the one labeled in the stromal loop domain. Its first step is somewhat slower (22 s) than those of the other labeled proteins (15 s) and comprises more than half of the total amplitude, whereas with the other proteins the second step is the predominant one (Table I). Moreover, the total signal change is somewhat larger when the dye is attached to the stromal loop in comparison to the other attachment sites. However, apart from these relatively small differences, the kinetic behavior of the differently labeled proteins during LHCIIb reconstitution is the same. We conclude that

2) The strong decrease in Bodipy 507/545 IA emission (Fig. 2a, dotted trace) is dependent on pigment-protein interaction, as no signal change is observed when only unbound dye and pigments are mixed (Fig. 2a, gray trace), and only a much smaller decrease is seen when the protein-bound dye is diluted into a detergent/lipid solution without pigments (Fig. 2a, continuous black trace), possibly due to partial aggregation of the nonpigmented protein.

The total amplitude of the decrease measured over a time range of 700 s is about 47% of the initial signal, which is less than what would be expected from the steady-state measurement. This is because part of the signal change is lost during the experimental dead time of \( \approx 2 \) s. Measurements over a time range of 50 s with an Applied Photophysics SX.17MV stopped-flow fluorometer with a dead time of about 1.4 ms revealed an additional apparent step in the decrease of dye fluorescence in the 10-ms range (Fig. 2a, inset, black dots). This decrease also is dependent on the presence of protein and pigments, as it is not seen in the absence of pigments (Fig. 2a, inset, black line) or in the absence of protein, i.e., when dye and pigments are mixed (gray line).
the dye does not significantly interfere with protein folding or pigment binding.

To test whether the decrease in the monitor dye fluorescence correlates with the formation of a functional LHCIIb, we labeled an apoprotein (ΔLhcb1) lacking most of its C-proximal trans-membrane helix and known to be able to form a stable pigment-protein complex (17). As expected, no energy transfer from Chl b to Chl a was observed during reconstitution attempts with this protein (not shown). However, if this protein binds either Chl a or Chl b or if it binds both such that they do not transfer energy, this binding would have escaped a monitor that is expected to pick up a single Chl molecule closely coordinated in the complete absence of pigments. Hence, a functional apoprotein is required for seeing pigment binding even with a fluorescent dye able to transfer its energy to any Chl. As shown in Fig. 4, the deletion mutant of Lhcb1 shows no signal change of the dye monitor exceeding the small one seen in the complete absence of pigments. Moreover, this observation clearly demonstrates that the quenching of Bodipy 507/547 1A fluorescence is not due to unspecific binding of Chls to the protein, as the truncated protein still contains two of its three hydrophobic α-helical membranes and most of the amino acids serving as ligands in native LHCIIb. We cannot exclude unspecific binding for the very fast quenching of the dye fluorescence with the time constant of 10 ms because no kinetic measurements over the time range of 50 s with a dead time of 1.4 ms were performed with the mutant.

**Fluorescent Dye Detects Early Pigment Binding Steps during LHCIIb Assembly in Vitro**—In the experiment described in Fig. 2, virtually the same kinetics of LHCIIb assembly was seen with energy transfer either from the fluorescent dye to Chls or from Chl b to Chl a. This was no longer the case when we lowered the Chl concentration in the reconstitution mixture. When the stoichiometric excess of Chls over protein dropped below 2 (protein and carotenoid concentrations were held constant), both apparent reaction phases slowed down when monitored by Chl-Chl energy transfer (Fig. 5a), in accordance with our earlier observations (14). On the other hand, when LHCIIb assembly was monitored by the fluorescent dye positioned in the stromal loop of the protein, both time constants τ1 and τ2 did not decrease but even decreased to some extent as the Chl stoichiometry dropped from 1.6- to 1.2-fold. This decrease was not significant in the case of the fluorescent dye monitor bound to the N or C termini or the luminal loop (Fig. 5b), but clearly, the fluorescent dye monitor did not indicate slower pigment binding at low Chl concentrations, as opposed to the Chl b monitor.

**Fluorescent Dye Monitor Detects Binding of Chl a in the Absence of Chl b**—Upon reconstituting LHCIIb at a low stoichiometric excess of Chls, the energy transfer from the fluorescent dye to Chls is established significantly faster than the energy transfer from Chl b to Chl a. This indicates that during the initial phase of pigment binding the fluorescent dye detects the binding of Chl a that do not yet take part in Chl-Chl energy transfer, presumably because only Chl a or only Chl b is bound. Therefore, we performed kinetic experiments in which either Chl was omitted from the pigment solution. As clearly shown in Fig. 6, Chl a in the absence of Chl b (Fig. 6, AC, black filled dots) binds faster to the protein than does Chl b in the absence of Chl a (BC, dark gray crosses). Exponential fits of these traces in comparison to that obtained with the full pigment complement (Fig. 6, ABC, dark gray trace) revealed that both reactions can still be described by two apparent phases, but the time constants and/or their amplitudes change (Table II). With Chl a as the only Chl, τ1 is the same as with both Chls, whereas τ2 doubles. However, in the AC trace the faster phase covers more than 2/3 of the total amplitude, whereas the slower phase predominates when both Chls are present, which makes the overall reaction even faster when Chl a is the only Chl, at least for the first 100 s. On the other hand, Chl b in the absence of Chl a binds at a reduced overall rate as compared with Chl a alone or the full pigment complement. This is mostly due to τ1 being doubled. The time constant of the slower phase, τ2, could only be determined with a relatively large uncertainty for this trace; it appears to be somewhere between that measured with Chl a only and that with both Chls. The same is true for the amplitude ratio A1/A2 (where A1 and A2 indicate amplitudes associated with the time constants τ1 and τ2).

When both Chls were present in the pigment solution but the carotenoids were omitted (Fig. 6, AB, light gray trace), the fluorescent dye exhibited a fast drop in its emission intensity and then a rise toward the signal level seen in the complete absence of pigments. This indicates transient Chl binding of Chls to the protein, confirming that carotenoids are structural components of the complex that are required for stable Chl binding. After mixing the Lhcb1 with carotenoids and Chlide a, no signal change could be observed at all (not shown), indicating that the Chl a precursor Chlide a is not able to interact specifically with the protein.

When the mixture containing labeled Lhcb1, Chl a, and carotenoids was diluted 2-fold with detergent solution, a rise in the dye-fluorescence was observed (Fig. 7), indicating partial dissociation of the pigment-protein complex. No such fluorescence increase was monitored when diluting the solution containing fully assembled LHCIIb after mixing the protein with the complete pigment set, i.e. Chl a, carotenoids, and Chl b (Fig. 7, inset).

Concomitantly with the binding of Chl a and carotenoid to

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**Table 1**

Kinetic data of LHCIIb folding and assembly from time-resolved fluorescence measurements of the monitor dye attached to four different sites of Lhcb1

| Site labeled | τ1 (s) | τ2 (s) | A1/A2 |
|--------------|--------|--------|-------|
| N terminus   | 15 ± 1 | 243 ± 24| 0.8 ± 0.1 |
| C terminus   | 15 ± 2 | 272 ± 23| 0.6 ± 0.2 |
| Stromal loop | 22 ± 3 | 229 ± 14| 1.2 ± 0.2 |
| Luminal loop | 16 ± 5 | 275 ± 32| 0.6 ± 0.1 |

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**Fig. 4. Kinetics of LHCIIb assembly with functional and non-functional apoprotein.** Time-resolved fluorescence measurement of the dye attached to the N terminus of full-length Lhcb1 and a C-terminal deletion mutant ΔLhcb1 (ΔC49), respectively, when mixed with pigments (Chl a, Chl b, and carotenoids; black trace, Lhcb1; dark gray trace, ΔLhcb1), and without pigments (light gray trace, ΔLhcb1). Concentrations are same as in Fig. 1.
Early Steps in LHCIIb Assembly

Fig. 5. Kinetics of LHCIIb assembly at different Chl stoichiometries. Time-resolved fluorescence measurement of LHCIIb assembly monitoring either the fluorescence of the dye attached to different sites of Lhcb1 or of Chl b, with increasing stoichiometric excess of Chl over protein (assuming 12 Chl molecules/LHCIb molecule; carotenoids 47 μM, 4-fold stoichiometric excess assuming 3 molecules/LHClIb molecule). a, open triangles, stromal loop; filled squares, Chl b. b, open triangles, N terminus; open squares, luminal loop; open circles, C-terminal; filled squares, Chl b. Time constants τ1 and τ2 are derived from the biexponential fit of the time-resolved dye signal recorded at 543 nm or the Chl b signal recorded at 660 nm.

Fig. 6. Kinetics of LHCIIb assembly with different pigment sets. Time-resolved fluorescence measurement at 543 nm of the monitor dye attached to the stromal loop of Lhcb1 after mixing the protein solution with different pigment sets: complete set with Chl a, Chl b, and carotenoids (dark gray trace); incomplete sets lacking Chl b (black filled dots), lacking Chl a (dark gray crosses), lacking carotenoids (light gray trace), and lacking all pigments (open black circles). (3.9 μM Chlb1: 71 μM Chls, 1.5-fold stoichiometric excess assuming 12 molecules/LHCIIb molecule; 47 μM carotenoids, 4-fold stoichiometric excess assuming 3 molecules/LHCIIb molecule.)

The Lhcb1 in the absence of Chl b, an increase in α-helical secondary structure was also observed by following the change of the CD signal at 222 nm (not shown). A biexponential fit of the signal change revealed a time constant of 26 s. The amplitude of the signal change comprised about 2/3 of the change observed for the formation of functional LHClIb (after mixing the Lhcb1 with carotenoids and Chl a + b). In this case two time constants of 21 and 165 s were resolved. No increase in α-helical structure was observed by CD when Lhcb1 and carotenoids but no chlorophylls were mixed under refolding conditions (not shown).

In contrast to LHClIb, which apart from the 50 N-terminal amino acids is resistant against protease digestion (18, 19), the Lhcb1 complex with pigments lacking Chl b is not. Treating the mixture containing Lhcb1, Chl a, and carotenoids with the protease trypsin led to the total degradation of the apoprotein as no fragments could be detected on an SDS gel (not shown).

Discussion

In this study, we investigated the assembly of Lhcb1 with pigments by time-resolved measurements of the energy transfer between a protein-bound fluorescent dye and chlorophylls. The fluorescent label enabled us to detect the first Chl molecule(s) to be bound during this process, whereas the monitor we used earlier, the energy transfer from Chl b to Chl a, requires that both Chl species are already present in the assembling complex. The critical Förster distance (that is the distance between donor and acceptor at which the energy transfer efficiency is expected to be 50%) between the dye and Chl a or b is 47 or 57 Å (as calculated for a situation with one donor and one acceptor molecule, respectively). This should be large enough for the dye to be able to detect any Chl bound, regardless of its binding site. The chlorophyll would only be “invisible” to the dye if their transitions dipoles were orthogonal to each other, which is unlikely given the high mobility of the hydrophilic protein domains to which the dye is attached.

Table II

| Pigment set | τ1 (s) | τ2 (s) | A1/A2 | Atotal (%) |
|-------------|-------|-------|-------|-----------|
| ABC         | 24 ± 3| 182 ± 12| 0.3 ± 0.1| 100 ± 3 |
| AC          | 24 ± 3| 372 ± 57| 2.3 ± 0.5| 95 ± 11 |
| BC          | 49 ± 15| 280 ± 123| 1.8 ± 1.1| 72 ± 10 |
| AB          | 16 ± 1| None |       |           |

The sets used are as follows: ABC, complete set; AC, lacking Chl b, BC lacking Chl a; AB, lacking carotenoids. Time constants τ1 and τ2 and amplitudes A1 and A2 are derived from the biexponential fit of the time-resolved dye signals (shown in Fig. 3, protein labeled in the stromal loop) recorded at 543 nm after mixing with different pigment sets (Fig. 6).
We can exclude that the fluorescent label interfered with the assembly kinetics by altering the protein structure and/or mobility. We have seen virtually the same assembly kinetics regardless of whether the dye was attached to either terminus or the stromal or luminal loop domains of the protein (Fig. 3). If the dye had an influence on the folding or pigment binding of the protein, then this would clearly be expected to be dependent on its position in the protein structure.

The data presented here confirm our earlier observations that assembly of the Lhcb1-pigment complex takes place in the range of 10 s to several minutes. We did see some energy transfer appear in a very fast step of about 10 ms (Fig. 2, inset). However, we cannot exclude at this point that this reflects nonspecific Chl attachment to the protein. On the other hand, the kinetic phases in the second and minute time range clearly reflect specific interaction between protein and pigments, since a mutant version of the protein that is unable to form stable complexes with pigments, but still contains most of its chlorophyll-binding amino acids, established no energy transfer between the dye and chlorophylls and, hence, did not bind any chlorophylls in our kinetic experiments (Fig. 4).

The expected advantage of the dye monitor to detect Chl binding prior to energy transfer between Chl $b$ and Chl $a$ was most obvious when assembly kinetics were measured at low pigment concentrations. Although the establishment of energy transfer between the Chls slowed down as the pigment concentration decreased, the Chl binding as seen by the fluorescent dye monitor did not (Fig. 5, a and b). The simplest explanation is that the reaction monitored by the energy transfer between the fluorescent dye and some Chl kinetically resembles a first-order process where rate constants are independent of pigment concentrations, whereas the reaction monitored by Chl-Chl energy transfer, requiring at least 2 Chls to be bound, does not resemble a first-order process and therefore exhibits concentration-dependent reaction times. Clearly, the binding of several Chl and carotenoid molecules and possibly additional molecules, such as lipids to an apoprotein, cannot be expected to result in first-order reaction kinetics; therefore, we treated the exponential signal changes in this and previous work as apparent kinetic phases rather than first-order kinetic traces. On the other hand, not all components for LHCCIb formation are necessarily rate-limiting to the same extent, and in fact the rate constants become independent of Chl and carotenoid concentrations when these are raised to a certain level (14), indicating pseudo-first order kinetics.

The energy-donor dye as a monitor for Chl binding has the major advantage that it can be used in experiments with either Chl $a$ or Chl $b$, whereas the energy transfer from Chl $b$ to Chl $a$ as a monitor obviously requires the presence of both Chls. Such experiments show that Chl $a$ binding is faster than that of Chl $b$ (Fig. 6 and Table II). We conclude that in the initial phase of pigment assembly Chl $a$ is bound to the protein earlier than Chl $b$, at least under conditions of low pigment concentrations.

Upon the initial Chl $a$ binding, Chl $b$ needs to be bound to Lhcb1 for Chl $b$-Chl $a$ energy transfer to occur. However, Chl $b$ appears to be less rate-limiting for the onset of energy transfer than Chl $a$. In a previous study (14) we observed an accelerated establishment of Chl $b$-to-Chl $a$ energy transfer as the Chl $a/b$ ratio in the pigment mixture was raised (with the total Chl concentration being held constant). Hence, Chl $a$ binding not only precedes Chl $b$ binding in the initial phase of LHCCIb assembly in vitro but also accelerates the formation of functional LHCCIb. We propose that the initial binding of Chl $a$ accelerates the subsequent binding of Chl $b$ (and possibly additional Chl $a$) molecules, possibly by triggering some protein folding (see below). Chl $a$ binding is not a prerequisite for Chl $b$ binding, as stable Lhcb1-pigment complexes can be assembled in the complete absence of Chl $a$ (20, 21).

In the absence of carotenoids, no more than a transient interaction of Chls with Lhcb1 is detected by the fluorescence dye monitor. This is in accordance with earlier observations that carotenoids are required for the formation of LHCCIb in vitro (8, 9) with lutein being the most efficiently stabilizing carotenoid (22–24). Unexpectedly, the fluorescent dye monitor detected specific Chl $a$ binding to Lhcb1 in the absence of Chl $b$, although it had been noted earlier that Chl $b$ is an absolute requirement for stable Lhcb1-pigment complex formation in vitro (21, 22) and stable insertion of Lhcb1 in etioplast membranes (25). It is well established that LHCCIb appearance in green plant tissue coincides with the accumulation of Chl $b$ (26–28). It should be noted, however, that all these reports refer to biochemically detectable LHCCIb. The complex described in the work presented here, consisting of Lhcb1, carotenoids, and Chl $a$, is so labile that, by contrast to native or recombinant LHCCIb, it dissociates upon mere dilution (Fig. 7); therefore, this complex cannot be isolated biochemically. Consistently, we observed rapid degradation of this complex by trypsin, whereas native or recombinant LHCCIb is known to be largely resistant toward proteases such as trypsin or thermolysin, except for part of the N-terminal hydrophilic domain (19, 29).

Although the binding of pigments minus Chl $b$ induces the formation of some α-helical structure in Lhcb1, we conclude that it leads to a more open structure than the binding of the full complement of pigments. The Chl $b$-dependent condensation of the LHCCIb structure is likely to be directly related to its increased stability (30).

It should be noted that carotenoids are required even for the labile binding of Chl $a$ to Lhcb1. At present, we cannot monitor the binding of lutein or other carotenoids into the complex. However, it can be assumed that carotenoid binding is coordinated with or even precedes Chl $a$ binding. If there is some weak interaction between Lhcb1 and carotenoids already in the absence of Chls, this would have escaped our earlier attempts to isolate such complexes (9). However, the addition of carotenoids to Lhcb1 under refolding conditions does not initiate the formation of α-helical secondary protein structure. Therefore, at least for triggering protein folding, both carotenoids and Chl need to be bound.

The environment in which LHCCIb assembly occurs in vivo,
the thylakoid membrane, or, according to observations made in C. reinhardtii, the inner envelope membrane (5, 31) clearly is different from the detergent micelles in which LHClIb reconstitution in vitro is achieved. However, because the structure resulting from either process is virtually the same (10) and because detergent micelles can be thought of as mimicking the hydrophobic phase otherwise provided by the membranes, it does not seem unreasonable to assume that mechanistically LHClIb reconstitution bears some similarities to LHClIb assembly in the thylakoid. To avoid photooxidative damage by unbound chlorophylls during greening, the newly made chlorophylls must be rapidly sequestered into complexes with proteins and carotenoids. LHClIb appears relatively late during the greening process, presumably because Chl b is accumulated and Lhcb1–3 are stabilized by chlorophylls and carotenoids only after other apoproteins exhibiting a higher affinity to chlorophyll a, such as the reaction center proteins, have been saturated (26, 32). Hence, the assembly of LHClIb in vitro takes place under conditions of limited Chl availability, particularly of Chl b which appears at the same time as LHClIb and when other Chl-a/b proteins accumulate (25, 33, 34). Therefore, in analogy to our observations in vitro, it is likely that during LHClIb biogenesis Chl a binding precedes that of Chl b.

The finding that the Lhcb1-Chl a-carotenoid complex is labile and susceptible to degradation by protease is in agreement with the observation that during the greening process the protein only accumulates in the thylakoid as Chl b appears. The fact, however, that Lhcb1 is able to interact with Chl a before Chl b is present opens the possibility that this LHcb1-bound Chl a is converted to Chl b in situ, as has been suggested before (35–37). For the formation of Chl b, Chloride-a oxidase (CAO) is needed (38, 39). The substrate of this enzyme is thought to be Chloride a rather than Chl a (40). By using our fluorescent dye monitor, we could not detect any binding of Chlide a to Lhcb1 in the presence of carotenoids; therefore, our observations do not support a scenario of LHcb1-bound Chloride a being converted to Chloride b by CAO. However, the possibility should be considered that although CAO does not accept Chl a in detergent solution as a substrate, it may still be able to convert protein-bound Chl a to Chl b. It is interesting that in a recent paper Nagata et al. (41) reported on a conserved A domain found in eukaryotic CAOs but not in a CAO of a prochlorophyte that does not contain LHClIb as a Chl b-binding protein; these authors suggest the A domain interacts with LHC proteins, which may be a prerequisite for the CAO to convert Chl a to Chl b in situ.

Although we show in this paper that Chl a binding to Lhcb1 precedes that of Chl b, we have not been able to dissect the two apparent phases of pigment binding in the time ranges of 10 s and several minutes. Both phases are seen, although to a different extent, regardless of whether only Chl a or only Chl b or both are present in the pigment mixture to be assembled with Lhcb1. Therefore, we still cannot distinguish whether these apparent phases in fact represent consecutive reaction steps or reflect a higher order reaction kinetics, or whether they are due to different populations of proteins folding and assembling at different rates. To address the latter possibility, we are presently studying the assembly of single Lhcb1 molecules.

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