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Assembly of Light-Harvesting Chlorophyll *a/b* Complex *in Vitro*. Time-Resolved Fluorescence Measurements†

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ABSTRACT: The assembly kinetics of a pigment-binding membrane protein, the light-harvesting chlorophyll *a/b* complex of green plants, have been determined *in vitro*. Time-resolved fluorescence spectroscopy, with millisecond time resolution, has been used to monitor changes in both protein and chlorophyll (Chl) fluorescence, as well as in energy transfer from Chl *b* to Chl *a*, during complex assembly. Three reaction steps could be resolved after rapid, stopped-flow, mixing of the apoprotein (light-harvesting Chl *a/b* protein, LHCP) and pigments, solubilized in sodium dodecyl sulfate (SDS) and octyl glucoside (OG), respectively. A fast step in the range of 10 ms was detected regardless of whether the reaction mixture contained pigments or protein, or both, and is interpreted as being connected with the formation of mixed SDS and OG detergent micelles. Two further steps were resolved: one with a time constant of about 1 min and another, slow step with a time constant of several minutes. Both of these were dependent on the presence of protein, Chls, and xanthophylls. Most, if not all, of the energy transfer from Chl *b* to Chl *a* was established during the slow step, indicating that the juxtaposition of these pigments, either by a structural rearrangement of the complex or by additional pigment binding, is the final stage in LHCII assembly *in vitro*.

Assembly of membrane-bound proteins is essential to membrane function; however, surprisingly little is known about the process. Photosynthesis is one process which relies on the correct assembly within the photosynthetic membrane of pigment-binding proteins. Indeed, the starting point for the whole photosynthetic process is the capture of light by light-harvesting complexes which bind many chlorophyll (Chl)[1] molecules. The most prominent of these so-called antenna complexes is the major light-harvesting chlorophyll *a/b* complex of photosystem II (LHCII) (Jansson, 1994). Not only is it the most abundant pigment-binding protein, containing about 50% of the total pigments involved in photosynthesis, but also there is a large body of information available about this particular complex (Paulsen, 1995). LHCII is one of the very few membrane proteins for which a high-resolution structure is known. The structural analysis of LHCII reveals that its apoprotein, light-harvesting chlorophyll *a/b* protein (LHCP), contains 3 α-helical trans-

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[1] Abbreviations: Chl, chlorophyll; LDS, lithium dodecyl sulfate; LHCII, light-harvesting complex of photosystem II; LHCP, light-harvesting chlorophyll *a/b*-binding protein; OG, octyl glucoside; PG, dipalmitoylphosphatidylglycerol; SDS, sodium dodecyl sulfate.

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membrane domains and noncovalently binds at least 12 Chl and 2 xanthophyll molecules (Kühlbrandt et al., 1994). A number of contact sites between protein and pigments have been resolved. However, it is still completely unknown how these interactions are established during the biogenesis of LHCII.

A helpful feature for studying membrane protein assembly is the ability to reconstitute the protein *in vitro*. While many water-soluble proteins can be refolded *in vitro* from a denatured state into their native structure with refolding occurring spontaneously (Matthews, 1993), to date this has only been achieved for a few membrane proteins; among them porins (Eisele & Rosenbusch, 1990), bacteriorhodopsin (London & Khorana, 1982), and LHCII. Stable LHCP−pigment complexes can be reconstituted *in vitro* in detergent solution by mixing denatured LHCP with pigments and subjecting it to renaturing conditions such as a number of freeze−thaw cycles (Plumley & Schmidt, 1987; Paulsen et al., 1990). Later experiments showed that reconstitution is also induced by adding a nonionic detergent like octyl glucoside (OG) to the pigment−protein mixture in lithium dodecylsulfate (LDS) and then removing most of the dodecyl sulfate by precipitation (Paulsen et al., 1993).

Crystallization experiments with reconstituted LHCII suggested that its structure is very similar to that of native LHCII (Hobe et al., 1994). The reconstituted complex is at least in part functional, as energy transfer from Chl *b* to Chl *a* is virtually 100% efficient (Paulsen et al., 1990). Measurements of the circular dichroism (CD) of LHCP before and after its reconstitution with pigments revealed that pigment binding is coupled to the folding of the protein, indicated by a significant increase in the apparent α-helix content during reconstitution (Paulsen et al., 1993). The refolding of LHCP is dependent on the presence of the complete set of pigments which is consistent with earlier observations that formation of stable LHCII depends on highly cooperative interactions of pigments and protein (Cammarata & Schmidt, 1992; Paulsen & Hobe, 1992). Moreover, these findings suggested that the binding of pigments triggers the folding of LHCP, at least during reconstitution *in vitro* but possibly also during LHCII biogenesis.

In a recent study, the regeneration of the membrane protein bacteriorhodopsin from its denatured apoprotein bacterioopsin and its retinal pigment has been studied in a time-resolved fashion using a stopped-flow technique (Booth et al., 1995). Transient folding intermediates were detected which form before noncovalent and, finally, covalent binding of retinal. Therefore, substantial folding of bacterioopsin can occur in the absence of its retinal pigment, implying that retinal does not initiate folding. This conclusion is in contrast to the situation with LHCP *in vitro* where folding seems to be triggered by the binding of pigments (see above).

Here, we investigate the folding and assembly kinetics of LHCII, using the approach developed for studying bacteriorhodopsin assembly. We show that LHCII assembly is induced by mixing SDS-solubilized apoprotein, LHCP, with pigments dissolved in OG, thus diluting out (rather than precipitating) the denaturing detergent SDS. This mixing step can be performed very rapidly in a stopped-flow apparatus, enabling us to measure the kinetics of LHCII assembly in the millisecond to second range.

MATERIALS AND METHODS

Proteins, Pigments, and Lipids. LHCP and pLHCP were overexpressed in bacteria carrying constructs of the *lhcb*1 gene "AB80" (Cashmore, 1984) from pea (Paulsen et al., 1990). Chls were obtained from Serva (Heidelberg, Germany) or Sigma (Poole, U.K.) or isolated from pea or spinach thylakoids by acetone extraction, dioxane−water precipitation (Iriyama & Shiraki, 1979), and subsequent separation on RP-18 (μ-Bondapak, 15−20 μm, Waters, Eschborn, Germany) with acetone/water, 82:18 (v/v), as the eluent. Xanthophylls were purified from the supernatant according to Davies (1976). In most folding experiments, a total pigment extract from thylakoids was used, complemented with isolated Chl *b* to obtain a Chl *a/b* ratio of 1, and with isolated xanthophylls (Paulsen et al., 1990). Sodium dipalmitoyl-L-α-phosphatidyl-DL-glycerol (PG) was obtained from Sigma (Munich, Germany).

Reconstitution of LHCII. The assembly of LHCP−pigment complexes was initiated by mixing equal volumes of protein and pigment solutions. Protein solutions contained 6.5 μM LHCP or pLHCP and 0.2% (w/v) SDS (or LDS) in reconstitution buffer (100 mM sodium borate, pH 9.0, 12.5% sucrose, and 5 mM dithioerythritol). Pigment solutions containing Chl *a* and Chl *b* (each 65 μM) and 20 μM xanthophylls were prepared by dissolving the pigments in ethanol [ethanol concentration in pigment solution 2% (v/v) or less] and adding them to a solution of 2% (w/v) OG, 0.075% (w/v) PG in reconstitution buffer. The OG/PG solution was sonicated for 15 min in a waterbath sonicator at room temperature in the dark prior to addition of pigments. In some experiments, some of the pigments were omitted as indicated. Chl concentrations were calculated from absorption measurements according to Porra (Porra et al.; 1989). Xanthophyll concentrations were calculated using an extinction coefficient of 240 mL (mg cm)$^{-1}$ (Davies, 1976). Complexes were analyzed on a partially denaturing polyacrylamide gel (Paulsen et al., 1990).

Fluorescence Measurements. For steady-state fluorescence measurements of energy transfer between Chl *a* and Chl *b*, 100 μL each of protein and pigment solutions was mixed at room temperature in a 5 mm cuvette, and fluorescence spectra were measured using a Hitachi F-2000 fluorometer with the excitation wavelength set to 460 nm, excitation and emission bandwidths at 5 nm, and a neutral density filter (2% transmission between 400 and 650 nm) in the excitation beam in order to reduce the excitation light intensity.

Time-resolved fluorescence measurements were performed using an Applied Photophysics SX.17MV stopped-flow fluorometer (deadtime ≈1.4 ms), at 22 °C. The path length for excitation was 2 mm; emission was measured at an angle of 90° with a path length of 10 mm. Complex assembly was initiated by mixing equal volumes of protein and pigment solutions, as described above. For measurements of protein fluorescence, excitation was at 295 nm (0.5 nm bandwidth), and emission between 305 and 390 nm was selected with appropriate optical filters. For measurements of Chl fluorescence, excitation was at 410 nm for Chl *a* and at 460 nm for Chl *b* (1 nm bandwidth), and emission selected with band-pass filters (10 nm, full width, half-maximum) centered at wavelengths ranging from 670 nm (for Chl *b*) to 700 nm (for Chl *a*). Chl *a* and Chl *b* excitation wavelengths

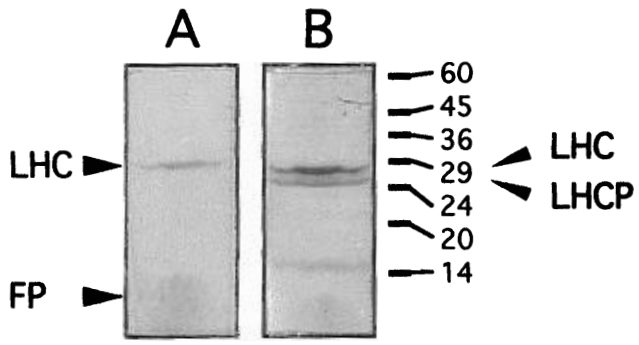

FIGURE 1: Partially denaturing gel electrophoresis of LHCP−pigment reconstitution product. 20 μL of the reconstitution assay (Materials and Methods) was applied per lane. Either the unstained gel was photographed, showing Chl-containing bands (A), or protein bands were visualized by staining with Coomassie (B). The approximate positions of protein molecular weight markers are shown in the right margin. The arrows labeled LHC, LHCP, and FP give the approximate positions of LHCP−pigment complex, noncomplexed LHCP, and noncomplexed "free" pigments, respectively.

were slightly shifted from their absorption maxima (of about 435 and 455 nm, respectively, in LHCII) in order to minimize coexcitation. Chl *a* emission was detected on the long-wavelength side of the emission maximum (about 680 nm) in order to minimize the contribution of Chl *b* and reabsorption of emitted light. Optical densities of the reaction mixture (1 mm path length) were: 0.06 (295 nm), 0.09 (320 nm), 0.16 (410 nm), 0.19 (460 nm), 0.05 (670 nm), and 0.004 (700 nm). Thus, the absolute level of the fluorescence signal at 320 nm (protein fluorescence) and 670 nm (Chl *b* fluorescence) was lowered due to reabsorption of emitted light, while at 700 nm reabsorption of emitted light was negligible.

Data Analysis. Time constants are the reciprocals of the experimentally determined rate constants which were calculated from fluorescence data by iterative deconvolution based on the Marquardt fitting algorithm, assuming multiexponential kinetics. Time constants were determined from measurements on different time scales (4000 data points per scale): 50 ms or 200 ms full scale for the fast components (data were the average of eight transients) and 200 s, 500 s, 1000 s, or 2000 s full scale for τ_1 and τ_2 (data were average of one, two, or four transients, with 10 ms electronic filtering). Where necessary, data corresponding to rises or decays in fluorescence were analyzed separately.

RESULTS

Reconstitution of LHCII and Energy Transfer from Chl b to Chl a. Figure 1 shows the result of a reconstitution experiment under the conditions used in the kinetic studies. The polyacrylamide gel electrophoresis system used separates reconstituted LHCP−pigment complexes from nonpigmented protein. From the comparison of the LHCII band with the band of nonpigmented LHCP, it is obvious that the yield of reconstituted protein (estimated to be about 50%) is far from the 90% or more reported in earlier studies (Paulsen et al., 1990). Far-UV circular dichroism spectra showed that under the reconstitution conditions described here the α-helical content of the reconstituted LHCII sample was about 45% (data not shown), also indicating that about half of the LHCP is properly folded. This lower yield of reconstituted protein is due to two differences in the experimental conditions

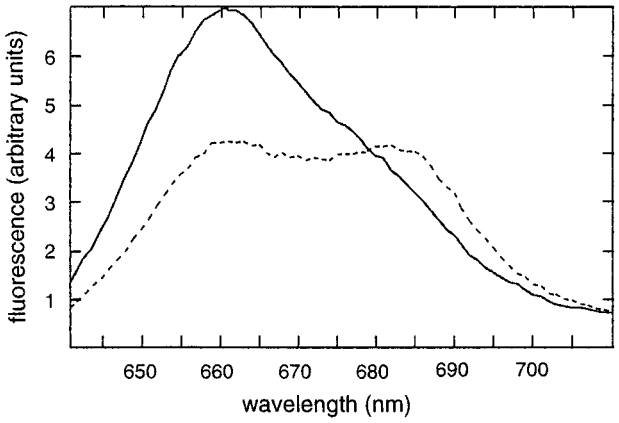

FIGURE 2: Steady-state fluorescence emission spectra. Spectra were taken as described under Materials and Methods for a standard reconstitution mixture (dashed line) and for a reconstitution mixture lacking LHCP (solid line).

used: (1) the protein concentration is lower than in earlier experiments in order to keep the optical density of solutions low enough for fluorescence measurements; this lower concentration results in a reduced yield of reconstituted complexes. (2) In order to maximize the relative changes in Chl fluorescence, the Chl present in the reaction mixture is only 1.5−2-fold the stoichiometric amount necessary to complex the protein present; in previous experiments, a more than 3-fold excess amount of pigments was used in order to increase the yield of reconstituted protein.

Fluorescence measurements on the green LHCII band from the gel shown in Figure 1, with excitation of Chl *b* at 460 nm, resulted in a Chl *a* emission spectrum with virtually no contribution from Chl *b* (not shown), indicating 100% energy transfer from Chl *b* to Chl *a* in the pigment fraction that had been successfully reconstituted.

Figure 2 shows that under the experimental conditions used in the stopped-flow experiments, the energy transfer from Chl *b* to Chl *a* upon excitation of Chl *b* is easily detectable in the steady state. Without LHCII formation, fluorescence emission is mostly from Chl *b* at about 660 nm. Upon reconstitution of Chl−protein complexes, Chl *b* fluorescence decreases as that of Chl *a* at about 680 nm increases, indicating that energy transfer from Chl *b* to Chl *a* is established as LHCII assembles. The apparent efficiency of energy transfer upon reconstitution, estimated roughly from the quenching of Chl *b* fluorescence, is 30% (Figure 2). This is consistent with an estimated 30% of the Chl *b* present being assembled into LHCII and, thus, transferring its energy with 100% efficiency, while the remaining Chl *b* is not involved in energy transfer. The increase in Chl *a* emission upon complex formation is partially obscured by the concomitant decrease in Chl *b* emission, due to the overlapping emission spectra of these two Chl species. Nevertheless, an increase in Chl *a* fluorescence can be detected.

Kinetics of Complex Formation. Upon mixing LHCP and pigment solutions in the stopped-flow cuvette, we monitored the assembly of pigment−protein complexes using three different measuring parameters: (1) Chl *b* fluorescence; (2) sensitized Chl *a* fluorescence upon excitation of Chl *b*, due to Förster energy transfer from Chl *b* to Chl *a*; and (3) intrinsic protein tryptophan fluorescence. Figure 3 shows the signal changes of all three parameters. At least three

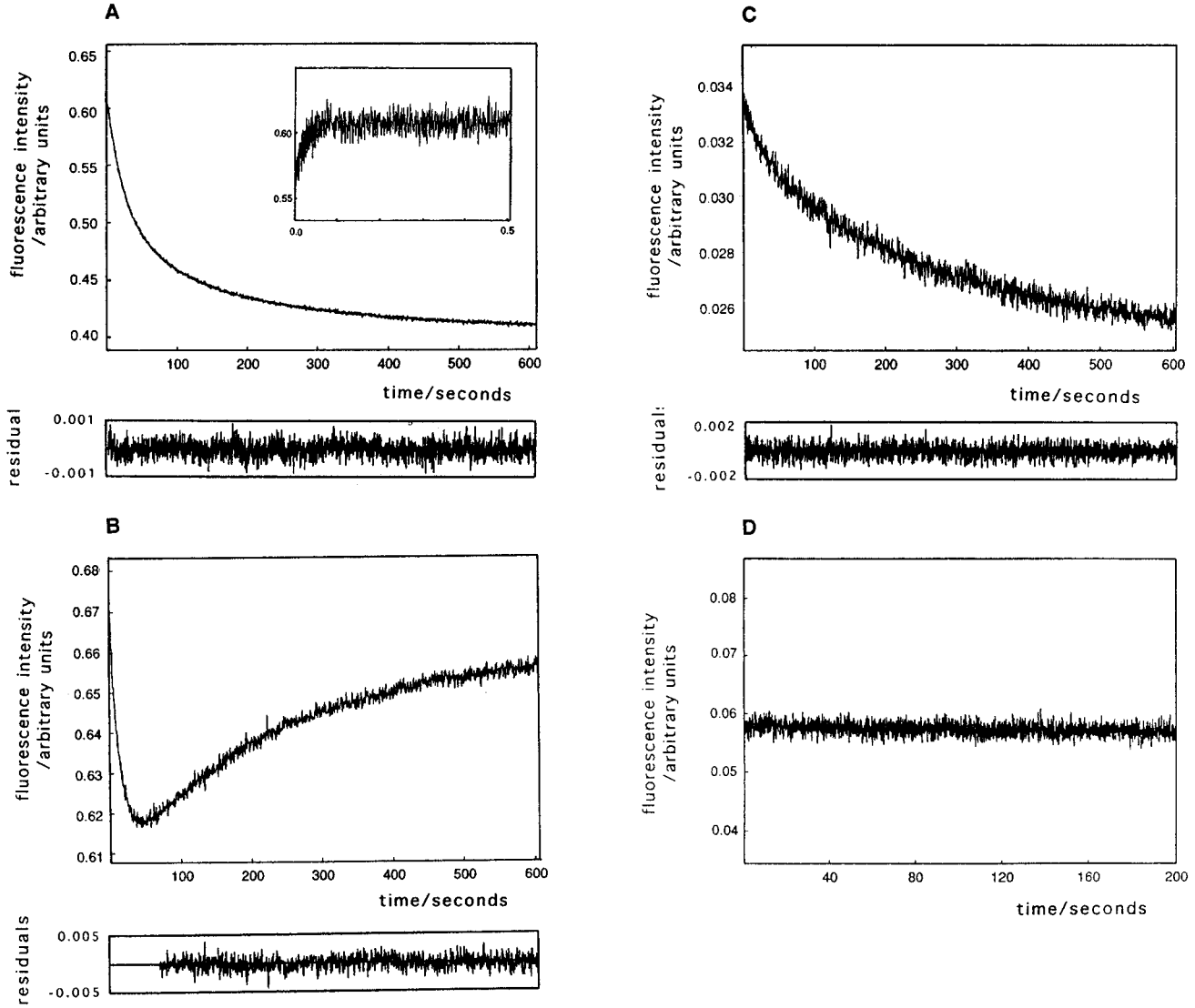

FIGURE 3: Time-resolved fluorescence data. Changes in (A) Chl *b* fluorescence, (B) sensitized Chl *a* fluorescence (upon excitation of Chl *b*), and (C) protein fluorescence, during LHCII assembly. Inset to (A) shows data taken on a shorter time scale to resolve the fast component observed during assembly of LHCII, shown here for Chl *b* fluorescence. In all cases, LHCP in SDS was mixed with OG/PG solutions containing the full complement of pigments. Residuals are shown below curves: (A) and (B), for a biexponential fit to the data; and (C), for a monoexponential fit to data over the range shown. (D) Example of a control experiment: monitoring protein fluorescence when LHCP in SDS was mixed with OG/PG containing only xanthophylls. Fluorescence intensity is in arbitrary units that are not directly comparable between figures.

Table 1: Kinetic Components Resolved by Monitoring the Assembly of LHCP−Pigment Complexes Using Various Fluorescence Signals

	fast component (s)[a]	τ_1 (s)[b]	τ_2 (s)[b]
Chl *b* fluorescence	0.016	$67_{(0.1)}$	$350_{(0.15)}$
Chl *a* fluorescence	0.025	$68_{(0.09)}$	$232_{(0.07)}$
sensitized Chl *a* fluorescence[c]	0.025	$31_{(0.05)}$	$354_{(-0.04)}$
protein fluorescence	0.019	$54_{(0.003)}$	$424_{(0.005)}$
proposed origin of component	micelle mixing	formation of nonfunctional pigment−protein complex	energy transfer from Chl *b* to Chl *a* and formation of functional LHCII

[a] A decay in fluorescence for Chl *b* and Chl *a* fluorescence, and a rise for protein fluorescence. Time constant and amplitude varied by ∼±30% and ∼±55%, respectively, between LHCP preparations. Amplitude of fast component was ∼55% of that of τ_1, for Chl *b* fluorescence. [b] Time constants τ_1 and τ_2 determined from biexponential fits to data with 1000 or 2000 s full scale. Amplitudes are given in parentheses as subscripts to time constants, and in arbitrary units which are not directly comparable between measurements of Chl *b*, Chl *a*, and protein fluorescence. Time constants varied by ∼±30% for τ_1 and ∼±20% for τ_2 between LHCP preparations (error to one standard deviation within an LHCP preparation was ±14% for Chl *b*, τ_2). Greatest variation in amplitude was ∼±55% for sensitized Chl *a* fluorescence τ_2. Other amplitudes varied by ∼±15%. Within experimental error, no difference was found whether SDS or LDS was used as protein denaturant. [c] Excitation of Chl *b* at 460 nm and measurement of Chl *a* emission at 700 nm.

time constants are apparent in all measurements (Table 1): a fast component seen as a rise in Chl *a* and Chl *b* fluorescence and a decrease in protein fluorescence (10−30 ms); a component (τ_1) of about 30−60 s, seen as a decrease in Chl *b*, Chl *a*, and protein fluorescence; and a slow component (τ_2), about 300−450 s, seen as a decrease in Chl *b* and protein fluorescence and a rise in sensitized Chl *a* fluorescence.

If Chl *a* fluorescence is monitored upon excitation of Chl *a* at 410 nm, the signal decreases during the slow step (τ_2), indicating a decrease in fluorescence yield, probably due to some change in the molecular environment of Chl *a* during this step (Table 1). However, if Chl *a* fluorescence is measured upon excitation of Chl *b* at 460 nm, the signal increases during the slow step, indicating that the lowering of the Chl *a* fluorescence yield is overcompensated by an increase of sensitized Chl *a* fluorescence, due to energy transfer from Chl *b* to Chl *a* (Figure 3B).

The major decrease in Chl *b* emission occurs during the slow step (Table 1), and this is the only step for which a concomitant increase in sensitized Chl *a* emission, following excitation of Chl *b*, is observed. Thus, most if not all (see Discussion) of the energy transfer between Chl *b* and Chl *a* during the formation of LHCII *in vitro* is established with a time constant of several minutes. Owing to the overlapping emission spectra of Chl *a* and Chl *b*, in addition to the above-mentioned decrease in Chl *a* emission which occurs during the slow step, the relative amplitudes of the decrease in Chl *b* fluorescence during τ_2 and the increase in sensitized Chl *a* fluorescence depended on the wavelength used to detect Chl *a* emission. Data are shown for measurements centered at 700 nm, where Chl *a* fluorescence is still easily detected but the contribution of Chl *b* is negligible. Measurements at 680, 690, and 710 nm were also consistent with the slow step arising from Chl *b* to Chl *a* energy transfer (data not shown).

Correlation of Kinetic Components with the Formation of Stable LHCII. The three time constants resolved by following changes in Chl *b*, Chl *a*, and protein fluorescence are very similar (Table 1), suggesting that all three fluorescence probes detect the same steps in LHCII assembly. In control experiments, conditions were changed such that no stable LHCII complexes formed, and either Chl *a* or Chl *b* fluorescence was measured when LHCP was omitted from the reaction mixtures (not shown), or protein fluorescence was measured when the reaction mixture contained less than all pigments (Figure 3D). In all these control experiments, only the fast step in the 10 ms range was observed. A change in fluorescence intensity for each individual fluorophore was observed on going from OG/PG micelles to OG/PG micelles containing 0.1% SDS (or vice versa for protein). In the case of Chl *a* and Chl *b*, the fluorescence intensity was lower by about 5% in the reconstitution mixture as compared to the reconstitution mixture without SDS (not shown). The protein fluorescence did not exhibit any slower changes in the absence of pigments, when protein was mixed with only xanthophyll or only xanthophyll and Chl *b* in the pigment solution. No increase in energy transfer from Chl *b* to Chl *a*, as monitored by a decrease in Chl *b* and an increase in sensitized Chl *a* emission upon excitation of Chl *b*, was detected when no xanthophylls were present.

Effect of the Transit Peptide. Time-resolved measurements of complex formation detected a difference between LHCP and its precursor form, pLHCP. The two slower reaction steps (τ_1 and τ_2) observed with the precursor are slower than the ones with the mature protein, both time constants being increased by about 50% (not shown).

DISCUSSION

The kinetic experiments on LHCII assembly resolve three steps: a fast event in the 10 ms range, and two slower steps of 30−60 s and several minutes, respectively. The fast step is observed both in protein and in Chl fluorescence even when the reaction mixture contains only protein or only pigments, respectively, and thus cannot be attributed to the formation of pigment−protein interactions. This step most likely reflects changes of the environment of the fluorophores due to the formation of mixed OG/PG/SDS micelles (Booth et al., 1995). Although pigment−protein complexes are not formed, it is possible that some folding of LHCP does occur during this micelle mixing step, and this will be investigated in future experiments. A similar fast step with a time constant of 4 ms has been observed in folding studies of the membrane protein bacterioopsin, whether bacteriorhodopsin's retinal chromophore was present or not, and has been assigned to the mixing of SDS and DMPC/CHAPS micelles (Booth et al., 1995).

Both two later kinetics (τ_1 and τ_2) are involved in formation of pigment−protein complexes, and experiments thus far indicate they are only observed when all the pigments required for assembly of stable LHCII are present. Formation of functional LHCII is indicated by the appearance of efficient energy transfer from Chl *b* to Chl *a*. Such energy transfer does not seem to be established during the step we observe in the range of 30−60 s (τ_1), as no increase of Chl *a* fluorescence, on excitation of Chl *b*, is detected during this step. We cannot exclude, however, that energy transfer does occur, as the expected increase in Chl *a* fluorescence may be obscured by the quenching of Chl *a* fluorescence due to a change in the molecular environment of Chl *a*.

It is possible that this step (τ_1) corresponds to formation of an assembly intermediate with a loosely folded protein structure and in which (at least some of) the pigments are bound but such that the majority of Chl *a* and Chl *b* molecules are not coupled in energy transfer. However, in view of the fact that under our experimental conditions only 50% of the LHCP present is assembled into functional LHCII, this step may equally correspond to formation of protein−pigment aggregates which account for the remaining 50% of uncomplexed protein (Figure 1), and in which Chl *a* and Chl *b* fluorescence (as well as that of the protein) is quenched but no energy transfer occurs between Chls. These two possibilities are currently under investigation.

In the folding of bacterioopsin, reaction steps with time constants of up to 15 s have been observed independently of the presence or absence of the retinal chromophore. This suggests that substantial protein folding occurs prior to pigment binding. Subsequent retinal binding then occurs in the minute range (Booth et al., 1995). By contrast, in our kinetic experiments with LHCP, folding steps in the range of seconds have only been detected in the presence of pigments. Thus, either the folding of LHCP takes place entirely in the 10−30 ms range (i.e., during the fast component, Table 1) in these experiments, or (at least part of) LHCP folding is initiated by pigment binding, confirming earlier observations of pigments inducing the renaturation of LHCP *in vitro* (Paulsen et al., 1993). Observations similar to the latter possibility have been made on a water-soluble protein, cytochrome *c*, whose folding is dependent on stabilizing interactions with the heme group (Hamada et al., 1993; Elöve et al., 1994).

Energy transfer from Chl *b* to Chl *a* is established during the slowest step (τ_2) we observe for LHCII assembly, as indicated by a decrease in Chl *b* emission and a concomitant

increase in Chl *a* emission upon excitation of Chl *b*. It follows that the juxtaposition of Chls is a slow process (time constant of several minutes *in vitro*) and is the last stage we detect during assembly of monomeric LHCII.

Formation of LHCII in the time range of several minutes may appear slow, compared to the folding of water-soluble proteins which usually takes place in less than a second (Matthews, 1993). However, time constants in the minute range have been observed during assembly not only of another membrane protein, bacteriorhodopsin, but also of some water-soluble chromophore-binding proteins (Chiba et al., 1994; Booth et al., 1995). The proper positioning of the 12 or so Chls may be a rate-limiting factor in LHCII assembly as ligand exchange reactions have been seen to slow down the binding of another porphyrin cofactor, heme-CN, to its apoprotein, apomyoglobin (Yee et al., 1991; Chiba et al., 1994). On the other hand, in heme-CO binding to apohemoglobin, the formation of the iron−histidine bond does not appear to be rate-limiting (Rose & Olson, 1983). The slowest step in cytochrome *c* folding is interpreted as a proline cis-trans isomerization (Elöve et al., 1994); in general, proline isomerization is rate-limiting in the last folding step(s) of a number of proteins (Schmid, 1993). LHCP has prolines in all hydrophilic domains, most of them bordering the α-helical trans-membrane domains (Kühlbrandt et al., 1994). Some of these prolines may undergo slow isomerization during LHCII assembly. However, if protein folding is rate-limiting in LHCII formation, our results suggest that such folding is triggered by pigment binding, as no slow protein folding has been observed by monitoring intrinsic protein fluorescence in the absence of pigments.

The slower folding kinetics observed with the precursor pLHCP compared to the mature form LHCP may reflect the increased length of the polypeptide of the precursor; alternatively, they may play a role in biogenesis of LHCII. The precursor pLHCP forms stable pigment−protein complexes *in vitro* (Paulsen et al., 1990). Upon the import of barley pLHCP into plastids isolated from greening barley, both the mature form and the precursor form of the protein have been found in the LHCII fraction, suggesting that pLHCP can be assembled into LHCII (Chitnis et al., 1988); however, *in vivo* only the mature protein is found in the thylakoid. The processing enzyme which converts pLHCP into LHCP is a soluble protein located in the stroma (Oblong & Lamppa, 1992); it is not known whether this protease cleaves pLHCP during its translocation across the envelope membrane, during its passage through the stroma compartment, or after its insertion into the thylakoid membrane. Slower folding and/or pigment binding of pLHCP as compared to LHCP may favor processing of the protein before it is assembled into LHCII.

We have determined the assembly kinetics of the major photosynthetic light-harvesting protein. Not only does this study show that the method developed for studying folding and assembly kinetics of bacteriorhodopsin can be applied to a second membrane protein, opening the way for detailed studies of membrane protein assembly, but it also has implications for the *in vivo* biogenesis of LHCII. The mechanism of LHCP insertion into the thylakoid membrane is unknown; in particular, the temporal sequence of events such as protein translocation into the membrane, pigment binding, and protein folding is still unclear. It is likely that during its transit through the stroma compartment, LHCP is kept in an unfolded conformation, possibly stabilized by a protein factor (Li et al., 1995). The data presented in this paper are consistent with the notion that protein folding and pigment binding occur in a concerted fashion. Whether the insertion of LHCP into the membrane is also mechanistically coupled with pigment binding as has been hypothesized (Paulsen et al., 1993; Hoober et al., 1994; Dahlin & Timko, 1994; Paulsen, 1995) remains to be seen. Time-resolved measurements of LHCII assembly *in vitro* will help to solve this question by giving more detailed information about the mechanism of protein folding and pigment binding and the structural parameters that affect these processes.

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