The Light-Harvesting Chlorophyll *a*/*b* Complex Can Be Reconstituted *in Vitro* from Its Completely Unfolded Apoprotein†

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ABSTRACT: The major light-harvesting chlorophyll *a*/*b* protein (LHCIIb) of higher plants is one of the few membrane proteins that can be refolded *in vitro*. During folding, the apoprotein is assembled with pigments to form a structurally authentic and functional pigment−protein complex. All reconstitution procedures used so far include solubilization of the apoprotein in sodium dodecyl sulfate (SDS) where the protein adopts approximately half of its α-helical folding present in the native structure. This paper shows that this preformed α-helix is not a prerequisite for LHCIIb folding *in vitro*. The apoprotein can also be reconstituted starting from a solution in guanidinium hydrochloride (Gnd) where the protein contains no detectable helical structure. Reconstitution yields are somewhat lower in the Gnd than in the SDS procedure, but the reconstitution products exhibit very similar biochemical and spectroscopic properties. The kinetics of LHCIIb assembly, as assessed by time-resolved fluorescence measurements, are virtually the same in both reconstitution procedures. This demonstrates that the initiation of α-helix formation is not a rate-limiting step in LHCIIb apoprotein folding.

Our knowledge about the mechanisms of membrane protein folding is rather limited compared to the wealth of information available on the folding pathways of water-soluble proteins (*1*). One reason for this is the relatively small number of membrane proteins that have successfully been refolded *in vitro*. Among these are *β*-barrel proteins (*2*, *3*) and proteins containing α-helical transmembrane domains (*4*). The *β*-strand proteins, such as the bacterial outer membrane proteins OmpA and OmpF, can be fully unfolded in urea or guanidinium hydrochloride (Gnd)[1] and then renatured with the help of detergent micelles, mixed lipid/detergent micelles, or lipid vesicles (*5*−*7*). Some α-helix proteins, including lactose permease (*8*) and diacylglycerol kinase (*9*), have also been refolded by following a similar procedure, albeit at a lower yield than *β*-strand proteins. However, most α-helical proteins cannot be refolded *in vitro* from a urea or Gnd solution. The preferred starting condition for folding these proteins is a solution in sodium dodecyl sulfate (SDS). Bacteriorhodopsin (bR) in SDS solution has been the first membrane protein that has been renatured in detergent micelles or mixed lipid/detergent micelles (*10*, *11*); it can also be refolded in lipid vesicles (*12*, *13*). The major light-harvesting chlorophyll (Chl) *a*/*b* protein (LHCIIb) (*14*, *15*) as well as all other members of the Chl *a*/*b* protein family (*16*−*20*) can be refolded and reconstituted with their pigments in a solution of SDS (or the corresponding lithium salt, LDS) or SDS mixed with other detergents. Only two helical membrane proteins have successfully been folded without SDS by dissolution of the proteins in organic solvents mixed with lipids and then evaporation of the organic solvent; these are bR (*1*) and EmrE, a 110-amino acid efflux transporter in *Escherichia coli* (*21*).

It is not surprising that choosing an SDS solution as a starting point for helical protein refolding has been particularly successful. As has been known for many years, SDS stabilizes α-helix structures (*22*, *23*). In fact, membrane proteins such as bR (*24*) and an LHCIIb apoprotein (*Lhcb1*) (*25*) show a substantial amount of helix structure (50 and 60%, respectively, of the partition present in the native structure) when they are "denatured" in SDS. One might speculate that this preformed α-helix in SDS solution is a prerequisite for successfully refolding these proteins *in vitro*.

In this paper, we show that for the renaturation of LHCIIb *in vitro*, a preformed α-helix in the dissolved apoprotein is not necessary. *Lhcb1* can be folded into functional LHCIIb starting from a solution in Gnd where the protein shows no detectable order of secondary structure.

MATERIALS AND METHODS

Preparation of Recombinant LHCIIb. The protein used in all experiments was a bacterially expressed *Lhcb1* containing a C-terminal hexahistidyl tag [clone C3.2h (*26*), a derivative of the Chl *a*/*b* protein gene AB80 (*Lhcb1**2) from tobacco (*27*)]. The protein and a pigment extract from pea thylakoids were isolated as described in ref *15*.

For preparative reconstitutions of LHCIIb, thylakoid pigments containing 60 *μ*g (67 pmol) of Chl were dissolved

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[1] Abbreviations: bR, bacteriorhodopsin; Car, carotenoid; CD, circular dichroism; Chl, chlorophyll; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Gnd, guanidinium hydrochloride; LDS, lithium dodecyl sulfate; LHCIIb, major light-harvesting chlorophyll *a*/*b* protein complex of photosystem II; *Lhcb1*, apoprotein of LHCIIb; LM, dodecyl *β*-D-maltoside; OG, octyl *β*-D-glucopyranoside; PG, phosphatidylglycerol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TX, Triton X-100.

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in 10 μL of ethanol and mixed by vortex with 100 μL of reconstitution buffer 1 containing 50 mM sodium borate (pH 9.0), 12.5% (w/v) sucrose, 10 mM β-mercapthoethanol, 2.5% (w/v) octyl β-D-glucopyranoside (OG) (Bachem, Heidelberg, Germany), and 0.55 mM phosphatidylglycerol (PG) (Avanti Polar Lipids, Alabaster, AL). Then 10 μL of *Lhcb1* (1.57 pmol) dissolved in 1% (w/v) SDS (Roche, Mannheim, Germany) or 6 M Gnd (Biomol, Hamburg, Germany) was added to the mixture under vortexing.

The reconstitution mixture was incubated at 25 °C for 10 min and centrifuged at 23000*g* for 15 min at 4 °C. The supernatant was collected, applied to a Ni^{2+}-chelating Sepharose fast flow column (0.8 cm × 4 cm) (Bio-Rad, Hercules, CA) equilibrated with OG buffer [1% (w/v) OG, 0.1 M Tris (pH 9) (Gerbu Biotechnik, Gailberg, Germany), and 12.5% (w/v) sucrose], and then incubated in darkness at 4 °C for 30 min. The column was washed with 2 mL of OG buffer and 2 mL of Triton X-100 (TX) buffer [0.05% (w/v) TX (Roche), 0.14 mM PG, and 0.1 M Tris (pH 7.5)]. The complexes were eluted with elution buffer [0.05% (w/v) TX, 0.14 mM PG, 10 mM Tris (pH 7.5), and 0.3 M imidazole (Sigma, St. Louis, MO)].

The material eluted from Ni^{2+}-chelating Sepharose fast flow columns was further purified either by ultracentrifugation through a sucrose density gradient or by partially denaturing polyacrylamide gel electrophoresis (PAGE). The sucrose density gradient (from ~0.1 to 1 M sucrose) was generated by one freeze−thaw cycle (freeze and thaw at −20 and 4 °C, respectively) applied to 4 mL solution containing 0.6 M sucrose, 0.1% (w/v) dodecyl β-D-maltoside (LM) (Biomol), and 5 mM sodium phosphate (pH 7.8) in an 11 mm × 60 mm centrifuge tube (Beckman, Palo Alto, CA). The eluate from the Ni^{2+}-chelating Sepharose column was loaded on the gradient, and the mixture was centrifuged at 370000*g* and 4 °C for 16 h. Native LHCIIb, isolated according to the method described in ref *28*, was used as a control. The band containing trimeric LHCIIb was extracted from the sucrose density gradient for further analysis.

Two partially denaturing polyacrylamide gel electrophoresis systems were used to analyze reconstituted pigment−protein complexes: (1) a 15% polyacrylamide gel run in LDS buffer [24 mM Tris, 192 mM glycine (Biomol), 0.1% (w/v) LDS (Serva, Heidelberg, Germany), and 0.5 mM ethylenediaminetetraacetate (EDTA) (pH 8.0) (Merck, Darmstadt, Germany)] (*29*) and (2) a 10% polyacrylamide gel run in Deriphat buffer containing 12 mM Tris, 0.15% (w/v) Deriphat (Henkel, Ambler, PA), and 48 mM glycine (*30*).

Pigment Analysis in Reconstituted LHCIIb. Sucrose density gradient bands containing trimeric LHCIIb were analyzed for their pigment contents by using the 2-butanol extraction method described in ref *31*. Then 10 μL of the 2-butanol extract was mixed with 20 μL of 80% (v/v) acetone, applied to an RP-18 HPLC column (Chromolith SpeedROD, Merck), and eluted with a gradient from 70 to 100% (v/v) acetone, with a flow rate of 1.5 mL/min. The pigments were quantitated as described in ref *15*.

Absorption and Fluorescence Spectra at 77 K. Fractions (150 μL) of sucrose density gradients containing trimeric LHCIIb were diluted with 450 μL of 80% (v/v) glycerol in 50 mM HEPES/NaOH (pH 7.5). The samples were frozen in a 1 cm × 0.4 cm plastic cuvette (ratiolab, Dreieich, Germany) by immersing it in liquid nitrogen. Absorption spectra were measured between 600 and 750 nm on a Shimadzu (Kyoto, Japan) UV-2101PC spectrophotometer. The measurement was repeated nine times, and the average of the spectra was calculated. Fluorescence emission spectra between 650 and 720 nm were recorded on a FluoroMax 3 instrument (Spex/ISA, Grasbrunn, Germany) with the excitation wavelength set to 470 nm, slit widths of 3 nm for both excitation and emission, and an integration time of 0.5 s.

Circular Dichroism (CD). CD spectra were recorded on a Jasco (Gross-Umstadt, Germany) J-810-S instrument. Far-UV CD spectra of *Lhcb1* dissolved in SDS or in Gnd were measured at 24 °C. *Lhcb1* was dissolved either in SDS or in Gnd at a concentration of 4 mg/mL. The spectrum for SDS-dissolved *Lhcb1* was measured from 260 to 200 nm, while the spectrum for the Gnd-dissolved apoprotein could only be measured from 260 to 207 nm because of the intrinsic UV absorption of Gnd. The measurement was repeated five times and averaged. CD spectra of trimeric LHCIIb from sucrose density gradients were recorded from 750 to 300 nm at 4 °C.

Time-Resolved Fluorescence Measurements of LHCIIb Formation. For time-resolved fluorescence measurements, reconstitution was performed under conditions slightly different from those for preparative reconstitutions. The SDS-denatured protein solution contained 0.72 μg/μL (28 μM) *Lhcb1* and 0.2% (w/v) SDS in reconstitution buffer 2 [100 mM lithium borate (pH 9.0), 12.5% (w/v) sucrose, and 10 mM dithiothreitol (DTT)]. The Gnd-denatured protein solution contained 4 μg/μL (157 μM) *Lhcb1*, 6 M Gnd, and 10 mM DTT. The pigment solution for reconstitutions of the SDS-solubilized protein contained 0.39 μg/μL (437 μM) Chl, 0.17 μg/μL (336 μM) carotenoids (Car), and 4% (w/v) OG in reconstitution buffer 2, whereas the one for reconstitutions of the Gnd-solubilized protein contained 0.22 μg/μL (244 μM) Chl, 0.09 μg/μL (171 μM) Cars, and 2% (w/v) OG in reconstitution buffer 2. The reconstitution was initiated by manually mixing the pigment solution and SDS- or Gnd-denatured protein solution at a ratio of 1:1 or 10:1, respectively, in a 1.5 mL reaction vessel by using a vortex mixer at 24 °C. The solution was then transferred to a fluorescence cuvette; the dead time between mixing and the start of the measurements was 10 ± 1 s. Chl and Car concentrations were quantitated according to the methods of Porra (*32*) and Davies (*33*), respectively. The Chl and Car concentrations that were employed corresponded to an excess over protein of 1.3 (assuming 12 Chls/LHCIIb) and 4 (assuming 3 xanthophylls/LHCIIb), respectively.

Fluorescence data were collected on a FluoroMax-2 spectrometer at 24 °C 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, an integration time of 0.1 s, and slit widths set to 0.5 and 6 nm for excitation and emission, respectively. Fluorescence emission upon excitation at 470 nm was assessed at 660 nm (Chl *b* emission) and 680 nm (sensitized Chl *a* emission). The kinetic traces were fitted to a sum of two exponentials by using the software Table Curve (version 2D 4.0, SPSS Inc., Chicago, IL). Time constants were the reciprocals of the calculated rate constants. The presented data reflect the average over at least eight independent measurements. Where appropriate, kinetic data were corrected by subtracting data from control experiments lacking the protein component.

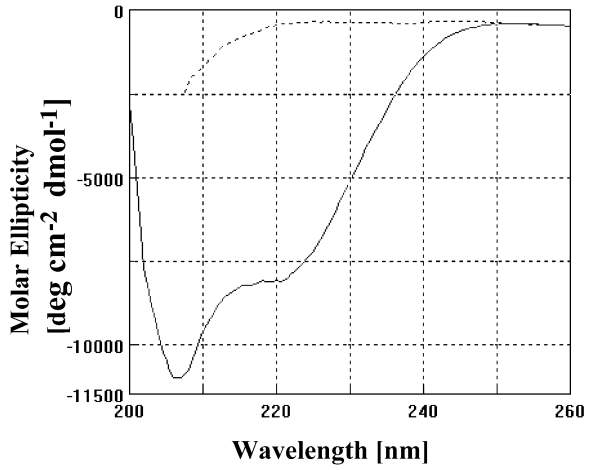

FIGURE 1: Far-UV CD spectra of *Lhcb1*. *Lhcb1* was dissolved either in 1% SDS (—) or in 6 M Gnd (- - -) at a concentration of 4 mg/mL. Spectra, recorded at 24 °C, represent an average of five measurements each.

RESULTS

LHCIIb Can Be Reconstituted from a Fully Unfolded Apoprotein. Recombinant LHCIIb can be reconstituted *in vitro* starting out from the apoprotein *Lhcb1* solubilized either in 1% SDS or in 6 M Gnd, although these two denaturants lead to significantly different secondary structures of the solubilized apoprotein. Figure 1 shows CD spectra in the far-UV region of both *Lhcb1* solutions. The same CD spectrum of SDS-dissolved *Lhcb1* was obtained when the protein solution was made either of washed protein inclusion bodies or of the acetone-precipitated material. Protein CD spectra can be evaluated to estimate the contributions of α-helix and *β*-turn to the protein structure if the spectral range at least down to 200 nm is measured. We have used this approach previously to detect 27% α-helix in SDS-solubilized *Lhcb1* (*25*). The CD spectrum of the protein dissolved in Gnd could only be measured down to 207 nm because of the intrinsic UV absorption of this reagent. However, Figure 1 clearly shows that the negative CD signal at 220 nm characteristic of α-helical proteins is not present when the protein is dissolved in Gnd. This demonstrates that virtually no α-helix is present in Gnd-solubilized *Lhcb1*.

Reconstitution *in vitro* of Gnd-solubilized *Lhcb1* yielded a mixture of monomeric and trimeric LHCIIb, very similar to reconstitutions conducted under the same conditions but starting out from the SDS-dissolved protein. The monomeric and trimeric reconstitution products can be separated by partially denaturing gel electrophoresis (Figure 2) or ultracentrifugation through a sucrose density gradient (not shown). Panel A in Figure 2 shows a nonstained Deriphat polyacrylamide gel. Both the SDS and Gnd reconstitution procedures (lanes 2 and 3, respectively) resulted in pigment−protein complexes that comigrated with monomeric and trimeric native LHCIIb (lane 1). Coomassie staining of the same gel (panel B) shows the protein component in the green bands of panel A. Deriphat electrophoresis is a mild separation technique, preserving trimeric LHCIIb complexes. Partially denaturing LDS electrophoresis (panel C in Figure 2), on the other hand, dissociates most of the trimeric LHCIIb but, unlike Deriphat electrophoresis, is able to separate LHCIIb monomers from the nonpigmented apoprotein, allowing

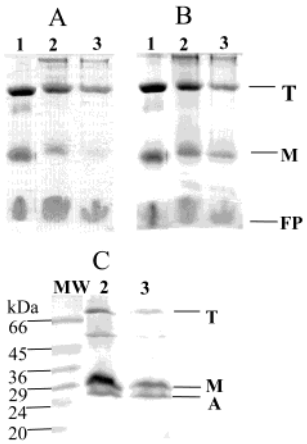

FIGURE 2: PAGE analysis of reconstituted LHCIIb. (A) Deriphat PAGE unstained, (B) Deriphat PAGE stained with Coomassie Blue, and (C) partially denaturing LDS−PAGE stained with Coomassie Blue: lane 1, native LHCIIb; lane 2, LHCIIb reconstituted from SDS-dissolved *Lhcb1*; and lane 3, LHCIIb reconstituted from Gnd-dissolved Lhcb1. Lane MW contained the molecular mass standards, and the approximate relative molecular masses in kilodaltons (kDa) are given in the left margin. T is trimer, M monomer, A apoprotein, and FP free pigments.

Table 1: Pigment Stoichiometries of Native and Recombinant LHCIIb Trimers[a]

sample	Chl *a*:*b*	Chl:(2 lutein)
native LHCIIb	1.27 ± 0.04	14.42 ± 0.21
SDS	0.92 ± 0.04	12.26 ± 0.31
Gnd	0.96 ± 0.08	11.50 ± 0.70

[a] The recombinant complexes were obtained by reconstitution either from SDS-dissolved or from Gnd-dissolved *Lhcb1* (SDS or Gnd, respectively). The data represent the averages ± standard deviation of six independent measurements.

quantitation of the reconstitution product. Clearly, the reconstitution yield (protein amounts in the monomer and trimer bands compared to nonreconstituted apoprotein amounts) of the SDS reconstitution procedure (lane 2 in panel C) is higher, but the yield of the Gnd procedure (lane 3) also significantly exceeds 50%. The same results were obtained when reconstitution products were analyzed by sucrose density gradient ultracentrifugation. The pigment−protein complexes resulting from either reconstitution procedure cosedimented with native LHCIIb monomers and trimers (not shown).

Specific Chl binding to *Lhcb1* during reconstitution of the Gnd-solubilized protein was verified by further analysis of the reconstitution products. The pigment stoichiometry [Chl *a*:*b* ratio and Chl:(2 lutein) ratio] was very similar between LHCIIb reconstituted from either SDS- or Gnd-dissolved *Lhcb1*. Recombinant LHCIIb reconstituted in the presence of all thylakoid Cars invariantly contains two lutein molecules; thus, the Chl:(2 lutein) ratio gives the number of Chls bound per apoprotein. From the data in Table 1, we calculated that in native LHCIIb each apoprotein bound approximately eight Chl *a* and six Chl *b* molecules, while in reconstituted LHCIIb from both the SDS and Gnd procedure, the numbers were approximately six Chl *a* and six Chl *b* molecules.

The low-temperature absorption spectra (Figure 3) are also quite similar between the recombinant complexes reconstituted from either SDS- or Gnd-dissolved *Lhcb1*. When the

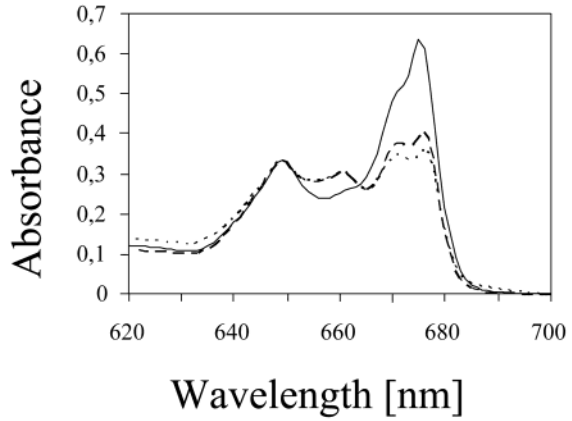

FIGURE 3: Absorption spectra of LHCIIb at 77 K. Absorption spectra of native LHCIIb trimers (—) and LHCIIb trimers reconstituted by the SDS method (- - -) and by the Gnd method (···). The spectra represent an average of nine measurements each. The spectra are nomalized to their Chl *b* absorption maxima at 649 nm.

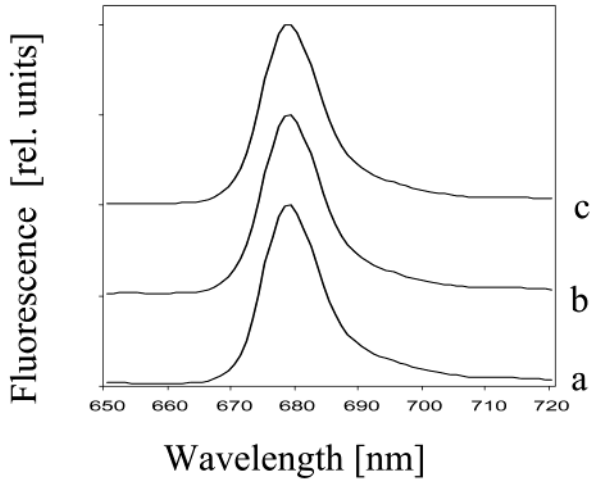

FIGURE 4: Fluorescence emission spectra of LHCIIb at 77 K. Fluorescence emission spectra of (a) native LHCIIb trimers, (b) LHCIIb trimers reconstituted from SDS-denatured *Lhcb1*, and (c) LHCIIb trimers reconstituted from Gnd-denatured *Lhcb1*.

spectra are normalized to the peak at 649 nm (Chl *b*), the signals at 675 and 670 nm (Chl *a*) are lower in the recombinant complexes than in native LHCIIb. Consistent with the pigment stoichiometries, this reflects a Chl *a*:*b* ratio that is lower in reconstituted than in native LHCIIb. On the other hand, both recombinant LHCIIb versions exhibit a peak at 660 nm which is only visible as a shoulder in the spectrum of native LHCIIb.

The fluorescence emission spectra of the two reconstituted LHCIIb versions and their native counterpart (Figure 4) are virtually identical. Although at the excitation wavelength of 470 nm predominantly Chl *b* is excited, all spectra show a Chl *a* emission band at 679 nm and no visible Chl *b* emission at shorter wavelengths, demonstrating complete energy transfer from Chl *b* to Chl *a*. Thus, all Chl *b* molecules were coupled to Chl *a* and, thus, presumably bound into specific Chl binding sites.

A structural similarity between the various LHCIIb preparations is also supported by their CD spectra in the visible range (Figure 5). The recombinant complexes from the two different reconstitution procedures exhibit virtually identical CD signals. The CD spectrum of native LHCIIb

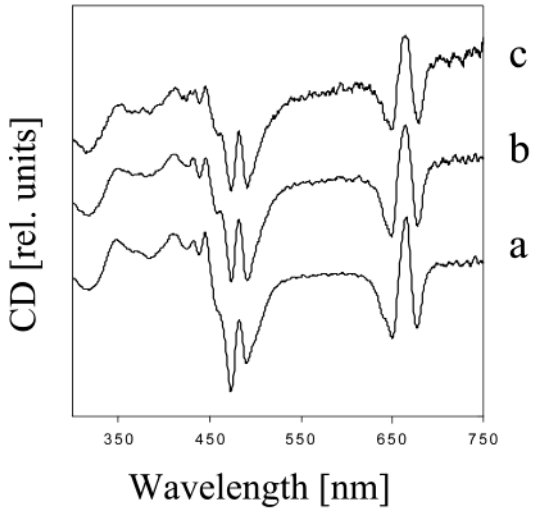

FIGURE 5: CD absorption spectra of LHCIIb. CD absorption spectra of (a) native LHCIIb trimers, (b) LHCIIb trimers reconstituted from SDS-denatured *Lhcb1*, and (c) LHCIIb trimers reconstituted from Gnd-denatured *Lhcb1*.

shows all the same signals except a more strongly negative band at 472 nm which may reflect the higher Chl *a* content of the native LHCIIb preparation. The similarity of the CD spectra demonstrates that in the three LHCIIb versions there is little or no difference in those pigment interactions that give rise to CD signals.

Initiation of α*-Helix Formation Is Not a Rate-Limiting Step in LHCIIb Assembly*. *Lhcb1* dissolved in SDS exhibits a substantial amount of α-helical conformation, whereas *Lhcb1* dissolved in Gnd does not (Figure 1). To determine whether these different initial structures influence the rate of LHCIIb formation, we measured the kinetics of LHCIIb reconstitution in time-resolved fluorescence measurements. Like in the preparative reconstitutions described before, complex formation was initiated by mixing the protein solution (either in SDS or in Gnd) with the pigment solution containing OG. To detect the formation of functional LHCIIb, the reconstitution mixture was illuminated with light at the excitation wavelength of Chl *b*. The LHCIIb function considered here is the intracomplex energy transfer from Chl *b* to Chl *a*. When Chl *b* was excited during the reconstitution procedure, the establishment of resonance energy transfer from Chl *b* to Chl *a* resulted in a decrease in Chl *b* emission and a concomitant increase in sensitized Chl *a* fluorescence. In previous studies, we had shown that functional LHCIIb forms in two apparent kinetic steps in the time range of 10 s to several minutes. Figure 6 shows the kinetic traces in the same time range of the decrease in Chl *b* emission (panel A) and the rise of sensitized Chl *a* emission (panel B). Neither of these signal changes was seen in mock reconstitutions lacking the protein component (not shown), demonstrating that the changes observed in Chl *b* and Chl *a* fluorescence were associated with LHCIIb reconstitution. Clearly, the reconstitution procedures starting with either SDS (black traces)- or Gnd-solubilized (gray traces) *Lhcb1* showed similar kinetics.

Two-exponential fitting of the traces shown in Figure 6 yielded the apparent time constants listed in Table 2. The corresponding residuals are shown in the bottom panels of Figure 6. All traces could be fitted reasonably well by two

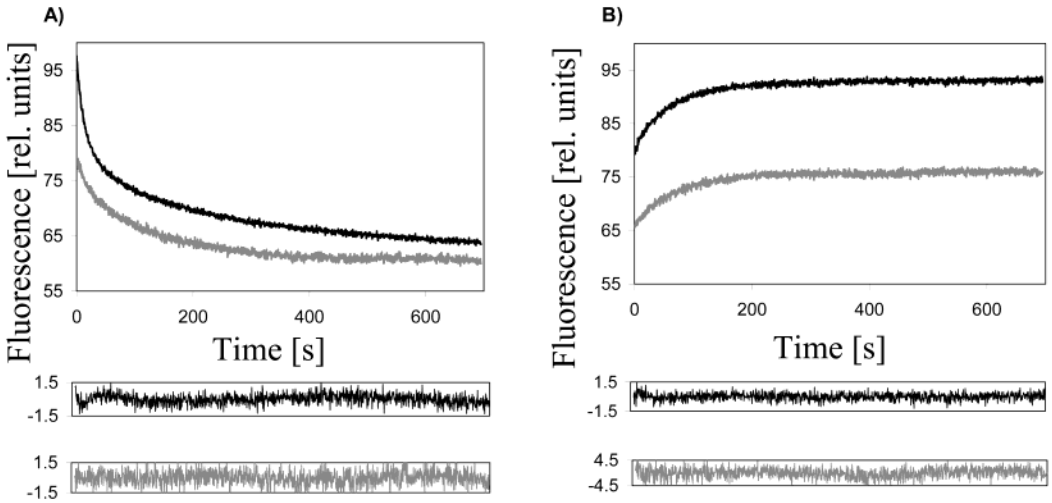

FIGURE 6: Time-resolved fluorescence measurements of LHCIIb formation. (A) Chl *b* fluorescence emission and (B) sensitized Chl *a* fluorescence upon excitation of Chl *b* during the assembly of the *Lhcb1*−pigment complex. The black and gray traces show the kinetics of a reconstitution experiment starting from SDS- and Gnd-denatured *Lhcb1*, respectively. Kinetic traces of a mock reconstitution containing only pigments were subtracted from the Gnd traces. The residuals resulting from a biexponential fit are shown below the traces.

Table 2: Kinetics of LHCIIb Folding and Assembly[a]

sample	τ_1 (s)	τ_2 (s)	A_1/A_2
Gnd	21 ± 4 (24 ± 8)	135 ± 17 (105 ± 25)	0.4 ± 0.1 (0.4 ± 0.2)
SDS	17 ± 2 (29 ± 6)	241 ± 19 (123 ± 56)	1.7 ± 0.2 (0.7 ± 0.3)

[a] The time-resolved measurements were started from *Lhcb1* dissolved in Gnd or SDS. Time constants τ_1 and τ_2 and amplitudes A_1 and A_2 are derived from the biexponential fit of the time-resolved measurements of the decrease in Chl *b* and increase in sensitized Chl *a* fluorescence (in parentheses) upon excitation of Chl *b*, during the assembly of the *Lhcb1*−pigment complex. Data reflect the average over eight (Gnd) and nine (SDS) independent measurements.

exponentials except at the initial 10 or 20 s where the fit was distorted presumably by carryover of the preceding step (found in earlier experiments as a decrease in both Chl *a* and Chl *b* emission in the time range of 1−10 s). The comparison of apparent time constants between the SDS and Gnd reconstitution kinetics showed no significant difference (except τ_2 of the decrease of Chl *b* fluorescence in the SDS reconstitution procedure; the signal exhibited a very slow, almost linear decrease which we cannot explain at present). Thus, the kinetics of the assembly of functional LHCIIb *in vitro* were not dependent on whether the protein was partially folded into α-helix before its reconstitution.

DISCUSSION

All procedures for reconstituting Chl *a*/*b* proteins with pigments include solubilization of the apoprotein in SDS or LDS (*34*). We show here that the major Chl *a*/*b* protein *Lhcb1*, an apoprotein of LHCIIb, can be refolded without SDS. Since the reconstitution procedures for all Chl *a*/*b* proteins are rather similar, it is likely that the other members of the Chl *a*/*b* protein family, too, can be refolded starting from a Gnd solution. This is an improvement in Chl *a*/*b* protein refolding *in vitro* since SDS, a strongly destabilizing detergent (*35*), is often difficult to remove from proteins.

The yield of Gnd-based LHCIIb reconstitution is somewhat lower than that of reconstitution starting from SDS-dissolved *Lhcb1*. Some of the Gnd-dissolved *Lhcb1* is probably lost by precipitation during the reconstitution procedure. Whereas recombinant LHCIIb is soluble in 2% OG (or the mixture of 2% OG and 0.6 M Gnd resulting from the reconstitution procedure), the nonpigmented apoprotein is not. So the formation of the pigment−protein complex upon mixing of the protein and pigment solutions competes with protein precipitation. We expect that improved yields should be obtained in the Gnd reconstitution procedure when protein precipitation is avoided or slowed by the addition of any component acting as a chaperone in this system.

According to its biochemical and spectroscopic characterization, Gnd-reconstituted LHCIIb appears to be authentic except that, compared to native LHCIIb, it lacks two Chl *a* molecules. The same is true for the product of the SDS reconstitution procedure chosen here. Other reconstitution procedures, including SDS or LDS, lead to Chl *a*:*b* ratios more similar to the native one of 1.3−1.4 and a total of 12−14 Chls per apoprotein, amounting to seven to eight Chl *a* and five to six Chl *b* molecules (*36*−*39*). The number of Chls per apoprotein and the Chl *a*:*b* ratio in recombinant LHCIIb depend on both the Chl *a*:*b* ratio present in the reconstitution mixture and the denaturing stringency applied during reconstitution and complex isolation (*36*, *39*). In a systematic titration of Chl binding sites in LHCIIb with Chl mixtures over a wide range of Chl *a*:*b* ratios, we recently found five binding sites absolutely specific for Chl *b*, whereas all other binding sites have only gradual preferences for Chl *a* or Chl *b* (*40*). Consequently, in the recombinant LHCIIb complexes with six Chl *a* and six Chl *b* molecules, one ambivalent Chl binding site is filled with Chl *b*. In fact, the absorption band at 660 nm that is more prominent in recombinant LHCIIb than in the native complex may represent a Chl *b* bound to a long-wavelength Chl *a* binding site, shifting the Chl *b* absorption, too, to longer wavelengths. Presumably, at a higher Chl *a*:*b* ratio in the reconstitution mixture, the promiscuous binding site now occupied by a Chl *b* would be filled with Chl *a*, shifting the Chl *a*:*b* ratio to 1.4. However, when higher Chl *a*:*b* ratios were used in the Gnd reconstitution, the yield of pigment−protein complexes decreased sharply. This may be due to Chl aggregation. In the Gnd reconstitution procedure, there is less detergent present than in SDS reconstitutions to keep the water-insoluble Chls in solution until they are complexed

with the protein. Chl *a* has an even stronger tendency to aggregate than Chl *b*; thus, at high Chl *a*:*b* ratios in the reconstitution mixture, the formation of Chl−protein complexes may fail because too much Chl *a* is lost due to aggregation.

The pigment−protein complexes resulting from either the SDS or Gnd reconstitution procedure are virtually identical as documented by all spectroscopic and biochemical measurements. The pigment stoichiometries are approximately the same; the absorption and CD spectra are virtually congruent, and fluorescence spectra show complete energy transfer from Chl *b* to Chl *a* in both cases. However, the starting conditions for complex assembly are quite different in the two procedures. The SDS-solubilized apoprotein adopts an α-helical conformation comprising ∼27% of the polypeptide, roughly half of the α-helical conformation present in the native protein structure (ref *25* and Figure 1). This is independent of whether washed inclusion bodies of *Lhcb1* or an acetone precipitate of the protein is dissolved in SDS. We cannot confirm the notion of Meyer et al. (*41*) that an acetone precipitate of the LHCIIb apoprotein, dissolved in SDS, adopts a random coil conformation. The Gnd-denatured protein on the other hand, according to its CD signal, does not exhibit any α-helix. Therefore, the successful reconstitution of Gnd-dissolved *Lhcb1* clearly shows that a partial α-helix conformation in *Lhcb1* prior to its exposure to pigments is not a prerequisite for its folding and pigmentation *in vitro*.

The different initial protein conformations do not significantly influence the kinetics of LHCIIb formation. In both reconstitution setups, energy transfer from Chl *b* to Chl *a* was established in two apparent kinetic steps with time constants of ∼20 s and 2−3 min. These time constants are similar to the ones observed previously in LHCIIb assembly experiments starting from SDS-dissolved *Lhcb1* (*42*, *43*). Only the relative amplitudes of the faster and the slower step show a significant difference between the two reconstitution procedures. In the SDS reconstitution procedure, the major part of the total signal change is associated with the faster kinetic step, whereas in the Gnd reconstitution, most of the energy transfer occurs during the slower phase. In earlier experiments, we had seen a similar shift in relative amplitudes toward one of the slower phase when the overall reaction was slowed by, for example, lowering the reactant concentration or increasing the detergent concentration. In contrast, in the experiments discussed here, we saw quite different contributions of the two apparent kinetic steps to the total amplitude, but their rate constants were about the same. We do not know the molecular events represented by the kinetic phases; therefore, at present we cannot interpret the significance of their different amplitudes.

What is the initial step in LHCIIb formation *in vitro*? According to the LHCIIb crystal structure, the pigments interact mainly with the α-helical transmembrane domains of the protein. Therefore, our working hypothesis has been so far that some α-helical protein segment, preformed in SDS solution, provides a binding site for one or several pigment(s) and this interaction then triggers the cooperative binding of more pigments and the completion of α-helical secondary structure. This notion may still be valid, although LHCIIb can be reconstituted from the entirely unfolded protein. The helical protein conformation is stabilized by a hydrophobic protein environment (*4*). Thus, it is likely that upon mixing of Gnd-dissolved *Lhcb1* with a detergent solution, the detergent micelles forming around the hydrophobic protein domains induce the formation of α-helix. This may then bind pigments and serve as a starting point for further assembly. The possible effect of OG micelles on the secondary structure of *Lhcb1* cannot be measured by a control experiment lacking pigments, as, unlike LHCIIb, the nonpigmented apoprotein is insoluble in OG solution. Clearly, the initiation of α-helix formation is not rate-limiting in the overall process, since the apparent kinetics of complex formation are the same regardless of whether the apoprotein contains some α-helix at the starting point. Experiments with the soluble protein cytochrome *c* have shown that the appearance of helical structure in folding proteins can be a fast process taking place in the millisecond range (*44*). In bR, part of the α-helix forms early in the folding process (*24*). In fact, the two-stage model of membrane protein folding, proposed by Popot and Engelman (*45*), is based on the concept of α-helices autonomously folding in a first step. In the second step, according to this model, the protein arrives at its final structure by establishing helix−helix interactions and interactions with cofactors. Our previous experiments show that Chl as well as Car concentrations can be rate-limiting in LHCIIb formation *in vitro* (*43*); therefore, the rate-limiting process is likely to involve the cooperative binding of several pigment components, associated with the extension of α-helical protein structure (*25*). If in fact the initial α-helix formation in these experiments is a fast process, then our observations are in line with the two-stage folding model (*45*), at least for this part of helix formation.

How do these observations of LHCIIb folding and assembly *in vitro* relate to LHCIIb biogenesis? The LHCIIb apoproteins are synthesized in their precursor form in the cytoplasm, post-translationally imported into chloroplasts, and then inserted into the thylakoid membrane. On their way across the chloroplast stroma, the proteins are chaperoned by the chloroplast signal recognition particle (*46*). The latter consists of the subunit cpSRP54, thought to bind to a hydrophobic domain of the LHCIIb apoprotein, and a dimer of cpSRP43, which has been shown to bind specifically to the stromal loop domain of the LHCIIb apoprotein (*47*−*49*). Delivery of the protein to the thylakoid is then mediated by the soluble receptor cpFtsY and the putative membrane receptor ALB3 (*50*−*52*). Nothing is known about the folding state of the LHCIIb apoprotein during its interaction with cpSRP and upon its integration into the thylakoid membrane. More significantly, we know nothing about where and how the protein becomes exposed to pigments. Clearly, the molecular mechanism of LHCIIb assembly *in vivo* needs to be elaborated in more detail before we can relate the folding behavior *in vitro* of its apoprotein to LHCIIb biogenesis in the chloroplast.

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REFERENCES

1. Booth, P. J., Templer, R. H., Meijberg, W., Allen, S. J., Curran, A. R., and Lorch, M. (2001) *Crit. Rev. Biochem. Mol. Biol. 36*, 501−603.

2. Buchanan, S. K. (1999) *Curr. Opin. Struct. Biol. 9*, 455−461.
3. Schulz, G. E. (2000) *Curr. Opin. Struct. Biol. 10*, 443−447.
4. Popot, J. L., and Engelman, D. M. (2000) *Annu. Rev. Biochem. 69*, 881−922.
5. Surrey, T., Schmid, A., and Jähnig, F. (1996) *Biochemistry 35*, 2283−2288.
6. Kleinschmidt, J. H., Wiener, M. C., and Tamm, L. K. (1999) *Protein Sci. 8*, 2065−2071.
7. Kleinschmidt, J. H., den Blaauwen, T., Driessen, A. J. M., and Tamm, L. K. (1999) *Biochemistry 38*, 5006−5016.
8. He, M. M., and Kaback, H. R. (1998) *Mol. Membr. Biol. 15*, 15−20.
9. Nagy, J. K., Lonzer, W. L., and Sanders, C. R. (2001) *Biochemistry 40*, 8971−8980.
10. Huang, K.-S., Bayley, H., Liao, M.-J., London, E., and Khorana, H. G. (1981) *J. Biol. Chem. 256*, 3802−3809.
11. London, E., and Khorana, H. G. (1982) *J. Biol. Chem. 257*, 7003−7011.
12. Popot, J.-L., Gerchman, S.-E., and Engelman, D. M. (1987) *J. Mol. Biol. 198*, 655−676.
13. Curran, A. R., Templer, R. H., and Booth, P. J. (1999) *Biochemistry 38*, 9328−9336.
14. Plumley, F. G., and Schmidt, G. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 146−150.
15. Paulsen, H., Rümler, U., and Rüdiger, W. (1990) *Planta 181*, 204−211.
16. Giuffra, E., Cugini, D., Croce, R., and Bassi, R. (1996) *Eur. J. Biochem. 238*, 112−120.
17. Schmid, V. H. R., Cammarata, K. V., Bruns, B. U., and Schmidt, G. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 7667−7672.
18. Pagano, A., Cinque, G., and Bassi, R. (1998) *J. Biol. Chem. 273*, 17154−17165.
19. Ros, F., Bassi, R., and Paulsen, H. (1998) *Eur. J. Biochem. 253*, 653−658.
20. Schmid, V. H. R., Potthast, S., Wiener, M., Bergauer, V., Paulsen, H., and Storf, S. (2002) *J. Biol. Chem. 277*, 37307−37314.
21. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1995) *J. Biol. Chem. 270*, 6856−6863.
22. Reynolds, J. A., and Tannford, C. (1970) *J. Biol. Chem. 245*, 5161−5165.
23. Montserret, R., McLeish, M. J., Böckmann, A., Geourjon, C., and Penin, F. (2000) *Biochemistry 39*, 8362−8373.
24. Riley, M. L., Wallace, B. A., Flitsch, S. L., and Booth, P. J. (1997) *Biochemistry 36*, 192−196.
25. Horn, R., and Paulsen, H. (2002) *J. Mol. Biol. 318*, 547−556.
26. Kosemund, K., Geiger, I., and Paulsen, H. (2000) *Eur. J. Biochem. 267*, 1138−1145.
27. Cashmore, A. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2960−2964.
28. Krupa, Z., Huner, N. P. A., Williams, J. P., Maissan, E., and James, D. R. (1987) *Plant Physiol. 84*, 19−24.
29. Paulsen, H., and Hobe, S. (1992) *Eur. J. Biochem. 205*, 71−76.
30. Sarvari, E., and Nyitrai, P. (1994) *Electrophoresis 15*, 1068−1071.
31. Martinson, T. A., and Plumley, F. G. (1995) *Anal. Biochem. 228*, 123−130.
32. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta 975*, 384−394.
33. Davies, B. H. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., Ed.) Vol. 2, pp 38−165, Academic Press, London.
34. Melkozernov, A. N., Schmid, V. H. R., Lin, S., Paulsen, H., and Blankenship, R. E. (2002) *J. Phys. Chem. B 106*, 4313−4317.
35. Ide, J. P., Klug, D. R., Kühlbrandt, W., Giorgi, L. B., and Porter, G. (1987) *Biochim. Biophys. Acta 893*, 349−364.
36. Kleima, F. J., Hobe, S., Calkoen, F., Urbanus, M. L., Peterman, E. J. G., van Grondelle, R., Paulsen, H., and van Amerongen, H. (1999) *Biochemistry 38*, 6587−6596.
37. Rogl, H., and Kühlbrandt, W. (1999) *Biochemistry 38*, 16214−16222.
38. Remelli, R., Varotto, C., Sandonà, D., Croce, R., and Bassi, R. (1999) *J. Biol. Chem. 274*, 33510−33521.
39. Yang, C. H., Kosemund, K., Cornet, C., and Paulsen, H. (1999) *Biochemistry 38*, 16205−16213.
40. Hobe, S., Fey, H., Rogl, H., and Paulsen, H. (2003) *J. Biol. Chem. 278*, 5912−5919.
41. Meyer, M., Wilhelm, C., and Garab, G. (1996) *Photosynth. Res. 49*, 71−81.
42. Reinsberg, D., Booth, P. J., Jegerschöld, C., Khoo, B. J., and Paulsen, H. (2000) *Biochemistry 39*, 14305−14313.
43. Reinsberg, D., Ottmann, K., Booth, P. J., and Paulsen, H. (2001) *J. Mol. Biol. 308*, 59−67.
44. Akiyama, S., Takahashi, S., Ishimori, K., and Morishima, I. (2000) *Nat. Struct. Biol. 7*, 514−520.
45. Popot, J. L., and Engelman, D. M. (1990) *Biochemistry 29*, 4031−4037.
46. Li, X., Henry, R., Yuan, J. G., Cline, K., and Hoffman, N. E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3789−3793.
47. DeLille, J., Peterson, E. C., Johnson, T., Moore, M., Kight, A., and Henry, R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 1926−1931.
48. Tu, C. J., Peterson, E. C., Henry, R., and Hoffman, N. E. (2000) *J. Biol. Chem. 275*, 13187−13190.
49. Jonas-Straube, E., Hutin, C., Hoffman, N. E., and Schünemann, D. (2001) *J. Biol. Chem. 276*, 24654−24660.
50. Kogata, N., Nishio, K., Hirohashi, T., Kikuchi, S., and Nakai, M. (1999) *FEBS Lett. 447*, 329−333.
51. Moore, M., Harrison, M. S., Peterson, E. C., and Henry, R. (2000) *J. Biol. Chem. 275*, 1529−1532.
52. Yuan, J., Kight, A., Goforth, R. L., Moore, M., Peterson, E. C., Sakon, J., and Henry, R. (2002) *J. Biol. Chem. 277*, 32400−32404.

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