Spectroscopic Characterization of Three Different Monomeric Forms of the Main Chlorophyll *a/b* Binding Protein from Chloroplast Membranes†

Stephan Nussberger,§,‖ Jan P. Dekker,⊥ Werner Kühlbrandt,§ Bauke M. van Bolhuis,⊥ Rienk van Grondelle,⊥ and Herbert van Amerongen*,⊥

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany, and Department of Physics and Astronomy and Institute for Molecular Biological Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands

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ABSTRACT: A detailed comparison has been made between dichroic steady-state spectroscopic properties at 77 K of several trimeric and monomeric forms of the major chlorophyll *a/b* binding protein (LHC-II) from pea. Monomeric forms were obtained by applying high concentrations of nonionic detergents, by a lipase treatment, or by a chymotrypsin/trypsin treatment. The latter treatments removed phosphatidyl glycerol essential for trimer formation. The absorption and dichroism spectra indicate that for trimeric LHC-II the chlorophyll *b* absorption region is centered around 649 nm and is composed of at least five subbands near 640, 647, 649, 652, and 656 nm. The chlorophyll *a* absorption region is centered around 670 nm and is composed of at least five bands near 661, 668, 671, 673, and 676 nm. The chlorophyll *b* band near 647 and 652 nm and the chlorophyll *a* bands near 668 and 673 nm are absent in the circular dichroism spectrum after monomerization. A configuration in which pigments of the same nature located on different monomers become excitonically coupled in the trimer could explain these results. In monomers obtained in high concentrations of nonionic detergents, no additional bands have disappeared, but the absorption spectra of the other two types of monomers lack the bands at 640 and 661 nm. These monomers have lost some chlorophyll *a* and *b* according to the fluorescence emission spectra, which show contributions from free chlorophyll *a* and *b*. The results suggest that phosphatidyl glycerol not only is involved in trimer formation but also has a structural role within the monomers.

The spectra analysis of photosynthetic chlorophyll-binding proteins in relation to their structure is of fundamental importance for the understanding of the mechanism of electronic excitation transfer after absorption of light. Much attention has been paid to the spectroscopic properties of the bacterial photosynthetic reaction centers, the antenna complexes in phycobilisomes from cyanobacteria, and the BChl *a*-binding protein from *Prosthecochloris aestuarii* (the so called FMO complex) since the crystal structures of these complexes are known (Van Grondelle et al., 1994). This offered the possibility to study the relation between structure and spectroscopy (Huber, 1990). Pearlstein and co-workers (Pearlstein, 1992; Lu & Pearlstein, 1993) reported a number of theoretical studies on the FMO complex, in which they carefully related spectroscopy and structure. One of the most important results of these studies is that the aggregation state of the complex (monomeric vs trimeric) has a large impact on the spectroscopic properties.

The determination of the three-dimensional structure of the light-harvesting chlorophyll *a/b* complex LHC-II from thylakoid membranes at 6 Å resolution (Kühlbrandt & Wang, 1991) and recently at 3.4 Å resolution (Kühlbrandt et al., 1994) has now also opened the possibility to study structure−function relationships of LHC-II in more detail. LHC-II is the major light-harvesting complex of photosystem II in green plants and most likely binds seven or eight Chl *a*,[1] five or six Chl *b*, and two to four xantophyll molecules (Siefermann-Harms, 1985), which are noncovalently attached to the apoprotein. The crystal structure at 3.4 Å (Kühlbrandt et al., 1994) revealed 12 chlorophyll and 2 lutein molecules per monomer. The chlorophylls were tentatively identified as seven Chl *a* and five Chl *b* molecules and their positions could be determined as well as the approximate orientations of the heme planes.

So far, the spectroscopic properties of LHC-II were analyzed in detail for the trimeric form (which is most likely the native form of the complex) and for aggregates of trimers (Haworth et al., 1982; Ide et al., 1987; Zucchelli et al., 1990; van der Vos et al., 1991; Garab et al., 1991; Hemelrijk et al., 1992; Kwa et al., 1992a; Ruban & Horton, 1992; Krawczyk et al., 1993). For the trimeric form at least six and possibly nine Q_y electronic transitions in the red absorption region have been characterized by means of low-temperature absorption, linear dichroism, circular dichroism,

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§ European Molecular Biology Laboratory.

‖ Present address: Department of Medicine, Renal Division, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115.

⊥ Vrije Universiteit.

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[1] Abbreviations: Chl, chlorophyll; OG, *n*-octyl β,D-glycopyranoside; NG, *n*-nonyl β,D-glycopyranoside; DM, dodecyl maltoside; plA_2, phospholipase A_2; CT, chymotrypsin; A, absorption; LD, linear dichroism; CD, circular dichroism; PAA, polyacrylamide; fwhm, full width at half-maximum.

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and polarized excitation spectroscopy (Hemelrijk et al., 1992; Kwa et al., 1992a). The spectroscopic properties of monomeric LHC-II are largely unknown.

In the present study we investigate the monomeric form of LHC-II by a combination of several spectroscopic techniques (absorption, linear dichroism, circular dichroism, and fluorescence) in order to reveal which spectroscopic features are due to intramonomeric pigment interactions and which are due to intermonomeric pigment interactions. This knowledge will be of crucial importance for a detailed understanding of the relation between structure and spectroscopy. Monomeric LHC-II was obtained in three different ways: by a reversible dissociation of the trimer by the nonionic detergent *n*-octyl β,D-glycopyranoside as well as by the irreversible dissociation of the trimer by limited proteolysis or lipase treatment (Nussberger et al., 1993).

MATERIALS AND METHODS

Preparation of Trimeric and Monomeric LHC-II. Trimeric LHC-II was isolated and purified from pea thylakoid membranes by the method of Burke et al. (1978) as modified by Kühlbrandt et al. (1983). Stock solutions of the purified trimeric form of LHC-II in 0.6% (w/v) *n*-nonyl β,D-glycopyranoside (NG) (Calbiochem) in deionized water corresponding to a chlorophyll (*a* + *b*) concentration of 4.5 mg/mL were stored at −80 °C.

Monomeric LHC-II was generated in three different ways: by treatment with nonionic detergent, by incubation with phospholipase, or by limited enzymatic proteolysis as described in detail by Nussberger et al. (1993). In short, for dissociation by detergent, the trimeric complex was incubated with 1.8% (w/v) *n*-octyl β,D-glycopyranoside (Bachem, Bubendorf, Switzerland) at a chlorophyll concentration of 2 mg/mL at room temperature for 2−4 days. The dissociation by phospholipase was achieved by incubation of the trimers with 3 μg phospholipase A_2/mL from bee venom (Sigma) in 22 mM trishydroxy-methylamino-methane at a chlorophyll concentration of 1.4 mg/mL at room temperature overnight. For the proteolytic dissociation, the trimeric complexes were incubated with a 1:1 (w/w) mixture of immobilized trypsin and chymotrypsin (Sigma) in 0.46% (w/v) NG, 10 mM glycine (pH 7.8), 3 mM NaN_3 at a chlorophyll concentration of 2.3 mg/mL at room temperature for 24 h. Proteolytic cleavage with immobilized trypsin alone [3.3 mg/mL chlorophyll, 26 mM Hepes buffer (pH 8.0), 0.4% (w/v) *n*-nonlyl β,D-glycopyranoside, 45 min] removed only eight amino acid residues at the N-terminus, without causing the trimers to dissociate.

Monomeric LHC-II that had been dissociated by *n*-octyl β,D-glycopyranoside was partially reassociated into the trimeric form by dilution of the detergent below its critical micellar concentration by dialysis against 20 mM glycine buffer (pH 7.5), 3 mM NaN_3 at room temperature as described by Nussberger et al. (1993).

The purity and stability of LHC-II trimers was checked by denaturing (Laemmli, 1970) and partially denaturing (Anderson, 1980) polyacrylamide gel electrophoresis.

Steady State Spectroscopy. For the characterization of LHC-II by absorption, fluorescence, and circular dichroism spectroscopy, the complex was solubilized in 84% (v/v) glycerol (Merck), 0.8% (w/v) *n*-octyl β,D-glycopyranoside, 0.05% dodecyl maltoside (Sigma), 20 mM Hepes buffer, pH 7.5, centrifuged in a bench-top centrifuge for 2 min at 15000 rpm and then frozen to 77 K using an Oxford cryoholder (Oxford Instruments Ltd., UK). Absorption and fluorescence spectra were recorded in 1 × 1 cm acrylic cuvettes; circular dichroism samples were frozen in an open quartz 1 × 1 × 0.5 cuvette as described by Hemelrijk et al. (1992).

For recording linear dichroism spectra, the complex was oriented in two-dimensionally compressed polyacrylamide gels [20 mM Hepes buffer, pH 7.5, 0.6% (w/v) *n*-octyl β,D-glycopyranoside, 0.03% dodecyl maltoside, 55% (v/v) glycerol, 14.5% (w/v) acrylamide/0.5% N,N′-dimethylbisacrylamide] using the method of Abdourakhmanov et al. (1979). The chlorophyll concentration corresponded to an optical density of 0.6−0.7 measured at 670 nm. The gels were polymerized with 0.05% (w/v) ammonium persulfate and 0.03% TEMED (Sigma).

Absorption spectra were recorded with a double beam Cary 219 spectrophotometer. Linear dichroism and circular dichroism spectra were measured on a home-built spectrapolarimeter, essentially the same as that described by Visschers et al. (1991). Fluorescence measurements were performed on a home-built fluorimeter (Kwa et al., 1992b).

RESULTS

Trimeric Complexes and Aggregate Effects. The starting material for all complexes described in this report was trimeric LHC-II from pea. This material was isolated basically according to the method of Burke et al. (1978), which includes a high-salt precipitation step. The material was obtained in 0.6% nonly glucoside (NG) in deionized water and also served as the starting material for two- or three-dimensional crystals (Nussberger et al., 1993; Kühlbrandt et al., 1994). The absorption spectrum at 77 K is given in Figure 1a (dashed line). The peaks and shoulders near 640, 649, 662−663, 670, and 678 nm are similar to those in the spectrum of trimeric LHC-II from spinach, reported by Hemelrijk et al. (1992). The latter material was isolated in dodecyl maltoside and prepared without high-salt precipitation steps. The spectrum suggests that some aggregation has taken place in the LHC-II trimers from pea in view of the slightly red-shifted Chl *a* peak (678 nm) and the extension of the red wing to 700 nm and above (Ruban & Horton, 1992). The possible tendency to aggregate is very likely a result of the high concentration of glycerol (a well-known precipitating agent), which was added to obtain good glassy material at cryogenic temperatures. The aggregation was limited by raising the OG concentration to 0.8% and by adding 0.05% DM to the glycerol buffer, but apparently could not be prevented completely.

A good test for the aggregation state of LHC-II is provided by the fluorescence emission spectrum at 77 K. Trimeric complexes give rise to a sharp peak near 680 nm, whereas (strongly) aggregated LHC-II is characterized by a fluorescence maximum at 700 nm and a shoulder near 685 nm at 77 K (see Ruban and Horton (1992) and references therein). The 77 K fluorescence spectrum of our preparation of trimeric LHC-II from pea shows only a small shoulder at 700 nm Figure 2a, full line) and a peak at 679 nm, indicating that the extent of the aggregation was limited. The full width at half-maximum (fwhm) is 10 nm (spectral bandwidth 4 nm) which is very similar to results from spinach LHC-II at 77 K (Hemelrijk et al., 1992). Also, the linear dichroism

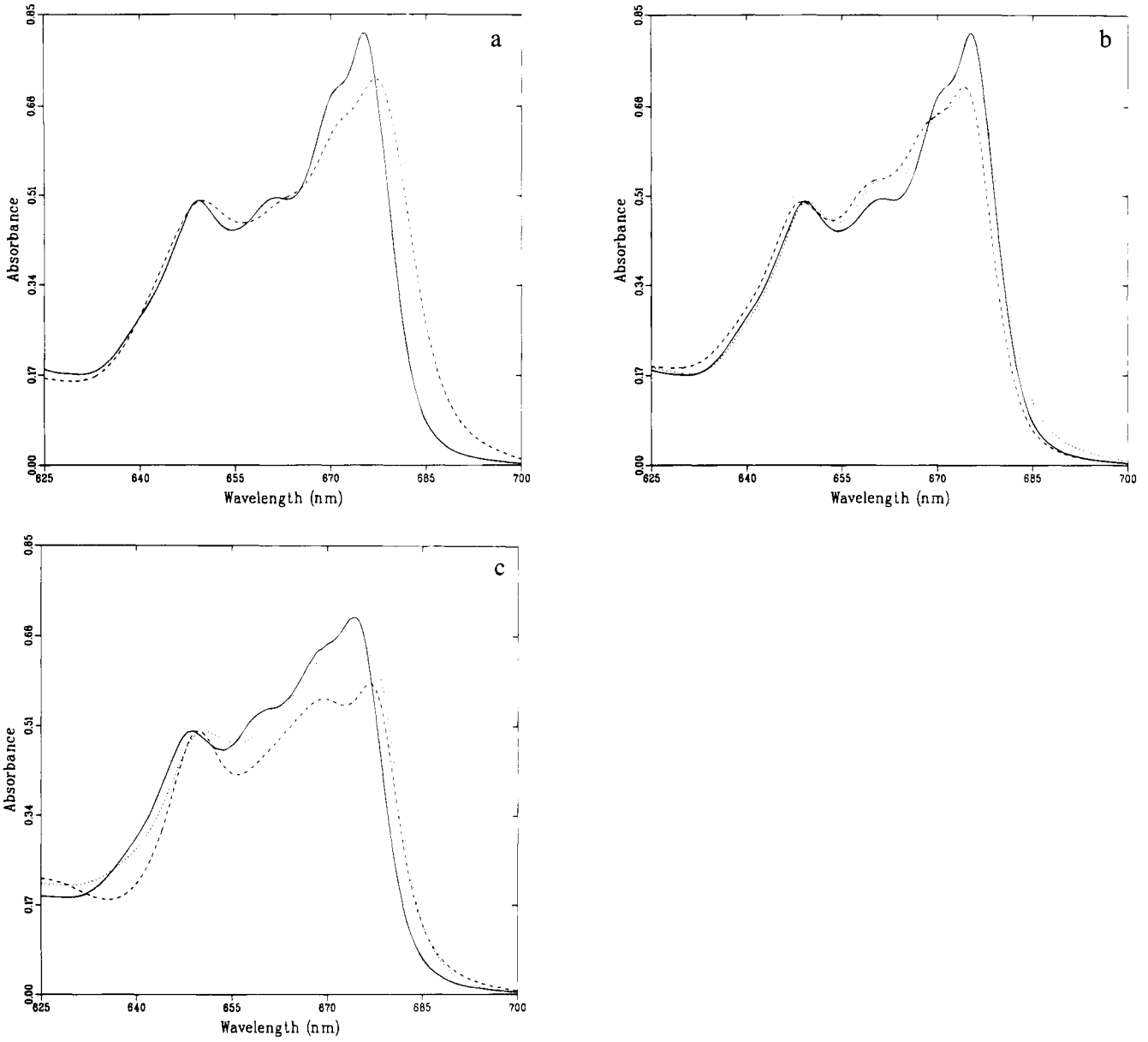

FIGURE 1: (a) Absorption spectra of trypsinized trimeric LHC-II (—) and nontrypsinized (slightly aggregated) LHC-II (– –) at 77 K. The spectra were recorded with an optical bandwidth of 1 nm and normalized to an optical density of 0.5 at 649 nm. (b) Absorption spectra of trypsinized trimeric LHC-II (—), octyl glucoside-induced monomers (OG-monomers) of LHC-II embedded in PAA gels (– –), and retrimerized OG monomers (••) at 77 K. The spectra have been normalized to an optical density of 0.5 at 649 nm. (c) Absorption spectra of octyl glucoside induced monomers of LHC-II embedded in PAA gels (—), phospholipase induced monomers (– –), and chymotrypsin/trypsin-induced monomers (••) at 77 K. The spectra have been normalized to an optical density of 0.5 at 649 nm.

spectrum (Figure 3a, full line) is essentially identical to that of spinach LHC-II (Hemelrijk et al., 1992). This suggests that the small aggregation effects do not have a very significant influence on the shape of the LD spectra.

Trypsin-Treated Trimeric LHC-II. Trypsin treatment of trimeric LHC-II results in the removal of eight amino acids from the N-terminus of the polypeptide. Since the phosphorylation site is included in this segment, the possibility to phosphorylate will be lost upon trypsinization (Allen, 1992), but the trimeric organization stays intact and pigments are not released as a result of this treatment (Nussberger et al., 1993). The 77 K OD spectrum of trypsinized trimeric LHC-II is shown in Figure 1a (solid line). The spectrum is characterized by bands near 640, 649, 660.5, 670, and 675 nm, which are better resolved than in all previously recorded 77 K spectra of trimeric LHC-II. Although the preparations were suspended in the same glycerol buffer as the non-trypsinized trimers, the glycerol-induced aggregation was largely absent, indicating that the presence of the eight N-terminal amino acids facilitates aggregation. The relative height of the Chl *b* peak as compared to the Chl *a* peak is somewhat higher than for the spectra presented by Hemelrijk et al. (1992) and Kwa et al. (1992a), which is in line with the slightly higher Chl *a*/*b* ratio in the latter material (1.47 and 1.33 for the spinach and pea preparations, respectively). This slightly changed ratio, however, does not significantly influence the total pigment organization, since the 77 K CD spectra are essentially identical Figure 4a, solid line, and Hemelrijk et al., 1992).

Detergent-Prepared Monomeric LHC-II. Incubation of trimeric LHC-II with octyl glucoside (OG) at concentrations of about 1.8% induces a reversible monomerization of the complexes (Nussberger et al., 1993). The reversible nature of this process makes the analysis of the resulting OG

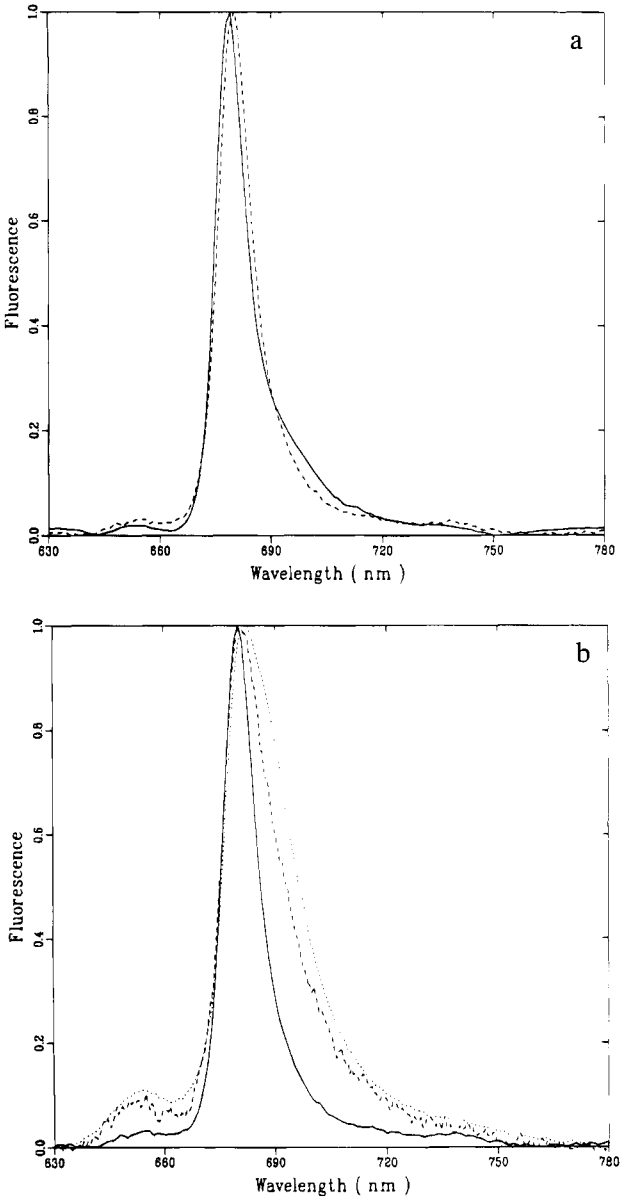

FIGURE 2: (a) Fluorescence emission spectra at 77 K of untrypsinized trimers (—) and octyl glucoside-induced monomers (in an uncompressed PAA gel) (– –) after excitation at 468 nm (excitation bandwidth of 18 nm, detection bandwidth of 4 nm). Spectra were normalized to 1 in the peak. (b) Fluorescence emission spectra at 77 K of octyl glucoside-induced monomers (in an uncompressed PAA gel) (—), phospholipase-induced monomers (– –), and chymotrypsin-induced monomers (••) after excitation at 468 nm. The excitation bandwidth was 18 nm and the detection bandwidth was 4 nm. The spectra were normalized to 1 in the peak.

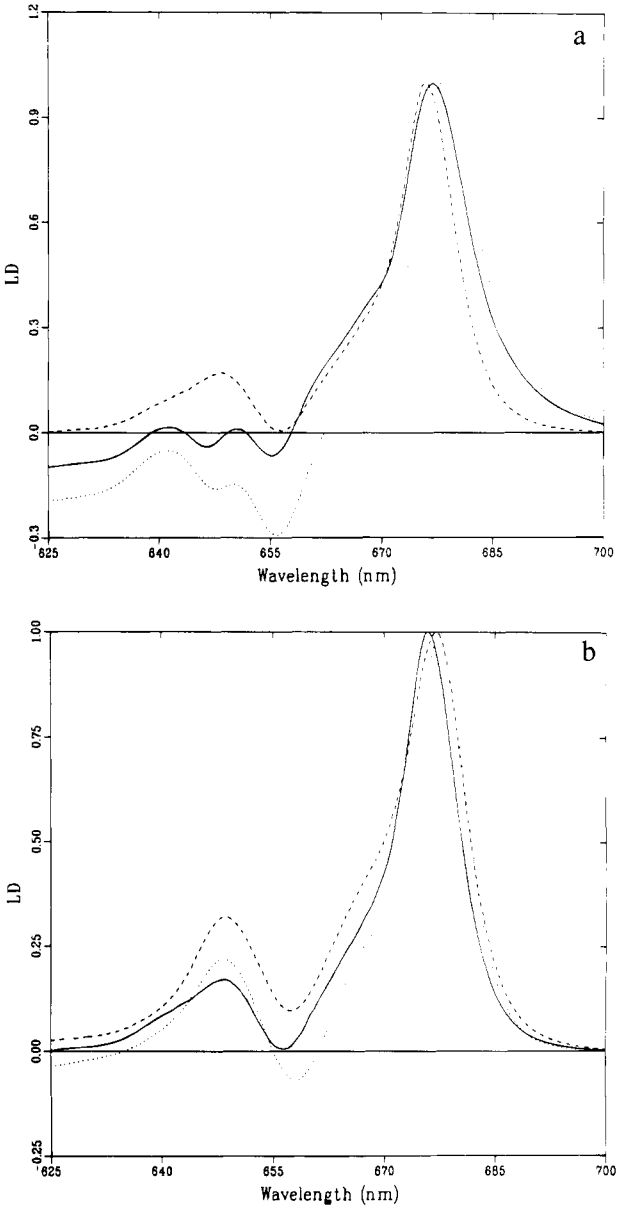

FIGURE 3: (a) Linear dichroism spectra of timeric LHC-II (—), octyl glucoside (OG) induced monomers (– –), and retrimerized OG monomers (••) at 77 K recorded with an optical bandwidth of 3 nm. After correcting all spectra to an optical density of 0.5 at 649 nm the spectra were multiplied with the following factors: 392 (—), 219 (– –), and 171 (••). (b) Linear dichroism spectra of octyl glucoside-induced monomers (—), phospholipase-induced monomers (– –), and chymotrypsin-induced monomers (••) at 77 K recorded with an optical bandwidth of 3 nm. After correcting all spectra to an optical density of 0.5 at 649 nm, the spectra were multiplied with the following factors: 219 (—), 59 (– –), and 290 (••).

monomers by low-temperature spectroscopy difficult, since the freezing/thawing conditions and the high amounts of glycerol can induce retrimerization. A clear indication that retrimerization indeed takes place under the particular conditions at which the low-temperature experiments were performed is the fact that a cooled sample of OG monomers shows a trimeric CD spectrum (not shown, see below for further details). However, it appeared that the retrimerization was prevented in (uncompressed) polyacrylamide gels (see also LD results). The corresponding absorption spectrum is shown in Figure 1b (dashed line). It resembles that of trypsinized trimers (Figure 1b, full line), although the monomer spectrum seems to be slightly blue-shifted. Note that the spectrum of the slightly aggregated, nontrypsinized trimers is *red*-shifted compared to that of trypsinized trimers (Figure 1a), due to which we may conclude that as a general tendency the spectrum red-shifts upon increasing the aggregation state.

The 77 K OD spectrum of the monomeric complexes is characterized by four clearly resolved bands at about the same positions as for the trypsinized trimers. A difference with the trimer solutions is the relative increase of the 660–661 nm band and the relative decrease of the 676 nm band (note that in Figure 1 all spectra have been normalized to

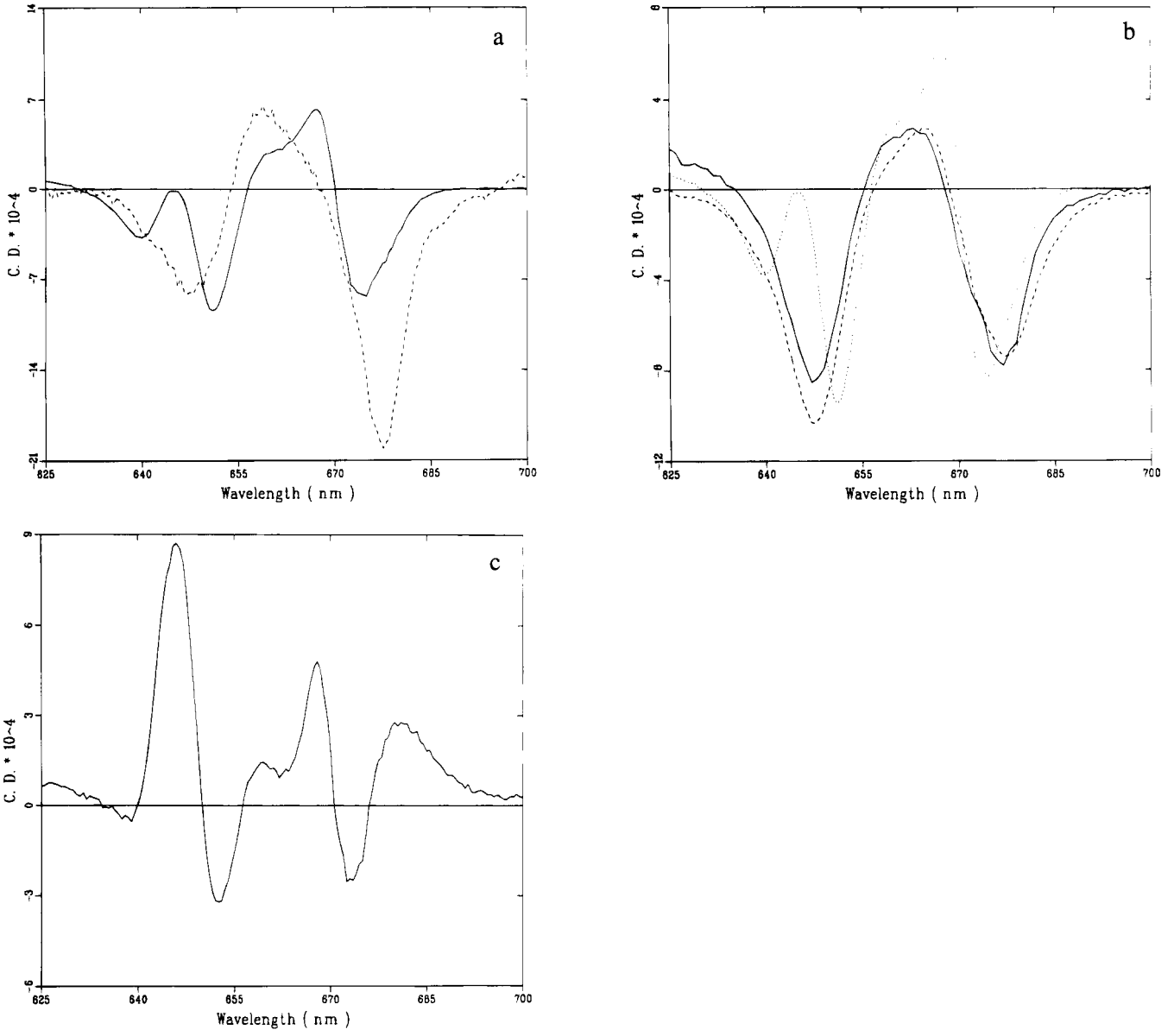

FIGURE 4: (a) Circular dichroism spectra at 77 K of trypsinized trimeric LHC-II (—) (normalized to an optical density of 0.6 at 649 nm) and octyl glucoside-induced monomers of LHC-II (— —) (normalized to an optical density of 0.5 at 649 nm) recorded with an optical bandwidth of 3 nm. (b) Circular dichroism spectra at 77 K of phospholipase-induced monomers (—), chymotrypsin/trypsin-induced monomers (— —) (both normalized to an optical density of 0.5 at 649 nm), and trypsinized trimeric LHC-II (••) (normalized to an optical density of 0.6 at 649 nm) recorded with an optical bandwidth of 3 nm. (c) Difference between the circular dichroism spectra of phospholipase-induced monomers and (trypsinized) trimeric LHC-II as given in Figure 2b.

an absorption value of 0.5 at 649 nm, the maximum of the Chl *b* band). Rather than stressing the small differences between the various OD spectra, however, we would like to point to the presence of the peaks and shoulders near 640, 649, 661, 670, and 676 in all preparations (monomers, trimers, and slightly aggregated trimers). This similarly contrasts with the pronounced differences observed by dichroism techniques (see below).

The 77 K LD spectrum of the OG monomers is shown in Figure 3a (dashed line). It reveals a large positive Chl *a* band around 676 nm, which is slightly blue-shifted compared to the LD spectrum of the trimeric complexes. However, the overall structure seems about the same in the region above 660 nm. A marked difference is observed in the Chl *b* absorption region, where the fine structure between 640 and 655 nm has been replaced by a positive band centered at 648.5 nm and the absence of LD at 656.5 nm.

In Figure 4a (dashed line) the 77 K CD spectrum of the OG monomers is shown. This spectrum was measured in an uncompressed polyacrylamide gel, which prevented the OG monomers from retrimerizing upon cooling (see above). The spectrum is characterized by a pronounced negative peak at 678 nm and by broad positive and negative peaks near 659 and 648 nm, respectively, and is rather different from that of the trypsinized trimers (Figure 4a, full line). The most obvious difference is observed in the Chl *b* absorption region, where in the trypsinized trimers a split signal with negative peaks at 651 and 640 nm occurs, while in the monomers a single negative band near 648 nm is observed.

A disadvantage of the measurements on gels is the fact that some orientation of the complexes may take place in the cuvette, even when the gels are not manually compressed. Such an orientation unavoidably leads to a LD contribution in the CD spectrum. Because the LD of the OG monomers

is only large around 676 nm, the "contamination" will only be significant in this absorption region. Thus, a LD contribution may be responsible for part of the negative feature at 678 nm in Figure 4a (dashed line). However, the split signal in the Chl *b* region as observed in the CD spectrum of the trypsinized trimers (Figure 4a, solid line) cannot be generated by making a linear combination of the "contaminated" CD spectrum (Figure 4a, dashed line) and the measured LD spectrum (Figure 3a, dashed line). We conclude from these observations that upon monomerization the spectral fine structure of Chl *b* in CD and LD disappears and is replaced by a single transition near 648 nm with positive LD and negative CD.

The fluorescence emission spectrum upon 468 nm excitation (mainly Chl *b*) of the OG monomers in an uncompressed PAA gel is shown in Figure 2a (dashed line). The spectrum is virtually identical to the one obtained with 436 nm excitation (mainly Chl *a*, not shown) and is also very similar to that of the (untrypsinized) trimers (Figure 2a, solid line), except that the shoulder around 700 nm is absent in the monomer spectrum. Very little fluorescence from Chl *b* (around 655 nm) and from free Chl *a* (around 673 nm) is detected for the OG monomers upon 468 or 436 nm excitation. This shows that no significant amount of chlorophyll molecules was removed from LHC-II upon monomerization after raising the OG concentration (no purification step was applied after the OG incubation).

Retrimerized Monomers. The 77 K OD spectrum of the retrimerized monomers is shown in Figure 1b (dotted line). The only significant difference with the spectrum of the OG monomers is a red-shift of the entire absorption spectrum by 1−1.5 nm. The same general red-shift is observed in the 77 K LD spectrum (Figure 3a, dotted line), from which it also becomes clear that the fine structure in the Chl *b* region reappears upon retrimerization. The reappearance of LD fine structure in the Chl *b* region indicates that the loss of this fine structure upon monomerization is reversible. The general red-shift may in part be caused by a partial aggregation beyond the level of the trimers. However, in extensively aggregated LHC-II the LD fine structure of Chl *b* is also observed (A. V. Ruban, S. L. S. Kwa, R. van Grondelle, P. Horton, and J. P. Dekker, unpublished results), due to which there is no reason to doubt the conclusion that the absence of the fine structure is a spectroscopic marker for the monomeric state and that the presence of the fine structure is a marker for the trimeric and aggregated states.

Chymotrypin/Trypsin Prepared Monomers. Treatment of trimeric LHC-II with a mixture of chymotrypsin and trypsin (CT) was shown to lead to a removal of the first 49 N-terminal amino acids and to monomerization of the complex (Nussberger et al., 1993). It was proposed that the monomerization is a result of the loss of a phosphatidyl glycerol (PG) molecule and that the region between amino acids 9 and 49 is important for the binding of this PG molecule (Nussberger et al., 1993). The N-terminal part of the polypeptide is located at the stromal side of the thylakoid membrane, and the first 24 N-terminal residues could not be traced in the crystal structure, presumably because this part of the protein is relatively flexible (Kühlbrandt et al., 1994).

In Figure 1c (dotted line) the 77 K absorption spectrum of the CT monomers is presented. The spectrum shows a relative decrease of the main Chl *a* band near 676 nm, a broadening of the 670 nm band, the disappearance of the 661 nm band, and a slight decrease of the absorption around 640 nm, which is also apparent from the second-derivative spectra (not shown). The disappearance of the 640 and 661 nm bands are not a consequence of monomerization, since these bands are still observed for the OG monomers; they must be caused by additional changes brought about by CT treatment. In the 77 K emission spectrum obtained upon 468 nm excitation, some Chl *b* fluorescence is observed (Figure 2b, dotted line); fluorescence from uncoupled Chl *a* (upon 436 nm excitation) could be observed as well (not shown), suggesting that some of the chlorophylls are released from their binding sites as a result of the CT treatment. The main Chl *a* emission band peaks at 683 nm and is broader (fwhm ~ 21 nm) than that of the other complexes.

Figure 4b (dashed line) shows the 77 K CD spectrum of the CT monomers. The spectrum is characterized by negative peaks near 677 and 648 nm and a broad positive peak near 664 nm. It is substantially different from that of the trypsinized trimers (Figure 4b, dotted line) but similar to that of the OG monomers (Figure 4a, dashed line), especially considering the fact that in the latter material the region around 676 nm is most likely distorted by LD contributions (see above). Also, the 77 K LD spectrum (Figure 3b, dotted line) is virtually identical to that of the OG monomers. These results demonstrate that upon monomerization by proteolytic treatment of Chl *b* fine structure disappears and is replaced by a broad transition at 648 nm with positive LD and negative CD. These results also demonstrate that the removal of chlorophyll by the CT treatment does not have a very significant influence on the overall conformation of the pigments.

Phospholipase-Prepared Monomers. Treatment of trimeric LHC-II with phospholipase A_2 in the presence of 0.6% NG was reported to result in a complete removal of PG from the complex and in an irreversible monomerization, which is in line with the hypothesis that PG is essential for trimer formation (Nussberger et al., 1993). Figure 1c (dashed line) shows the 77 K absorption spectrum of the plA_2 monomers. It shows the same general features as the spectrum of the CT monomers: decreased 676, 661, and 640 nm bands and increased or broadened 670 and 650 nm bands. Also the fluorescence (Figure 2b, dashed line), CD (Figure 4b, solid line), and LD (Figure 3b, dashed line) features are similar: emission bands of uncoupled Chl *b* and Chl *a* molecules, a braodened 77 K emission band at about 682 nm, and the absence of Chl *b* fine structure in the dichroism spectra. The latter result strongly suggests that the disappearance of the Chl *b* fine structure is a general feature of monomerization.

In Figure 4c the difference between the CD spectra of the trypsinized trimer (Figure 4b, dotted line) and the plA_2 monomer (Figure 4b, full line) is shown. The most prominent features are the positive and negative peaks at 646 and 653 nm in the Chl *b* region and (to a somewhat smaller extent) the positive and negative peaks at 668 and 673 nm in the Chl *a* region. A similar difference spectrum is obtained when the CT spectrum is taken instead of the plA_2 spectrum (not shown). There is some indication that also a weakly positive band just above 655 nm and a weakly negative band near 640 nm disappear upon plA_2 and CT treatment. It should be noted that a similar difference spectrum is also found when instead of the specturm of

trypsinized trimers that of one of nontrypsinized trimers is used.

DISCUSSION

Of the three types of monomers studied, the CT and plA_2 monomers show pronounced changes in the fluorescence emission spectra with contributions from detached Chl *a* and Chl *b* and broadened fluorescence emission bands with maxima at 682−683 nm. In contrast, the OG monomers show no fluorescence from uncoupled pigments or broadening of the Chl *a* emission band. Because also the absorption spectra of the plA_2 and CT monomers differ more from the trimer spectrum than that of OG monomers, we conclude that of all three monomers studied the OG monomers resemble the monomer subunits within the trimer most closely. This is consistent with the recent finding that the dissociation of native trimeric LHC-II by detergent treatment into monomers was almost fully reversible when the detergent was removed by dialysis (Nussberger et al., 1993), indicating that the complex has not been denatured by detergent treatment. In contrast to the detergent-monomerized complex, the dissociation of the native complex by using phospholipase A_2 to degrade phosphatidylglycerol enzymatically or by partial proteolysis with trypsin/chymotrypsin was irreversible. The observation that monomers generated by incubation with elevated levels of nonionic detergent are more intact than those generatd by lipase treatment is surprising. One would *a priori* expect that high levels of detergent are more likely to detach pigment molecules from the complex than the removal of a particular lipid associated with the complex. The fact that this is not the case indicates that this lipid, phosphatidylglycerol, is not only important for trimer formation (Nussberger et al., 1993) but also has a structural role within the monomer.

Despite the different degrees of intactness of the OG, plA_2, and CT monomers, no marked differences in the CD spectra were observed, indicating that the CD of the monomers is mainly determined by the Chl molecules that are least accessible from the outside of the protein, i.e. "far away" from the phosphatidyl glycerol. The differences between the monomer and trimer CD spectrum are highlighted in Figure 4c, where the CT monomer spectrum of Figure 4b has been subtracted from the trimer spectrum shown in the same figure. The difference spectrum can most easily be explained in the following way. Upon monomerization, excitonic interactions between 2 coupled Chl *b* molecules are broken, leading to the disappearance of a positive exciton CD band at 646 nm and a negative exciton CD band at 653 nm, whereas a similar uncoupling of 2 Chl *a* pigments leads to the disappearance of the positive and negative peaks at 668 and 673 nm, respectively. The center wavelengths of 649.5 and 670.5 nm are close to the absorption peaks of uncoupled Chl *b* and Chl *a*. Our results indicate that for the OG monomers no obviously related changes in the absorption spectrum occur. This is in line with the calculations of Pearlstein (1992) for the FMO complex, which demonstrated that the CD spectrum for monomers and trimers may differ appreciably, whereas the absorption spectra are hardly distinguishable. It should be noted that aggregation of LHC-II might in principle also influence the dichroic features around the absorption maxima due to dispersive effects (Krawczyk, 1981).

The results presented in this contribution strongly suggest that the split CD signal in the Chl *b* absorption region marks the trimeric and aggregated states, whereas the absence of the split signal marks the monomeric state of LHC-II. In trimers, the split CD signal is easily observed at 77 K, but also at room temperature the signal is observed to some extent. Earlier work by Gülen et al. (1986) and Ide et al. (1987) indicates that the split CD signal disappears upon raising the detergent concentration. The present work indicates that monomerization of LHC-II is responsible for this phenomenon.

Also in the LD spectrum the characteristic features in the Chl *b* region disappear upon monomerization and we attribute the changes around 650 nm to the same phenomena as described above for the CD spectra. The changes in the CD spectra in the Chl *a* region are not mirrored in the LD spectrum. One should realize, however, that bands that are observed in a CD spectrum need not necessarily be present in a LD spectrum.

Besides these characteristic changes due to monomerization there are some additional changes upon plA_2 and CT treatment which do not occur after raising the OG concentration. The weak positive Chl *b* band around 640 nm in the trimeric LD spectrum is still visible as a shoulder in the OG monomer LD spectrum, but it is absent in the absorption and LD spectra of plA_2 and CT monomers. This band has been ascribed to Chl *b* in the past (van der Vos et al., 1991; Hemelrijk et al., 1993). Additional support for the assignment of this band to Chl *b* (and not to a higher energy band related to the main Chl *a* transition at 676 nm) comes now from the fact that plA_2 and CT monomers still have significant absorption bands around 676 nm whereas the 640 nm shoulder has disappeared.

The position of the 640 nm band is blue-shifted with respect to that of free Chl *b*. Such a large blue-shift may arise from excitonic interaction with a Chl *b* band above 650 nm (656 or 661 nm). It can be argued that it is very unlikely that the 661 nm band is a Chl *b* band. From the Gaussian decomposition of the Q_y region by Krawczyk et al. (1993) the ratio between the integrated areas < 656 nm (Chl *b*) and > 665 nm (Chl *a*) can be calculated to be 0.72. Taking into account the number of pigments per monomer and the approximate ratio of the dipole strengths of 0.7:1 for Chl *b*: Chl *a*, a ratio of 0.52 ±.08 would be expected. Although the former ratio is a slight overestimation due to the neglecting of some vibrational and phonon side bands and although the absorption ratio might be influenced by some excitonic, hypo- and hyperchromic effects it seems unlikely that the 661 nm band is significantly contributed to by Chl *b*, in contrast to the suggestion by Krawczyk et al. (1993). From the Gaussian decomposition by van der Vos et al. (1991) the same conclusion can be drawn. Therefore, if the strong blue-shift of the 640 nm band would be due to exciton coupling, this 640 nm band would most likely be coupled to the 656 nm (Chl *b*) band. The difference CD spectrum in Figure 2c indicates that only weak CD bands near 640 and 656 nm disappear upon plA_2 treatment. The same changes are observed after CT treatment (results not shown). In the context of the explanation of the blue-shift by exciton interactions the small CD would imply that the transition dipoles of the pigments that give rise to 640 and 656 nm bands are oriented almost in one plane (see also below). For plA_2 and CT monomers this interaction is then

disturbed, possibly because at least one of the interacting pigments is released from the protein. This could explain the appearance of fluorescence from free Chl *b* for these monomers.

Alternatively, the blue-shifted position of the 640 nm band may also be explained through the presence of charged amino acids in the immediate environment of a Chl *b* molecule (Eccles & Honig, 1983). The recent structure at 3.4 Å (Kühlbrandt et al., 1994) indicates that indeed some chlorophylls are close to charged residues, such as the glutamate−arginine pairs that ligate some of the chlorophylls.

Also in the Chl *a* region the absorption spectra of plA_2 and CT monomers are different from the trimer and OG monomer spectra. The 661−662 nm band is absent, the 676 nm band is less intense, and a relative increase and broadening of the 670 nm band suggests a merging of the two outer bands into a center band for plA_2 and CT monomers. In light of the foregoing discussion, this is easily explained by disruption of excitonic interactons between Chl *a* bands (for instance due to loss of some chlorophyll molecules as suggested by the fluorescence spectra), but also a change in protein environment cannt be excluded as a possible cause of the changed absorption bands. A presumed disruption of excitonic interactions is not accompanied by a change in CD. As for the 639.5 and 656 nm bands, this would imply that the corresponding transition dipoles are oriented in one plane. All monomer LD spectra show a similar shoulder near 663 nm. This indicates that the 661 nm absorption band of OG monomers is not responsible for this feature since this absorption band is absent in the absorption spectra of the other monomers.

It is of interest to relate the structural information (Kühlbrandt et al., 1994) to the spectral properties observed in the present study. The 646 and 653 nm Chl *b* bands of the trimers disappear upon monomerization (Figure 4c). As was discussed above, this can be explained by two Chl *b* molecules on different monomers that are in relatively close contact within the trimers. The most likely candidates are chlorophylls b3 and b6 that are 19 Å apart (center to center distance) within the trimer. Both pigments are located at the lumenal side of the protein. This would imply that the 639.5 nm Chl *b* band, which is absent for plA2 and CT monomers is due to one or more of the three proposed Chl *b* pigments (b1, b2, b5) at the stromal side. In this respect it is interesting to note that the N-terminal part that has been cleaved off for CT monomers, which has led to the disappearance of the 639.5 nm band, is also located at the stromal side. From a recent study on CP26 it was concluded that the strongly blue-shifted 637 nm band has very similar spectroscopic properties as the 639.5 nm band for LHC-II (van Amerongen et al., in press). It was proposed that this band is due to one Chl *b* pigment bound at the same binding site as Chl b5 (the involved amino acids are conserved in both proteins) whereas the blue-shift is caused by charged residues in the immediate environment of this pigment (notably the glutamate and arginine residues in LHC-II). Alternatively, Chl b_2 could be responsible for the 639.5 nm band since it is located close to the N-terminal 49 amino acids that have been cleaved off in CT monomers which lack the 639.5 nm band. Note that for CP26 one additional Chl *b* band was observed near 649 nm and it was preliminary assigned to a pigment bound to an identical site as Chl b3. This is also in line with the present assignment.

Table 1: Summary of Q_y Absorption Bands of LHC-II

λ^a (nm)	techniques	refer-ences	tri-mers	monomers OG	monomers plA_2	monomers CT	sign CD	sign LD
639.5	A LD CD Stark	abcde	+	+	−	−	(−)	+
647	LD CD (Stark)	(a)bce	+	−	−	−	+	−
649	A(LD) CD Stark	abcde	+	+	+	+	−	0[c],+[d]
652	LD CD	bce	+	−	−	−	−	+
656	A LD Stark	abc(d)e	−	−	−	−	(+)	−
661	A (Stark)	abc(d)e	+	+	−	−	(+)	0
668	CD	bce	+	−	−	−	+	
671	A Stark	abcde	+	+	+	+		
673	CD	ce	+	−	−	−	−	
676	A LD CD Stark	abcde	+	+	+	+	−	+
680	hole burning	f	+	?	?	?		

[a] The positions of the absorption bands have an uncertainty of approximately 1 nm. The signs of the CD or LD bands that are given between brackets or are absent are (somewhat) uncertain due to significant overlap with other bands that give more pronounced features. [b] References: (a) Krawczyk et al., 1993. (b) Hemelrijk et al., 1992. (c) Kwa et al., 1992a. (d) van der Vos et al., 1991. (e) This study. (f) Reddy et al., 1994. [c] Trimers. [d] Monomers.

In the Chl *a* absorption region the disappearance of the coupling between the 668 and 673 nm bands is observed upon monomerization. In line with the above given arguments this could be caused by disruption of interactions between pigments a4 and a5 (center to center distance, 16.4 Å) or a3 and a7 (center to center distance, 16.9 Å) located on different monomers.

The absorption bands in the Q_y region that have been identified so far at low temperature with the use of different techniques are summarized in Table 1.

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SUPPLEMENTARY MATERIAL AVAILABLE

A description is given of the absorption bands in the Q_y region that have been determined so far with the use of different techniques at low temperatures and some of their characteristics (4 pages). Ordering information is given on any current masthead page.

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