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THE DETECTION, ISOLATION AND CHARACTERIZATION OF A LIGHT-HARVESTING COMPLEX WHICH IS SPECIFICALLY ASSOCIATED WITH PHOTOSYSTEM I

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10% of the chlorophyll associated with a 'native' Photosystem (PS) I complex (110 chlorophylls/P-700) is chlorophyll (Chl) *b*. The Chl *b* is associated with a specific PS I antenna complex which we designate as LHC-I (i.e., a light-harvesting complex serving PS I). When the native PS I complex is degraded to the core complex by LHC-I extraction, there is a parallel loss of Chl *b*, fluorescence at 735 nm, together with 647 and 686 nm circular dichroism spectral properties, as well as a group of polypeptides of 24–19 kDa. In this paper we present a method by which the LHC-I complex can be dissociated from the native PS I. The isolated LHC-I contains significant amounts of Chl *b* (Chl $a/b \simeq 3.7$). The long-wavelength fluorescence at 730 nm and circular dichroism signal at 686 nm observed in native PS I are maintained in this isolated complex. This isolated fraction also contains the low molecular weight polypeptides lost in the preparation of PS I core complex. We conclude that we have isolated the PS I antenna in an intact state and discuss its in vivo function.

Introduction

The existence of a specific antenna complex closely associated with PS I has been propsed on a theoretical basis by Butler and co-workers [1,2] and on the evidence of detergent fractionation and chloroplast developmental studies by Mullet et al. [3–5]. For their functional models, Butler and co-workers termed this component Chl a_1 and assigned the 735 nm fluorescence emission at 77 K to this complex. From the structural studies of Mullet [3–5] we have adopted the term LHC-I, indicating a light-harvesting complex serving PS I.

Mullet et al. [3] isolated a 'native' PS I complex which contained 110 Chl/P-700, with an apparent Chl a/b ratio of greater than 18. This native particle was composed of several polypeptides, including the reaction center core of molecular mass 68–66 kDa [6,7]. Fluorescence emission of this complex at 77 K showed a maximum at 735 nm. This long-wavelength fluorescence emission indicated that the LHC-I (or Chl a_1) was still present. A subsequent detergent fractionation of PS I-110 yielded a complex with approx. 65 antennae Chl/P-700 and was designated PS I-65 [3]. The PS I-65 was depleted in polypeptides of 24–19 kDa, underwent a shift in the 77 K fluorescence emission to approx. 722 nm, and showed a loss of chlorophylls absorbing between 700 and 710 nm. These changes in the properties of isolated PS I were interpreted as a specific loss of the LHC-I complex [3,5].

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Abbreviations: Chl, chlorophyll; PS, photosystem; LHC, light-harvesting complex; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Although it is possible to observe the loss of LHC-I from PS I, the original treatment with Triton X-100 did not allow recovery of LHC-I as a functional or structural entity. We recognized that the purification of the LHC-I was essential to verify the association of antennae chlorophyll with the 24–19 kDa polypeptides; therefore, in this paper we describe procedures for the isolation and characterization of a functional LHC-I.

Materials and Methods

Preparation of isolated complexes

Samples of PS I-110 and PS I-65 were prepared by Triton X-100 solubilization of pea chloroplasts as described elsewhere [3]. The PS I-110 isolation was modified by the addition of 5 mM $MgCl_2$ to the grinding media in thylakoid isolation, and 0.75 mM EDTA-NaOH, pH 7.8, to the first resuspension buffer. The procedure for isolating LHC-I from PS I-110 was developed from the initial studies of Mullet et al. [5]. The procedure requires extraction of the PS I-110 preparation by charged detergents. For this extraction, purified PS I-110 collected from a sucrose gradient [3] was dialyzed overnight at 4°C against 0.05 M sorbitol to remove Triton X-100 and sucrose. The particles were then pelleted by centrifugation at 40 000 × *g* for 10 min. The particles were resuspended to a Chl concentration of 0.5 mg/ml and treated with Zwittergent-16 (2 mg/ml) and dodecyl-β-D-maltoside (1.5 mg/ml) (Calbiochem-Behring Corp., La Jolla, CA) for 60 min at 4°C with continuous stirring. The treated PS I-110 (3 ml) was loaded on a 13 ml 0.1–1.0 M sucrose density gradient containing 20 mM Tricine-NaOH (pH 7.8) and 1% dodecyl-β-D-maltoside; this gradient was cast over a 2 ml cushion of 2 M sucrose. The gradients were centrifuged 15 h at 100 000 × *g* in a Beckman SW-28 rotor at 4°C.

Determination of Chl a/b ratios

In previous papers [3–5] Chl *a*/*b* ratios of PS I preparations were determined by fluorescence assays at 77 K performed on aqueous samples extracted directly in ethanol according to the method of Boardman and Thorne [8]. Excitation of the extract was with 478 nm light and Chl *b* and *a* emissions were measured at 658 and 678 nm, respectively. Pigment ratios were determined by the following equation (from Ref. 8):

$$\text{Chl } a/b = \frac{32.5(F_{678}/F_{658} - 0.20)}{(1 - 0.079F_{678}/F_{658})}$$

In our previous studies [3,5] we did not notice the suggestion [8] that this assay is sensitive to the presence of water. In the present study, in order to obtain anhydrous samples of chlorophyll from our PS I and LHC-I fractions for fluorescence analyses, aqueous pigment-protein samples were extracted with 80% acetone, followed by a transfer of the pigments to diethyl ether using phase separation. The ether extract was then dried over anhydrous sodium sulfate in the dark before evaporating the solvent in a dry nitrogen atmosphere. The pigments were redissolved in absolute ethanol and assayed by fluorescence at 77 K. Pure preparations of Chl *a* and *b*, used for calibration of water effects, were obtained from Sigma Chemical Co. (St. Louis, MO) and dried by the procedure described above before dissolution in ethanol.

Characterization of isolated pigment-protein complexes

Isolated complexes were characterized by several optical techniques: absorption, circular dichroism (CD) and 77 K fluorescence emission spectra. Absorption and first-differential spectra were determined on a Hitachi 110 spectrophotometer, CD was measured using a Jasco JA-40 spectrometer and low-temperature fluorescence was measured on a SLM-4800 fluorescence spectrophotometer. Samples used for optical analysis contained approx. 10 μg Chl/ml; chlorophyll concentrations were measured by the method of MacKinney [9]. The polypeptide composition of the isolated complexes was determined by SDS-polyacrylamide gel electrophoresis as described by Leto and Miles [10].

Results

Chl b content of PS I-110

Mullet et al. [3] described a procedure for the isolation of a native PS I complex, which contains 110 Chl/P-700 and is termed PS I-110. It was reported that this complex had a Chl *a*/*b* ratio of

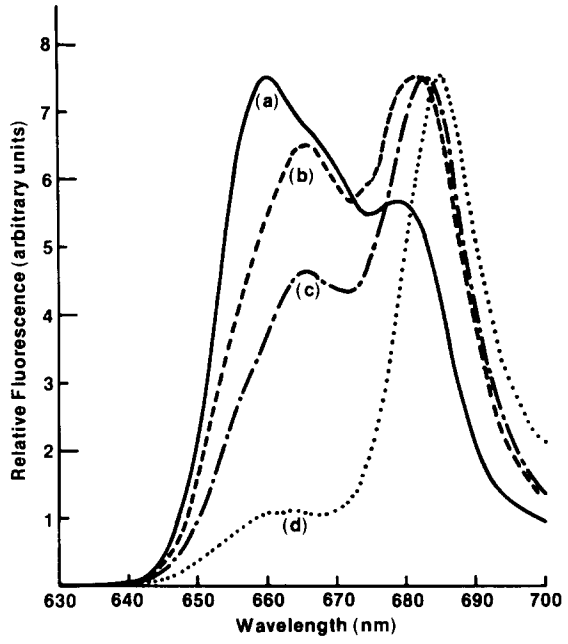

Fig. 1. The 77 K fluorescence emission spectrum of a Chl *a*/Chl *b* mixture (20:1) in: (a) 100%, (b) 97.5%, (c) 95% and (d) 90% ethanol. Excitation with 478 nm light. Spectra were corrected for phototube response, normalized and smoothed by computer.

greater than 18, as determined by low-temperature fluorescence. However, since these ratio determinations were made upon extracts of aqueous fractions and the presence of water is believed to affect this assay [8], we have reexamined the experimentally determined Chl *a*/*b* ratios.

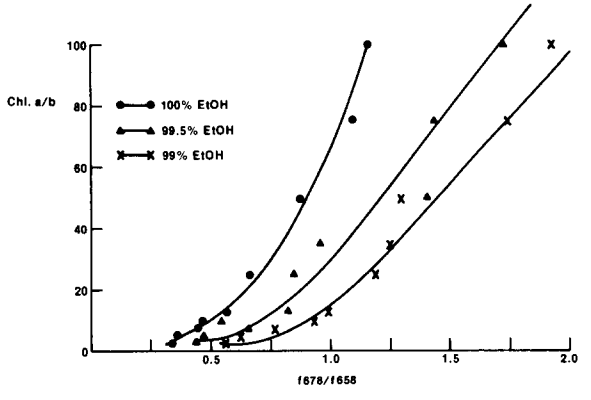

Fig. 2. Plot of Chl *a*/*b* ratio against F_{678}/F_{658}. Fluorescence at 678 nm and 658 nm was measured at 77 K, exciting beam 478 nm. Chlorophyll concentration of each sample = 25 μg/ml, path length 2 mm. Chlorophyll samples were prepared at indicated ratios from purified preparations of Chl *a* and Chl *b*.

Purified Chl *a* and *b* were mixed in absolute ethanol ± various amounts of water. Fig. 1 shows the effect of water addition on the 77 K fluorescence emission spectrum. As the proportion of water increases, the fluorescence emission at short wavelengths is depressed. This observation is confirmed by the data in Fig. 2, in which the ratio of F_{678}/F_{658} increases markedly with only 1% water contamination of the chlorophyll/ethanol solution. This fluorescence ratio is the basis for the Chl *a*/*b* ratio determinations (see equation 1) which were utilized in earlier PS I analyses [3–5]. Since those PS I preparations were not treated to remove water contamination, it seemed likely that earlier estimations of the Chl *b* content in PS I-110 were incorrect. Accordingly, we redetermined the Chl *a*/*b* ratio of a dried extracts of PS I-110, by the procedure described in Materials and Methods, and consistently obtained ratios between 9 : 1 and 10 : 1 for PS I-110 (from at least 10 separate experiments). When PS I-110 was degraded to PS I-65 by Triton X-100 extraction [3], we observed the Chl *a*/*b* ratio to increase to 15 : 1. We interpret this observation as indicating that Chl *b* is a component of PS I-110 and is rather specifically associated with the PS I antenna which is removed in the preparation of PS I-65.

Isolation of LHC-I

The fact that the measured Chl *b* content of PS I-110 was higher than that of PS I-65 suggested that the polypeptides of LHC-I bind both Chl *a* + *b*. This was investigated by developing procedures to isolate LHC-I (see Materials and Methods). Three steps were necessary. First, residual Triton X-100 had to be removed from the PS I-110 particles, since this detergent appears to be very effective in dissociating chlorophyll from protein in LHC-I when the latter is dissociated from the PS I core. Second, it was necessary to use low levels of charged detergents to dissociate LHC-I from PS I-110. Third, the dissociated LHC-I was isolated by sucrose gradient centrifugation.

The criteria by which LHC-I are defined [3] include: it has a long-wavelength (near 730 nm) fluorescence emission at 77 K, and has a polypeptide profile which includes protein species between 24 and 19 kDa but no 68–66 kDa (reaction center) polypeptides. Based upon the Chl *a*/*b*

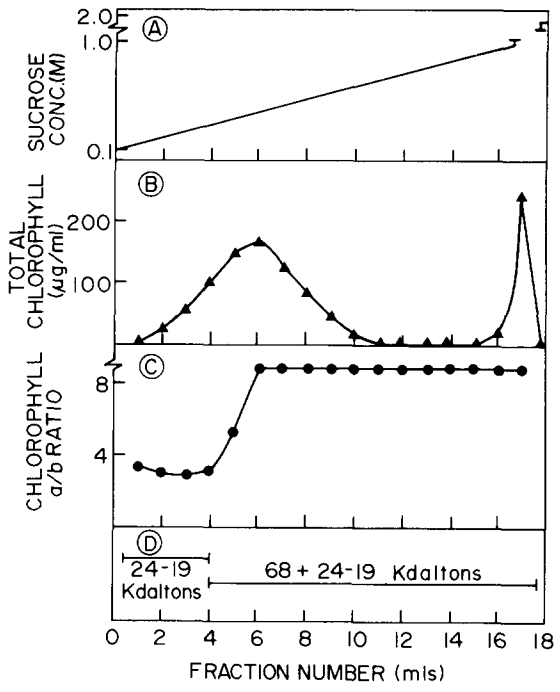

Fig. 3. Analysis of chlorophyll content in 1-ml fractions from a preparative sucrose gradient of 0.1–1.0 M sucrose (13 ml) on a 2 M sucrose cushion (2 ml) (A). Total chlorophyll content (μg/ml) (B) and Chl *a*/*b* ratio (C) are presented together with a summary of SDS-polyacrylamide gel electrophoresis analysis (D) of each fraction. The Chl *a*/*b* ratios were determined by the spectrophotometric procedure of MacKinney [9]. This assay system is unreliable for ratios greater than 8; we have therefore designated these as constant high values in C.

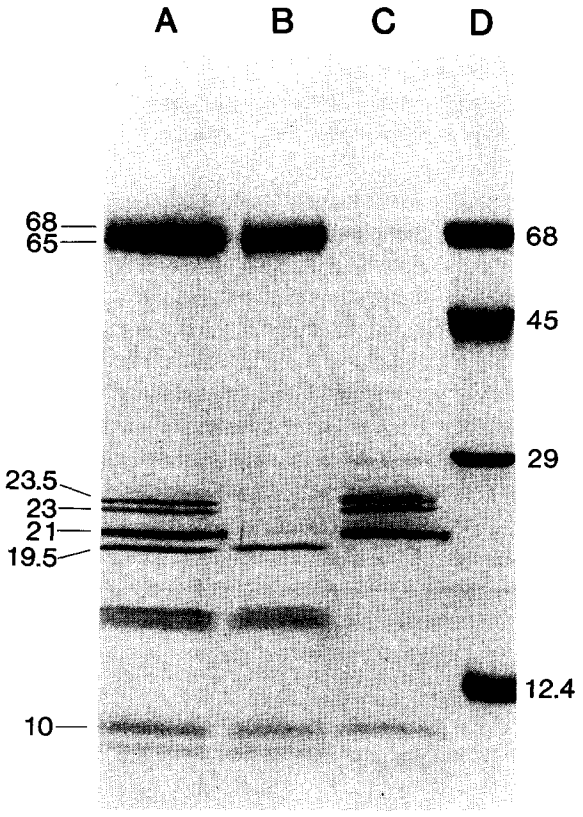

Fig. 4. SDS-polyacrylamide gel electrophoresis analysis of PS I-110, PS I-65 and LHC-I. The asterisk (*) indicates a stained band which, under different gel conditions (such as in LDS) runs as two distinct polypeptides. The apparent molecular mass (in kDa) of individual polypeptides is indicated on the left side of the figure. The molecular mass standards in lane D were bovine serum albumin, ovalbumin, carbonic anhydrase B, and cytochrome *c*.

determinations described above, the LHC-I should also be enriched in Chl *b*. We have therefore analyzed a sucrose gradient containing a solubilized PS I-110 preparation for chlorophyll and protein content. Fig. 3 presents a chlorophyll analysis (total chlorophyll and Chl *a*/*b* ratio) of 1-ml fractions from a sample sucrose gradient. Most Chl *b* was recovered in the top 5 ml of the sucrose gradient. The lower fractions (6–18) were rich in Chl *a*. We have analyzed individual fractions by SDS-polyacrylamide gel electrophoresis and have found fractions 2–5 to contain virtually only polypeptides of 24–19 kDa, whereas fractions 6–18 were enriched in the PS I reaction center polypeptides of 68–66 kDa (see Fig. 3D). Low temperature fluorescence emission spectra of the individual fractions indicated that the top 1–2 ml contained free pigment with an emission maximum at 675–680 nm. Fractions 2–5, on the other hand, exhibited fluorescence at 730 nm when excited with 440 nm light; this emission is indicative of LHC-I.

Spectral and polypeptide analysis of LHC-I

The polypeptide content of isolated PS I-110 (lane A) and PS I-65 (lane B) is shown in Fig. 4; these were isolated as previously described [3,4]. The latter are depleted of polypeptides of 24–19 kDa. These polypeptides, as well as a polypeptide of approx. 10 kDa, were recovered in the sucrose gradient fraction Nos. 2–5 (Fig. 4, lane C). In the following sections we refer to these pooled fractions as LHC-I.

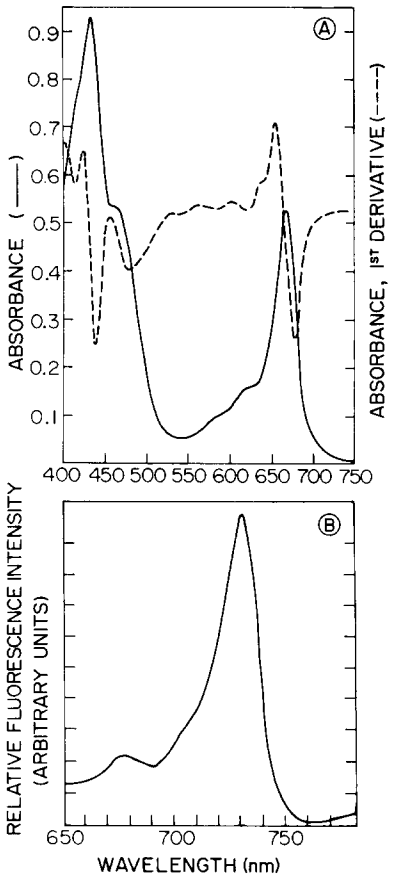

Fig. 5. Room-temperature absorption, first-differential ($\delta A/\delta\lambda$) and low-temperature (77 K) fluorescence emission spectra of isolated LHC-I.

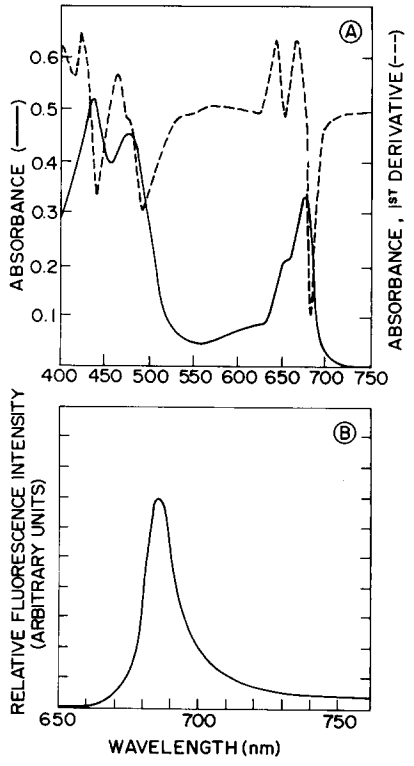

Fig. 6. Room-temperature absorption, first-differential ($\delta A/\delta\lambda$) and low-temperature (77 K) fluorescence emission spectra of isolated LHC-II.

Chlorophyll analyses of the isolated LHC-I band gave a Chl a/b ratio of 3.7 ± 0.3 (average of five analyses). This is consistent with the observation that extraction of PS I-110 to yield PS I-65 increases the Chl a/b ratio of the resultant PS I-65.

Fig. 5 shows the room-temperature absorption spectrum (and its first derivative) of the purified LHC-I. For comparison, Fig. 6 shows the spectral properties of LHC-II isolated as described by Burke et al. [11]. Although both of these antenna complexes contain significant amounts of Chl *b*, their absorption (Fig. 5A vs. Fig. 6A) and fluorescence (Fig. 5B vs. Fig. 6B) properties are markedly different. These data indicate that LHC-I and LHC-II are different complexes in the thylakoid membrane. The absorption spectrum of LHC-I (Fig. 5) does not appear to be very different from that of PS I-110 (not shown); however, the first derivative of the spectrum clearly reflects the increased proportion of Chl *b* in the LHC-I complex (cf. signal at approx. 648 nm). The low-temperature (77 K) fluorescence emission presented in Fig. 5B demonstrates that the long-wavelength Chl *a* forms of PS I-110 are preserved in isolated LHC-I. Unique chlorophyll species are also detectable by CD; the data presented in Fig. 7 are a comparison of the CD spectra of PS I-110, PS I-65 and LHC-I. There are several spectral features which change in the preparation of PS I-65 from PS I-110. The most important of these is the loss of the longest wavelength signal at 686 nm from PS I-110 which we believe to be a component of LHC-I, since it is recovered in that fraction (Fig. 7C). In addition, there is a reduced Chl *b* signal at 647 and 482 nm and some loss of carotenoid signal at 503 nm. While the long-wavelength CD feature at 686 nm is maintained in the LHC-I, no strong Chl *b* signal can be seen in this spectrum. This sensitive optical

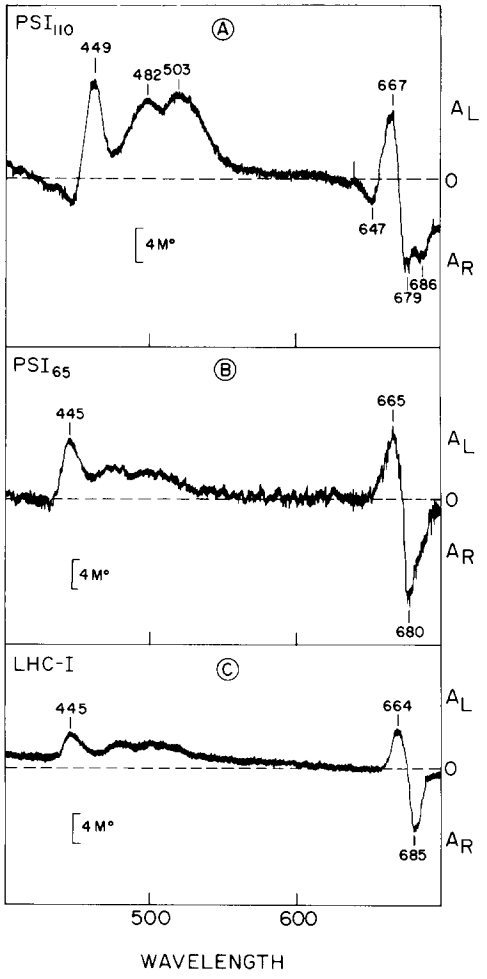

Fig. 7. Room-temperature CD spectra of PS I-110, PS I-65 and LHC-I. Chlorophyll concentration approx. 15 μg/ml, baseline determination with 10 mM Tricine-NaOH (pH 7.8).

probe provides us with a second line of evidence to show that the long-wavelength antenna of native PS I can be isolated in its intact form.

Discussion

Procedures for the preparation of PS I complexes, i.e., detergent-derived pigment-protein complexes which undergo reversible absorption changes at about 700 nm (P-700), and/or demonstrate PS I partial reactions, have been developed with wide range of compositions and properties. Early detergent fractionation studies achieved preparations with a large complement of light-harvesting chlorophyll antennae; the Chl/P-700 ratios ranged from 80:1 to 150:1 [12–14]. More recent attempts have been made to isolate 'reaction center' preparations of PS I, i.e., preparations containing a minimum Chl/P-700 ratio; these smaller complexes had Chl/P-700 ratios of 7:1 [15], 15:1 [16] to 40:1 [6]. The reaction center polypeptides of PS I can be isolated through the use of SDS or lithium dodecyl sulfate or Triton X-100. The 'P-700-Chl *a* protein' resulting from these procedures has a molecular mass of 110 kDa in a partially denatured form [7]. When run on denaturing polyacrylamide gels, as in this work, the reaction center polypeptides are of the 70–65 kDa size range [6,7,17] and bind approx. 40 Chl/P-700 [7]. These reaction center polypeptides are clearly visible in the SDS-polyacrylamide gel electrophoresis profiles of PS I-110 and PS I-65, as shown in Fig. 4 (lanes 4 and B). When isolated from chloroplast membranes via mild, uncharged detergent solubilization techniques, as in this work, PS I exists as an aggregate complex containing 13 or more polypeptides (Fig. 4, lane A). Analysis of various mutants has shown that PS I requires the concerted interaction of these multiple polypeptides for both functional expression as well as physiological stability within the membrane [18,19]. A summary of the probable role of these various polypeptides is given elsewhere [6,17,20]. It can be concluded that the native PS I, in the thylakoid membrane, exists as a structural complex of multiple polypeptides and chlorophylls.

Mullet et al. [3–5] have shown that Triton extraction of native PS I leads to a loss of chlorophylls, shown here to be both Chl *a* and Chl *b*, together with four polypeptides of 24–19 kDa. These polypeptides were hypothesized to bind the Chl $a + b$ and compose the light-harvesting complex serving as antenna for PS I (LHC-I). The information presented in this study shows that the LHC-I can be isolated, and is distinctly different from the previous characterized LHC-II [11,21].

We present two lines of evidence to demonstrate that the pigment organization of LHC-I is maintained in the isolated material; these include 77 K fluorescence emission and CD spectra. CD is the differential absorption of right- and left-handed circularly polarized light and is an indicator of chlorophyll-chlorophyll and/or chlorophyll-pro-

tein interaction. In this paper we observe that a characteristic CD feature, i.e., a signal at 685–686 nm, is maintained in both the PS I-110 complex and isolated LHC-I (Fig. 7A and C), suggesting that these pigments of LHC-I are not substantially disrupted by the detergent solubilization. Similarly, the 730 nm Chl *a* fluorescence peak at 77 K, which is diagnostic of PS I antennae, is maintained in LHC-I (Fig. 5B). Neither the long-wavelength CD signal nor the long-wavelength fluorescence emission is observed in the PS I-65 core complex (i.e., the PS I from which LHC-I was removed).

Wollman and Bennoun [22] have recently reported the existence of an isolatable pigment-protein complex, CP O, which is part of the PS I antenna in *Chlamydomonas*. As with the LHC-I, the absorption spectrum of CP O contains proportionally more Chl *b* and carotenoid [23] than PS I. Wollman and Bennoun [22] report a Chl *a*/*b* ratio of 6.3 for CP O. CP O is also a long-wavelength fluorescent species, with an emission at 720 nm at 77 K and is composed of several, relatively low (28–19 kDa) molecular mass polypeptides. Thus, it appears that the CP O isolated from *Chlamydomons reinhardii* is analogous in composition to that of LHC-I isolated from peas.

Wollman and Bennoun [22] have suggested that CP O could be directly involved in Mg^{2+} regulation of energy distribution. This is in agreement with an earlier speculation of Haworth et al. [23] who observed that fluorescence from PS I-110, but not PS I-65, was cation sensitive. Yamamoto and Ke [24] have also reported cation-sensitive fluorescence from isolated PS I fragments (TSF-I). A second possible in vivo function of the LHC-I could be deduced by comparison with purple photosynthetic bacteria. Many of these contain a small antenna component (usually less than 5% total pigment) which is tightly associated with the reaction center [25,26]. This specialized component usually absorbs and fluoresces at longer wavelengths than the bacterial reaction center itself [25–27]. Although light absorbed by this specialized antenna complex is of lower energy than the reaction center trap, it has been demonstrated that 'uphill' energy transfer to the reaction center does occur [26,27]. Indeed, it is believed that all exciton energy transfer from the major antenna complexes to the reaction center is mediated through this minor, long-wavelength antenna form. It is thought that the existence of this antenna, closely associated with the reaction center, may protect the trap from photodestructive processes [26,27]. Although the reaction center of PS I is considerably different from that of purple photosynthetic bacteria, we suggest that LHC-I serves a similar function. We have observed, during the course of this investigation, that the room-temperature fluorescence from LHC-I is of very low yield, unlike purified LHC-II [11]. This may be indicative of an efficient internal conversion process within this complex, which is consistent with the hypothesized role of a photoprotective trap.

As a last point, it is interesting to note that recent studies by Mullet et al. [28] have found that the polypeptides of LHC-I are synthesized in the cytoplasm as larger size-class precursor proteins. These are taken up and processed to mature form by isolated intact chloroplasts. These observations are essentially identical to the pattern of synthesis of the polypeptides of LHC-II [29]. It appears that the evolutionary strategy by which the light-harvesting antennae size was increased for both PS I and II was very similar; this includes the incorporation of both Chl *a* and *b* as well as polypeptides of similar size. It shall be of interest to see if any homologies in primary structure of the polypeptides of LHC-I and LHC-II are discovered.

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