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CHLOROPHYLL-PROTEINS OF TWO PHOTOSYSTEM I PREPARATIONS FROM MAIZE

by

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Two photosystem I (PSI) preparations were purified by non-denaturing SDS-PAGE or sucrose gradient ultracentrifugation and examined as to their chlorophyll-protein composition. In both preparations a minimum of two chlorophyll-proteins can be distinguished in addition to the 110 kD *P*-700 Chl_a-P1 complex. One (LHCI-730) is a chlorophyll-protein (Mr 40 kD) having a high chlorophyll *a/b* ratio, and a major 77 K fluorescence peak at 730 nm. It consists of three polypeptides with apparent molecular weights of 21, 22.5 and 24 kD. Another chlorophyll-protein (LHCI-680) with a lower molecular weight (Mr 25 kD) fluoresces at 77 K with a maximum at 680 nm. This chlorophyll-protein has a high chlorophyll *b* content and two constituent polypeptides of 20 and 25 kD. Absorption, 77 K fluorescence and circular dichroism spectra of the PSI related chlorophyll-proteins are presented and compared with those of photosystem II chlorophyll-proteins from maize thylakoids. We propose a model for energy transfer in the photosystem I reaction centre with the following sequence: LHCI-730 → LHCI-680 → Chl_a-P1. LHCI-680 acts as a connecting antenna which can also transfer energy from $Chl_{a/b}$-P2. This model was used to interpret the 77 K fluorescence emission from two barley mutants.

1. INTRODUCTION

In 1975 THORNBER reported the isolation of two chlorophyll-proteins by SDS-PAGE: CPI and CPII (39). CPI (Chl_a-P1) binds only chlorophyll *a* and has been identified as the photosystem I (PSI) reaction centre (3). CPII ($Chl_{a/b}$-P2) binds both chlorophyll *a* and *b*. It is the major light-harvesting protein and is mainly associated with photosystem II (PSII) in the appressed regions of grana thylakoids.

Improvement of analytical methods led initially to the detection of two other less stable chlorophyll-proteins which bind only chlorophyll *a* and which are associated with the PSII reaction centre: Chl_a-P2 and Chl_a-P3 (2, 7, 14, 29). Chlorophyll *a/b*-binding chlorophyll-

Abbreviations: CD = circular dichroism; LHCI = light-harvesting complex of photosystem I; LHCII = light-harvesting complex of photosystem II; PAGE = polyacrylamide gel electrophoresis; PS = photosystem ; SDS = sodium dodecylsulphate; tricine = N-(tris(hydroxymethyl)methyl) glycine; Tris = tris-(hydroxymethyl) amino methane.

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proteins, described as either oligomers of CPII (2, 7, 29) or as a new chlorophyll *a/b* complex (28, 29), were also resolved.

In the last few years the presence of another chlorophyll *a/b*-binding, light-harvesting chlorophyll-protein associated with PSI (LHCI), has been reported (21-24, 33, 36, 41) which has a high chlorophyll *a/b* ratio and apoproteins in the 20-25 kD molecular weight range. In a previous paper (11), the chlorophyll-proteins of bundle sheath and mesophyll plastids of maize were examined and a similar chlorophyll *a/b*-binding chlorophyll-protein was shown to be present in both types of plastids. The holoprotein had a relative molecular weight of about 40 kD and after re-electrophoresis under denaturing conditions was shown to contain three polypeptides with molecular weights of 21, 22.5 and 24 kD. This chlorophyll-protein has been successfully isolated in pure form by mild SDS-PAGE of bundle sheath thylakoids (11). Contamination by Chl_a-P2 and Chl_a-P3 which have similar electrophoretic mobilities is avoided, because they are both absent from maize bundle sheath chloroplasts (12). The chlorophyll-protein designated $Chl_{a/b}$-P3 in (11) according to the terminology of (29) fluoresces with a major peak at 730 nm at 77 K, has a red absorption peak at 674 nm, and is a light-harvesting chlorophyll-protein of PSI (also called LHCI).

In this study we have examined two PSI preparations isolated by two independent methods which preserve, as much as possible, the native conditions of the complex as determined by *P*-700 activity and low temperature fluorescence emission spectra. Chl_a-P1* was isolated by non-denaturing SDS-PAGE of octylglucoside-solubilized thylakoids (11, 12), and a second PSI preparation was isolated by sucrose gradient ultracentrifugation of Triton X-100 solubilized thylakoids as described by MULLET et al. (30). The chlorophyll-proteins of these preparations have been separated by SDS-PAGE under conditions which avoid the dissociation of chlorophyll-protein complexes into their component polypeptides. The chlorophyll-proteins were isolated from gels and analyzed for their polypeptide composition by SDS-PAGE in the presence of 6 M-urea and by spectroscopy. We conclude that the PSI complex contains two chlorophyll *a/b*-proteins which differ from the main light-harvesting complex of PSII.

In this paper we have reported, for the first time, the circular dichroism (CD) spectra of all the chlorophyll-proteins that can currently be resolved from thylakoids. CD is the differential absorption of right- and left-handed circularly polarized light and is an indicator of pigment-pigment and/or pigment-protein interactions important in the mutual orientation of the chromophores in the protein. This technique appears particularly promising in chlorophyll-protein studies because of its ability to distinguish between pigment species with similar absorption properties but different orientations.

2. MATERIALS AND METHODS

2.1. Thylakoids

Two weeks old, greenhouse-grown maize plants (Zea mays L.) were homogenized in 0.1 M-tricine/NaOH pH 7.8, 0.4 M-sorbitol. The slurry was filtered through two layers of 20 μm nylon net and pelletted at 5000 × g for 5 min. The pellet was washed twice with 10 mM-sodium pyrophosphate pH 7.4, then with 2 mM-tricine/NaOH pH 7.8. The washed thylakoids were resuspended at a chlorophyll concentration of 1 $mg \cdot ml^{-1}$ in 10% glycerol, 65 mM-Tris/HCl pH 6.8, 5 mM-dithiothreitol.

Membranes from bundle sheath thylakoids were prepared as previously described (12).

2.2. PSI preparation by sucrose gradient ultracentrifugation

A native PSI preparation was prepared by gradient ultracentrifugation of Triton X-100 solubilized thylakoids as described in (30), with the exception that 0.6% Triton X-100 instead of 0.7% was used in the solubilization step.

An antenna I-depleted PSI particle was prepared by dialyzing the native PSI preparation for 3 hrs against 5 mM-tricine pH 8.0, 150 mM-NaCl and then adding dodecylmaltoside to 1%. The PSI particles were recovered as an high mobility, non-fluorescent, apple-green band in the lower 2/3 of the tube, after 18 hrs sucrose

gradient ultracentrifugation at 40,000 × g in a Beckman SW 40 rotor at 4 °C. The gradient was 0.1 to 1 M-sucrose in 5 mM-tricine pH 8.0 including 1% dodecylmaltoside. The PSI-containing band was recovered with a syringe and used immediately or was frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.3. Electrophoresis

Solubilization of thylakoids with octylglucoside and non-denaturing, tube SDS-PAGE was carried out as previously described (11, 12). The Chl_a-P1* containing band was excised from the gel and incubated for 10 min at 0 °C in 150 mM-NaCl and loaded onto an identical gel tube with the exception that 150 mM-NaCl was included in the stacking gel. Gels were run for 2.5 hrs at 25 mA/18 tubes at 4 °C or lower. For re-electrophoresis, gels were not prerun. Green bands were excised from the second gel and used immediately for elution or frozen in liquid nitrogen until further analysis.

The PSI preparation (section 2.2) was dialyzed for 3 hrs against 150 mM-NaCl, 5mM-tricine pH 8.0 and concentrated to 0.5 mg chl · ml^{-1} in an Amicon ultrafiltration cell at 4 °C. 25 µl of the concentrated PSI suspension was loaded onto a tube gel with NaCl in the stacking gel as described above. Denaturing SDS-PAGE in the presence of 6 M-urea was as described (11). Apparent molecular weights were obtained by comparison with the following standards: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD), α-lactosidase (14.4 kD).

2.4. Spectroscopy

For absorption and circular dichroism spectroscopy, gel slices were ground in a small volume of glass distilled water and incubated overnight at 4 °C. Gel debris were removed by filtering through 20 µm nylon net and centrifuging 10 min in an Eppendorf microfuge. Absorption and circular dichroism spectra were taken as previously described (11) with the exception that a 10 mm optical pathlength, quartz cuvette was used instead of a 2 mm one, because of the low concentration of some of the samples eluted from gels. The accuracy of the wavelength calibration was checked at 490 ± 1 nm with $[Co(CN)_3]^{3+}$.

Fluorescence emission spectra at 77 K were taken directly from gel slices as described (11).

2.4. Other

Chlorophyll concentration and chlorophyll *a/b* ratios were determined as described by ARNON (9).

3. RESULTS

Table I presents an overview of the names and functions of the green bands obtained by electrophoresis after solubilization with the non-ionic detergent octyl β,D-glucopyranoside. The bands are listed in order of increasing electrophoretic mobility. For each green band, the subunit composition determined from re-electrophoresis, and Coomassie blue-staining polypeptide composition with apparent molecular weights obtained in this study are given in the last two columns of the table.

3.1. Preparation and fractionation of Chl_a-P1*

Solubilization of thylakoids with low amounts of the non-ionic detergent octyl β,D-glucopyranoside (detergent:chlorophyll = 20:1 w/w) in low concentration buffer yielded a high amount of chlorophyll associated with Chl_a-P1* (Figure 1A). In some experiments almost all of the chlorophyll associated with PSI was found in Chl_a-P1*, while the amount of Chl_a-P1 and $Chl_{a/b}$-P3 was very much decreased. The Chl_a-P1* band excised from the gel showed 730 nm fluorescence emission at 77 K (Figure 2). Its absorption spectrum showed shoulders at 472 and 480 nm, indicating the presence of both chlorophyll *b* and carotenoids. These features were particularly evident if the Chl_a-P1* and Chl_a-P1 spectra were compared (Figure 3), the latter showing no chlorophyll *b* contribution. The polypeptide composition of the Chl_a-P1* is shown in Figure 4B, track 8. The pattern included the 110 kD reaction centre of PSI, five

Table I. Overview of chlorophyll-protein bands obtainable from maize thylakoids.

Green bands	Function	Composition	Polypeptide content (Mr in kD)
Chl_a-P1* = CP1a (29)	Photosystem I	Chl_a-P1 + LHCI-680 +	67, 25, 24, 22.5, 21, 20.5
		LHCI-730 + 12 polypeptides	12 polypeptides between 19 & 5
Chl_a-P1 = CP1 (29)	Photosystem I reaction centre	Chl_a-P1	67
$Chl_{a/b}$-P2** = LHCP1 (29)	Photosystem II light-harvesting	$Chl_{a/b}$-P2 + 1 polypeptide	29, 28, 27, 7.8
Chl_a-P2 CPa (29)	Photosystem II	Chl_a-P2	50
Chl_a-P3	reaction centre	Chl_a-P3	43
$Chl_{a/b}$-P3 = LHCI-730 (11)	Photosystem I light-harvesting	LHCI-730	24, 22.5, 21
$Chl_{a/b}$-P1 (29)	Photosystem II antenna	$Chl_{a/b}$-P1	29.5
$Chl_{a/b}$-P2 = LHCP3 (29)	Photosystem I + II light-harvesting	$Chl_{a/b}$-P2	29, 28, 27
$Chl_{a/b}$-P4 = LHCI-680	Photosystem I connecting antenna	LHCI-680	25, 20.5
Free pigment (FP)	-	-	-

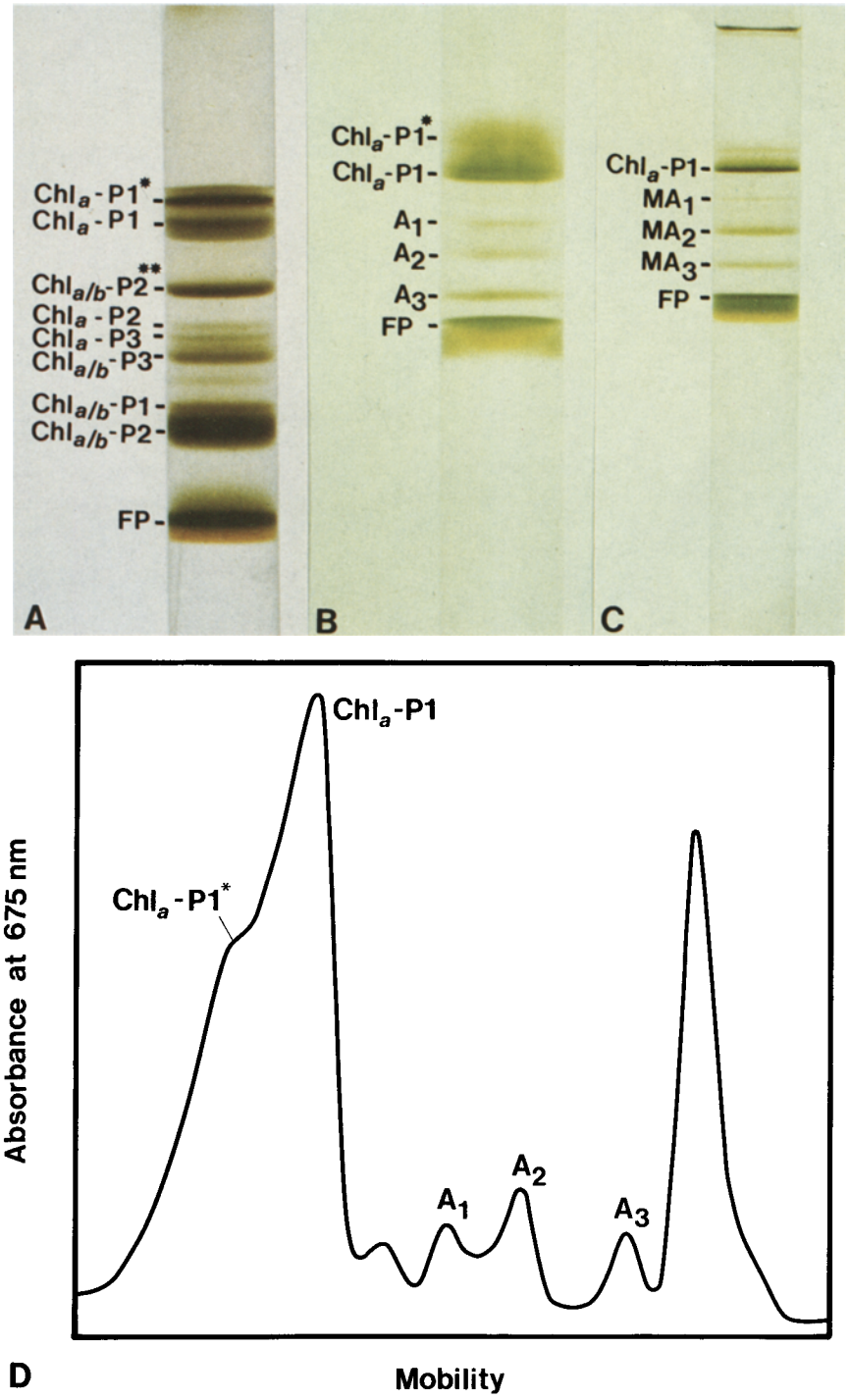

Figure 1. A: Non-denaturing SDS-PAGE of the chlorophyll-proteins from maize thylakoids. The designations and composition of the green bands are given in Table I. B: The uppermost band from (A) was cut from the gel and re-run in mildly dissociating conditions (150 mM-NaCl in the stacking gel). C: Mildly dissociating SDS-PAGE of PSI-110 particles. D: densitometer tracing of the gel in (B). A1 = dimer of A2: A2, MA2 = LHCI-730: A3, MA3 = LHCI-680.

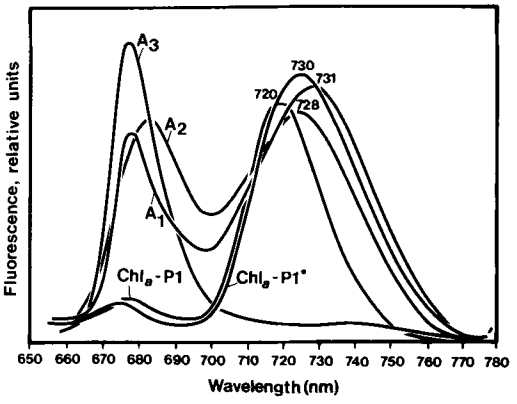

Figure 2. In situ 77 K fluorescence emission spectra of Chl$_a$-P1* and of the chlorophyll-proteins obtained by re-electrophoresis (Figure 1B). Fluorescence was excited with a broad band blue light. The slit width for emission was 5 nm.

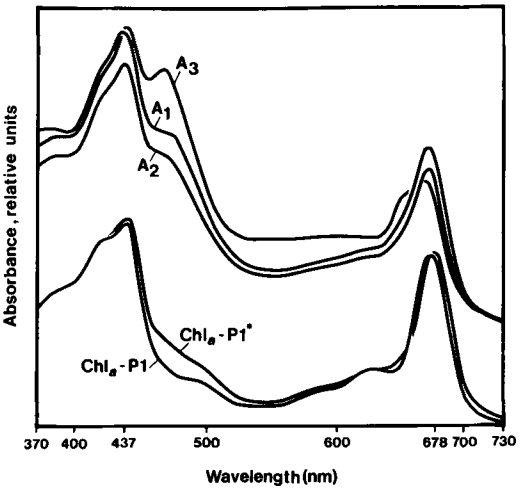

Figure 3. Room temperature absorption spectra of Chl$_a$-P1* and of the chlorophyll-proteins obtained from re-electrophoresis of Chl$_a$-P1* in mildly dissociating conditions, as in Figure 1B. Chlorophyll-proteins were eluted from gel slices with glass distilled water overnight at 4 °C, and spectra were obtained with an Aminco DW2a spectrophotometer. The slit width was 2 nm, pathlength 10 mm.

polypeptides with molecular weights between 20 and 25 kD and seven other polypeptides with lower molecular weights.

When the Chl$_a$-P1* band was excised, soaked 15 min in 150 mM-NaCl, and re-run in a tube gel with 150 mM-NaCl in the stacking gel, four green bands were present, plus the free pigment band at the front (Figure 1B). The second highest molecular weight band was identified as Chl$_a$-P1 from its 720 nm emission at 77 K, and the exclusive presence of chlorophyll *a* with a 678 nm red peak in the absorption spectrum. It contained only the 110 kD PSI reaction centre protein, which has a 67 kD apoprotein.

The two green bands with mobilities corresponding to about 60 kD (A1) and 40 kD (A2), respectively, had very similar 77 K fluorescence emission spectra with maxima at 731 and 728 nm (Figure 2). Their absorption spectra were identical and revealed a higher chlorophyll *b* concentration than that of the starting material (Chl$_a$-P1*) (Figure 3). SDS-PAGE in the presence of 6 M-urea, showed that the A1 and A2 bands both contained three polypeptides with molecular weights of 24, 22.5 and 21 kD (bands B, C and D, respectively, in Figure 4A, tracks 3, 4). It is likely, therefore, that A1 is a dimer of A2. The fact that the apparent molecular weight of A1 is not twice that of A2 is not surprising because of the known anomalous migration of chlorophyll-proteins in in SDS-PAGE (18). In view of the large differences in apparent molecular weights of these chlorophyll-proteins and their component polypeptides it is clear that all three polypeptides are present in the native complex. It is not obvious whether only one or all three bind chlorophyll.

The green band with the highest mobility (A3) had a 77 K fluorescence emission maximum at 680 nm and an absorption spectrum (Figure 3) showing a very high chlorophyll *b* content (chlorophyll *a/b* ratio of about 1). The interpretation of its polypeptide composition was complex because the short distance between this green band and the free pigment band at the front made it difficult to excise the chlorophyll-protein band without contamination from low molecular weight polypeptides migrating near the front. The green A3 band contained two major polypeptides with molecular weights of 25 and 20 kD (bands A and E, respectively, Figure 4A, track 5), neither of which was present in the green A1 and A2 bands, as well as other low molecular weight polypeptides that were also

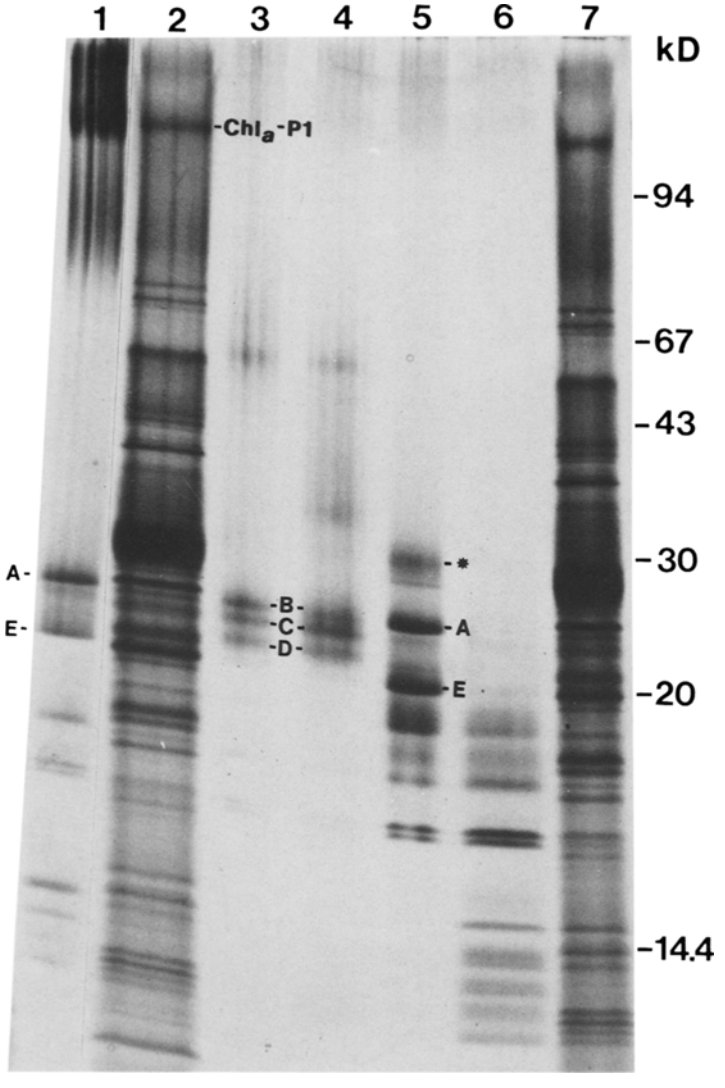

Figure 4A. SDS-PAGE in the presence of 6 M-urea of chlorophyll-proteins excised from tube gels (Figure 1B). Track 1: Chl$_a$-P1*. Track 2: thylakoids. Track 3: A1 band (dimer of A2). Track 4: A2 band (LHCI-730). Track 5: A3 band (LHCI-680). Track 6: FP band. Track 7: thylakoids. The uppermost band in track 5 (*) is considered a contaminant as it was not present in other experiments.

present in the electrophoresis of the free pigment band (Figure 4A, track 6). The similarity in the molecular weights of the A3 holoprotein and the two polypeptides found after re-electrophoresis of the excised gel band may mean that one of the polypeptides is a contaminant.

It is thus clear on the basis of electrophoretic mobility, chlorophyll *a/b* ratio, polypeptide composition and 77 K fluorescence, that there are two different light-harvesting chlorophyll *a/b*-proteins associated with photosystem I. These are named Chl$_{a/b}$-P3 and Chl$_{a/b}$-P4 (Table I) after the nomenclature system of (29), but for clarity will be referred to as LHCI-730 and LHCI-680 in this paper. The number refers to the wavelength of the 77 K fluorescence emission peak.

In some experiments an additional green

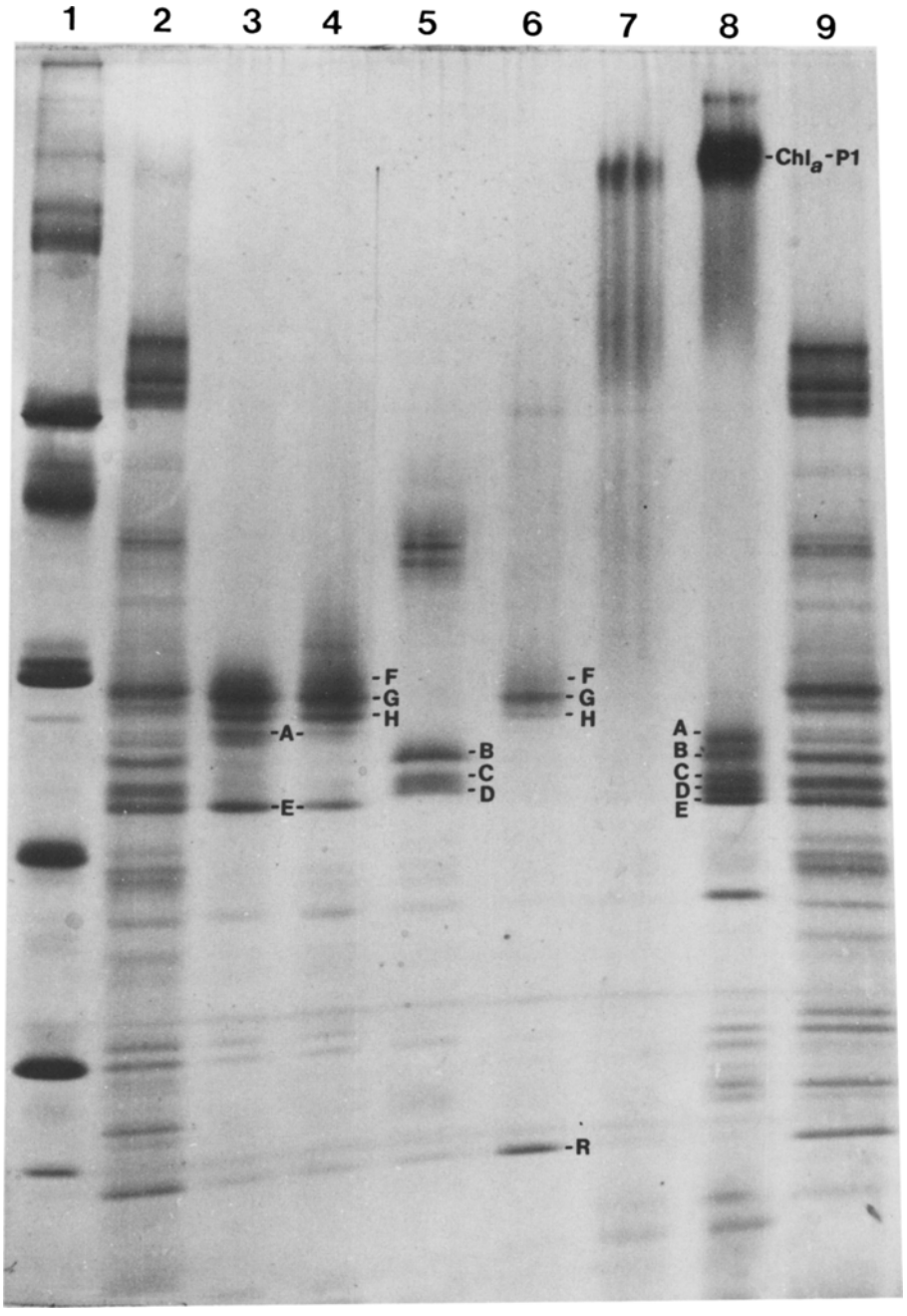

Figure 4B. SDS-PAGE in the presence of 6 M-urea of chlorophyll-proteins obtained from bundle sheath membranes by non-denaturing SDS-PAGE. Track 1: molecular weight markers. Track 2: bundle sheath thylakoids. Tracks 3 and 4: Chl$_{a/b}$-P2 + Chl$_{a/b}$-P4 (LHCI-680). Track 5: Chl$_{a/b}$-P3 (LHCI-730). Track 6: Chl$_{a/b}$-P2**. Track 7: Chl$_a$-P1. Track 8: Chl$_a$-P1*. Track 9: bundle sheath thylakoids.

band having a mobility between Chl_a-P1 and A1 was seen. This band had a fluorescence emission peak at 732 nm and an absorption spectrum and polypeptide composition identical to those of the A1 and A2 bands. This confirms the tendency of this chlorophyll-protein to be found as higher molecular weight forms. When gel slices from the free pigment front were re-electrophoresed in a urea gel system, the low molecular weight polypeptides of Chl_a-P1* were separated. Twelve bands with molecular weights between 19 and 5 kD were resolved (Figure 4A, track 6), indicating that the polypeptides of Chl_a-P1* were more numerous than shown in Figure 4B, track 8.

A small amount of Chl_a-P1* was still present after re-electrophoresis (Figure 1B and 1D). When this was re-run under denaturing conditions and compared with the starting material (Figure 4A, track 1; cf Figure 4B, track 8), a specific decrease was observed in the amounts of polypeptides B, C and D of LHCI-730 (= green bands A1 and A2). The 25 and 20 kD polypeptides (A and E) of LHCI-680 were apparently more strongly bound to the Chl_a-P1 reaction centre.

3.2. Fractionation of PSI particles from sucrose gradient ultracentrifugation (prepared according to MULLET et al. (30)).

The PSI preparation from sucrose gradient ultracentrifugation of Triton X-100 solubilized thylakoids showed characteristics similar to Chl_a-P1* in respect to 77 K fluorescence emission (730 nm peak) (Figure 5) and absorption spectrum (Figure 6). The chlorophyll *a/b* ratio was 5.5. When this preparation was electrophoresed under mildly dissociating conditions as described for Chl_a-P1*, there were four green bands as well as the yellow-green free pigment front (Figure 1C). The pattern was thus similar to that after the re-electrophoresis of Chl_a-P1*, although the second band in order of mobility (MA1) was much fainter than the A1 band.

The highest molecular weight band was the same as Chl_a-P1 with respect to 77 K fluorescence emission, absorption spectrum and polypeptide composition. The second band

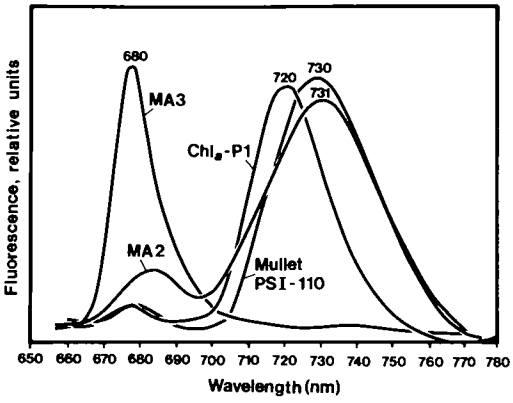

Figure 5. In situ 77 K fluorescence emission spectra of chlorophyll-proteins resolved after electrophoresis of the PSI-110 particles.

(MA1) fluoresced with a peak at 680 nm, its absorption spectrum showed only chlorophyll *a* and it contained a single protein with a molecular weight of 67 kD. In addition, the eluted band showed no optical activity when examined for circular dichroism, which was at variance with the properties of the A1 band from Chl_a-P1* (see below). This indicates that the chlorophyll was randomly organized, so that the MA1 band has no properties in common with the A1 band.

The third green band (MA2) was very similar to the A2 band (LHCI-730), with 730 nm fluorescence at 77 K and an absorption spectrum revealing the presence of both chlorophyll *a* and *b* contributions. Three polypeptides

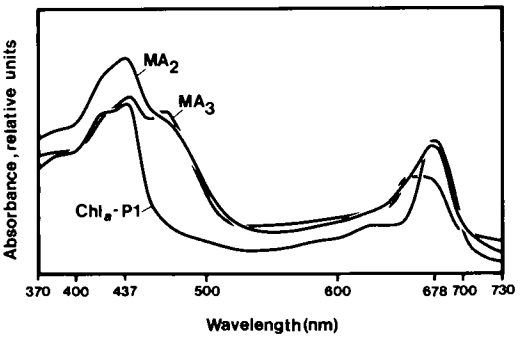

Figure 6. Absorption spectra of the chlorophyll-proteins eluted after electrophoresis of the PSI-110 preparation in mildly dissociating conditions.

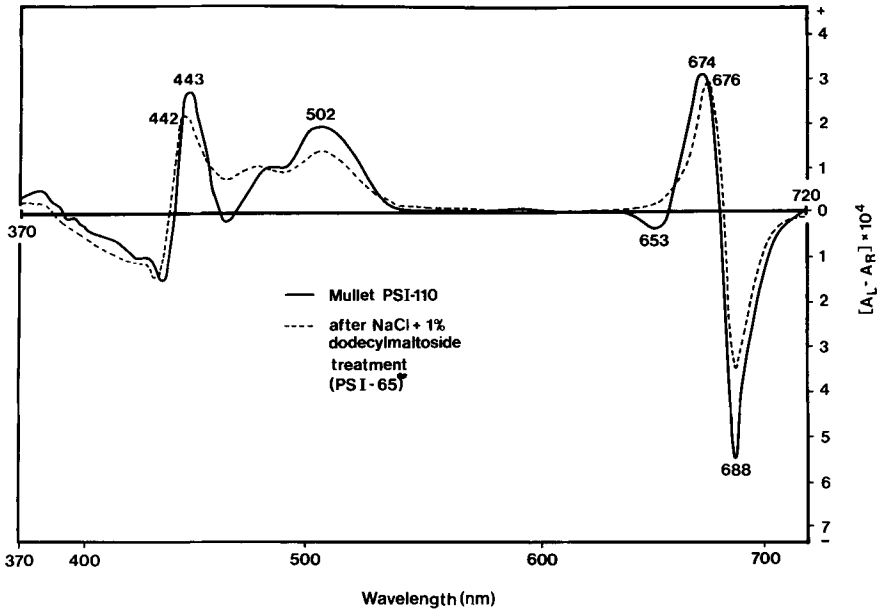

Figure 7. Circular dichroism spectra of the PSI-110 preparation before and after depletion of the antenna I by dialysis against NaCl and treatment with dodecylmaltoside. Slit width was 0.5 nm. The chlorophyll concentration was 20 $\mu g \cdot ml^{-1}$, pathlength 10 mm.

of 21, 22.5 and 24 kD were present in this band. In addition two other polypeptides of 38 and 42 kD were found. The fourth green band (MA3) was identical to the A3 band (LHCI-680) from Chl_a-P1* with respect to 77 K fluorescence emission, absorption and polypeptide composition.

When chlorophyll-proteins of bundle sheath thylakoids were separated by mild SDS-PAGE and the excised gel slices containing chlorophyll-proteins were re-run in the presence of 6 M-urea (11), the $Chl_{a/b}$-P3 band had the spectral characteristics of LHCI-730, i.e., those of A1, A2 and MA2, and also consisted of three polypeptides of 21, 22.5 and 24 kD (Figure 4B, track 5). We conclude that the green bands A2, MA2 and $Chl_{a/b}$-P3 represent the same chlorophyll-protein, A1 being a oligomeric form.

3.3. Circular dichroism of chlorophyll-proteins separated by non-denaturing SDS-PAGE from maize thylakoids and PSI preparations

CD spectra in the visible region have been reported from $Chl_{a/b}$-P2 preparations (37). More recently, CD spectra have been described for various PSI preparations (21) and for LHCI (21, 24). One of the interesting features of the published spectra was a negative peak at 647 nm from the native PSI preparation that appeared to be indicative of the presence of chlorophyll *b*. In order to check this assignment, we have examined different native and LHCI depleted PSI preparations, as well as the other PSI-associated chlorophyll-proteins. In addition, we have compared these spectra with those of other chlorophyll-proteins isolated from maize thylakoids by mild SDS-PAGE, to determine if a common pigment organization could be recognized in the light-harvesting chlorophyll-proteins compared with the reaction centre ones. Absorption and fluorescence emission spectra of the chlorophyll-proteins from maize thylakoids as well as their polypeptide composition have been published (11).

In Figure 7, CD spectra of PSI prepared by gradient ultracentrifugation are shown before and after treatment with NaCl and dodecylmaltoside. The antenna-depleted preparation fluoresced at 720 nm at 77 K and contained 5

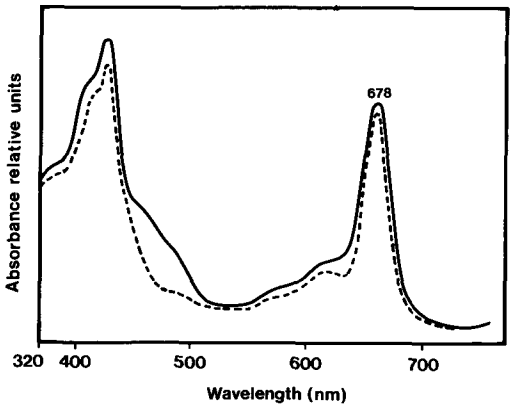

Figure 8. Absorption spectra of the PSI-110 preparation before and after depletion of the antenna I.

polypeptides of 110, 32, 19 kD and a doublet at about 17 kD exactly corresponding to PSI-65 (30). From the absorption spectrum (Figure 8), the chlorophyll *b* contribution was not present and the polypeptides in the 20-25 kD range were absent from the preparation.

The main differences between the native and antenna-depleted preparations were the disappearance of a negative signal at 653 nm and a reduction of the intensity of another one at 688 nm. Another reproducible feature was a small shift in opposite directions of the two positive peaks at 442 and 675 nm. They were also narrower, as if a component with a maximum near the main signal were removed. Very similar results were obtained when the CD spectra of Chl_a-P1* and Chl_a-P1 were examined (Figure 9). The Chl_a-P1* preparation was very similar to the PSI from gradient ultracentrifugation, while the Chl_a-P1 was much simpler in composition compared with the antenna-depleted gradient fraction since it only contained the 110 kD PSI reaction centre protein. In both antenna-depleted preparations, CD signals were decreased in intensity but still present at 480 and 503 nm, indicating the presence of carotenoids in the PSI reaction centre.

When the 730 nm fluorescing complexes, namely A1 and A2 from Chl_a-P1* and MA2 from the PSI-110 preparation, were eluted from the gel and examined for CD, all had the same spectra, as exemplified by MA2 (Figure 10). The spectra obtained from the 680 nm fluorescing chlorophyll-proteins (A3 and MA3) were identi-

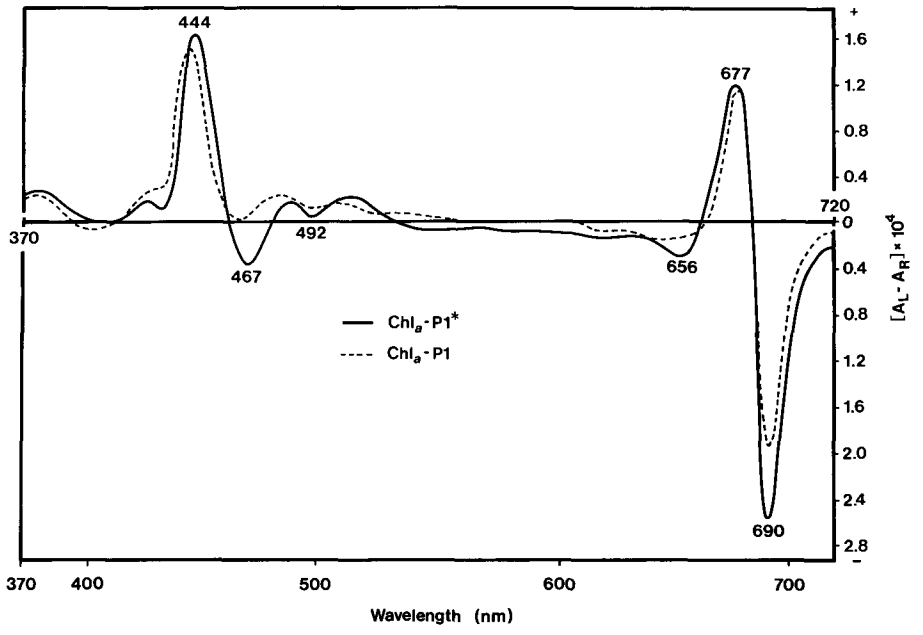

Figure 9. Circular dichroism spectra of the eluted Chl_a-P1* and Chl_a-P1 bands. Chlorophyll concentration was 10 $\mu g \cdot ml^{-1}$, slit width = 1 nm.

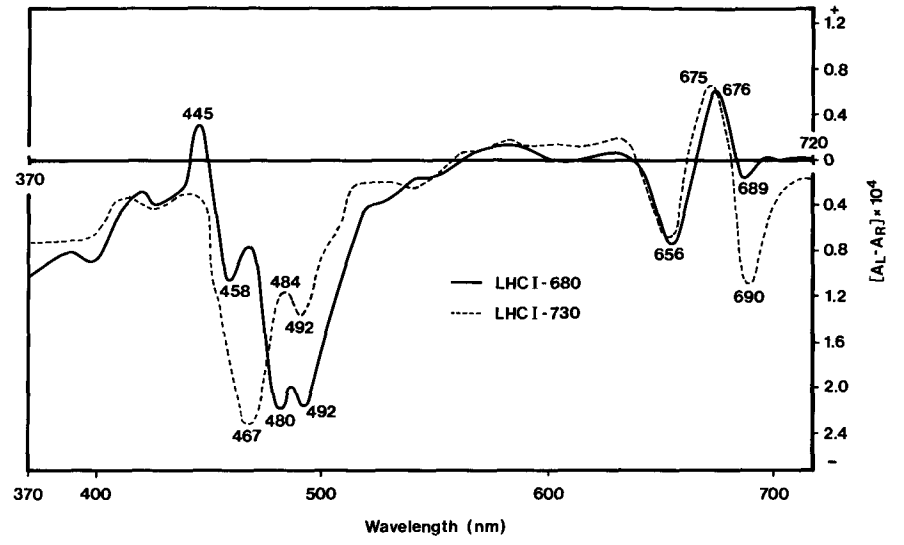

Figure 10. Circular dichroism spectra of LHCI-680 (MA3) and LHCI-730 (MA2). Bands A1, A2 and MA2 had the same spectra (LHCI-730); bands A3 and MA3 had the same spectrum as LHCI-680. Chlorophyll concentration was 10 μg · ml^{-1}, slit width = 1 nm.

cal to each other (Figure 10). The CD spectra from LHCI-680 and LHCI-730 were drastically different from those of the PSI reaction centre-containing preparations, mainly because of the stronger optical activity in the 450-500 nm spectral region. The main difference between LHCI-680 and LHCI-730 was in the 689-690 nm negative signal which was much smaller in

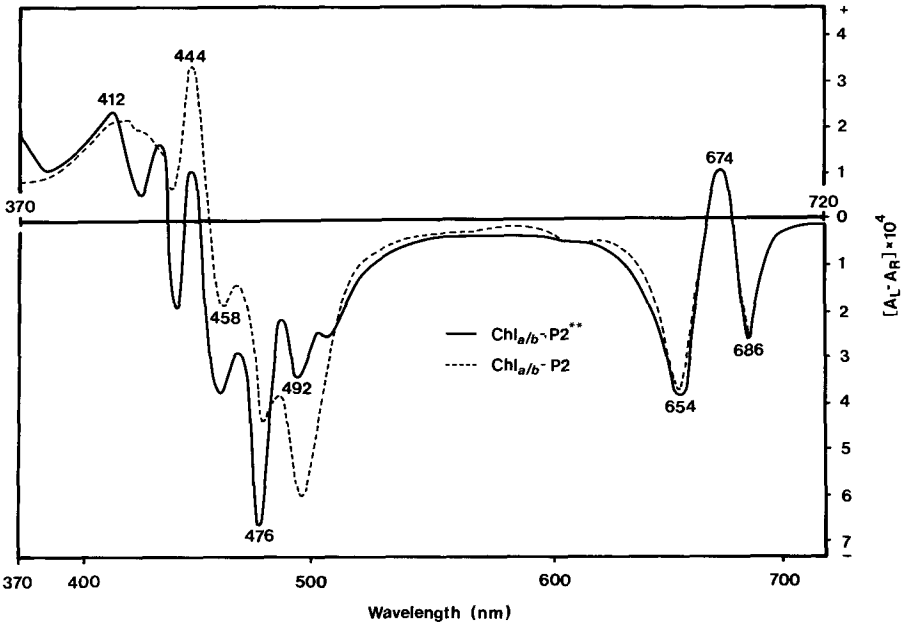

Figure 11. Circular dichroism spectra of both $Chl_{a/b}$-P2 and $Chl_{a/b}$-P2** are shown. Chlorophyll concentration was 20 μg · ml^{-1}, slit width = 0.5 nm.

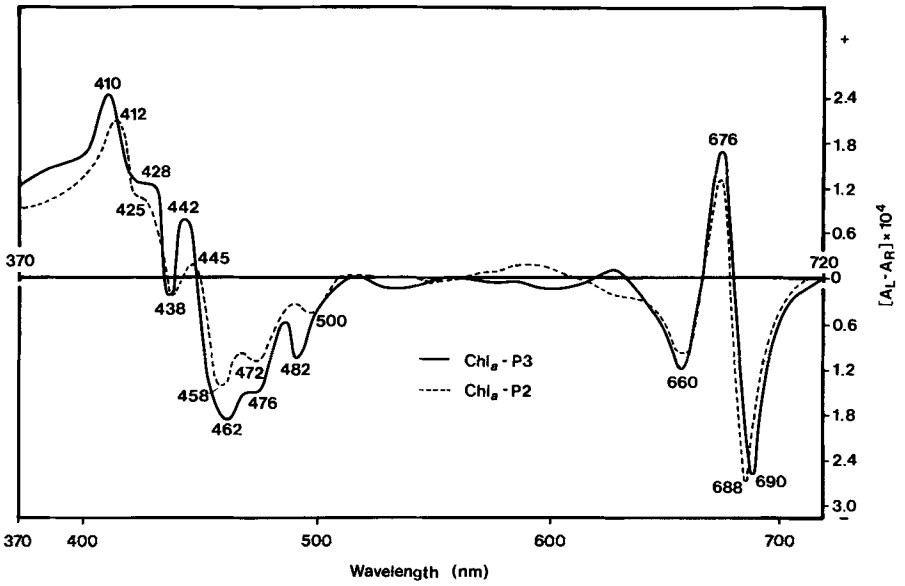

Figure 12. Circular dichroism spectra of Chl$_a$-P2 and Chl$_a$-P3 bands eluted from gel slices. Chlorophyll concentration = 10μg · ml^{-1}, slit width = 1 nm.

the former. The positive signal at 675-676 nm and the negative one at 655-656 nm were, in contrast, equally strong. LHCI-730 showed two strong negative peaks at 492 and 467 nm and a shoulder at about 460 nm. LHCI-680 did not show the 467 nm signal, while the 492 nm peak was more intense and a new peak was present at 480 nm. A minor peak was also seen at 458 nm and may correspond to the 460 nm shoulder in LHCI-730. A 445 nm positive signal which was almost absent in LHCI-730, was seen in LHCI-680 (Figure 10).

Chl$_{a/b}$-P2 appeared in two forms after non-denaturing SDS-PAGE, the higher molecular weight one (Chl$_{a/b}$-P2**) being an oligomer of the other (Chl$_{a/b}$-P2) (2, 11, 12, 29, 36). Both Chl$_{a/b}$-P2 containing bands were eluted from the gels and examined for CD spectra (Figure 11). The spectra of the two forms were very similar in the red region with two negative signals at 686 and 654 nm, and a weak positive one at 674 nm. Some differences in the fine structure were perhaps present in the 640-650 nm range. Although the same signals were present in the spectra of both Chl$_{a/b}$-P2 forms, large differences were seen in the relative intensity of the bands. In particular, the 476 nm signal was more intense in the oligomeric form, while the ones at 492 and 458 nm were increased in Chl$_{a/b}$-P2.

Chl$_a$-P2, Chl$_a$-P3 and Chl$_{a/b}$-P3 had similar electrophoretic mobilities and it was difficult to excise the bands without cross contamination. To reduce contamination of Chl$_a$-P2 and Chl$_a$-P3 by Chl$_{a/b}$-P3, gel slices were used only from those experiments in which most of the PSI-associated chlorophyll was in the Chl$_a$-P1* position so that the amount of the Chl$_{a/b}$-P3 band was very low. The CD spectra of Chl$_a$-P2 and -P3 were very similar with two negative signals (at 688-690 and 660 nm) and a positive one at 676 nm (Figure 12). The spectra were also similar in the 450-500 nm range, where three negative signals were seen in both cases, peaking at 482, 476 and 462 nm (Chl$_a$-P2) and 500, 472 and 458 nm (Chl$_a$-P3). Chl$_a$-P2 and/or Chl$_a$-P3 have been suggested to contain the PSII reaction centre. When their CD spectra were compared with those from the PSI reaction centre-containing chlorophyll-proteins, many differences could be seen, particularly the low intensity of the positive 445 nm signal in Chl$_a$-P2 and -P3, while the 660 nm negative signal was not present in PSI.

4. DISCUSSION

The chlorophyll-protein composition of the two native PSI preparations, Chl_a-P1* and PSI-110 (30), shows a common pattern characterized by the presence of three chlorophyll-proteins: Chl_a-P1, LHCI-730 and LHCI-680. Other green bands represent oligomers of LHCI-730 (e.g., the A1 band) or chlorophyll not bound specifically to the protein (MA1, FP) as indicated by the absence of extrinsic optical activity by CD analysis and by the shift of the 678 nm peak of the absorption spectra to 669 nm. A survey of the chlorophyll-proteins and their complexes is given in Table I.

Chl_a-P1, also called CP1, contains the PS1 reaction centre (*P*-700) (3) and is composed of apoproteins of about 67 kD which bind only chlorophyll *a*. The 77 K fluorescence emission is at 720 nm and the red absorption peak is at 678 nm.

LHCI-730 is the chlorophyll-protein in the A1 and A2 bands from Chl_a-P1*, the MA2 band from PSI-110 and the $Chl_{a/b}$-P3 band from bundle sheath thylakoids. Its 77 K fluorescence emission is around 730 nm and the absorption peak in the red is at 673-74 nm. It contains three polypeptides of molecular weights 24, 22.5 and 21 kD. Both chlorophyll *a* and *b* are bound in the ratio 3-4. The two proteins of 38 and 42 kD in the urea gel from the MA2 band are not thought to be components of the complex since they were not present in the A1 and A2 bands after re-electrophoresis of Chl_a-P1* (Figure 4A, track 4). These proteins have been removed from a PSI-110 preparation from spinach without any change in the chlorophyll-protein pattern (23). LHCI-730 has spectral characteristics and a polypeptide composition similar to previously described LHCI preparations (23, 30).

LHCI-680 isolated from either Chl_a-P1* or PSI-110, fluoresces at 680 nm and has a high chlorophyll *b* content (chlorophyll *a/b* ratio about 1-1.2). These spectral characteristics are similar to those of the main light-harvesting complex ($Chl_{a/b}$-P2), but the different CD spectra and polypeptide composition clearly distinguish between LHCI-680 and $Chl_{a/b}$-P2. SDS-PAGE of the excised LHCI-680 band in dissociating conditions, shows two polypeptides of 25 and 20 kD but one may be a contaminant. In non-denaturing SDS-PAGE, LHCI-680 has the same mobility as $Chl_{a/b}$-P2, so that when chlorophyll-proteins are solubilized and electrophoresed without prior separation of the photosystems, $Chl_{a/b}$-P2 and LHCI-680 are superimposed on the gel. This is confirmed by the different polypeptide pattern obtained when the two forms ($Chl_{a/b}$-P2 and $Chl_{a/b}$-P2**) are re-electrophoresed in denaturing conditions. $Chl_{a/b}$-P2 contains the same three polypeptides present in $Chl_{a/b}$-P2**, plus the 20 and 25 kD proteins also present after re-electrophoresis of the LHCI-680 band (Figure 4B, see also ref. 11). The CD spectra support this conclusion, since the very intense 492 nm negative signal characteristic of LHCI-680 is increased in the spectrum from $Chl_{a/b}$-P2 compared with the one from $Chl_{a/b}$-P2**. On the other hand, the changes in the intensity of the 492 nm signal might be caused by conformational changes due to the different aggregation states of $Chl_{a/b}$-P2 (15).

Resolution of the light-harvesting $Chl_{a/b}$ protein into two different chlorophyll-proteins $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 has been reported (28), but $Chl_{a/b}$-P1 is not the same as LHCI-680, since its apoprotein has a molecular weight very similar to one of $Chl_{a/b}$-P2 (see Table I). Furthermore, $Chl_{a/b}$-P1 is not present in the bundle sheath chloroplast of maize (11, 12) while LHCI-680 is, as confirmed by the presence of the 20 and 25 kD polypeptides after re-electrophoresis of $Chl_{a/b}$-P2 (Figure 4B, tracks 3,4).

Non-denaturing SDS-PAGE has been used as a quantitative method to determine the relative amounts of the PSI- and PSII-associated pigments, on the basis of the assignment of Chl_a-P1 and Chl_a-P1* to PSI and of all other chlorophyll-proteins to PSII (4-6, 26). The recent finding (11) that LHCI-730 (synonyms: $Chl_{a/b}$-P2*, LHCP2 or $Chl_{a/b}$-P3) is a PSI light-harvesting chlorophyll-protein, and that part of the chlorophyll migrating in the position of $Chl_{a/b}$-P2 (LHCP3) is actually bound to LHCI-680, indicates that this method would underestimate the amount of chlorophyll associated with PSI.

The separation of two LHCI forms fluorescing at 680 and 730 nm (LHCIa and LHCIb, respectively) by gradient ultracentrifugation, has been

recently reported (24). LAM et al. found less optical activity in the LHCIa compared with LHCIb, although the CD spectra were similar. They hypothesized a lower degree of pigment organization in LHCIa (24). In contrast, LHCI-730 and LHCI-680 show almost identical optical activity, but the CD signals peak at different wavelengths in the spectra from the two preparations (Figure 10).

A comparison of the CD spectra of the native and antenna-depleted PSI preparations shows that the negative peak at 653 nm is due to the LHCI contribution. Our spectra differ from those in (21) and (24) who report the wavelength of this signal at 647 nm. The wavelength accuracy of the instrument that we have used has been checked (see section 2.4) and the measurements repeated several times. An even longer wavelength peak, at 656 nm, was found in Chl_a-P1* (Figure 9), probably indicating a more native conformation. This negative peak probably includes the contribution of a short wavelength absorbing chlorophyll *a* species (16), comparable to that present in the spectra of Chl_a-P2 and -P3 which only bind chlorophyll *a*. This signal is also present in Chl_a-P2 and -P3 obtained from the barley mutant *chlorina f2* lacking chlorophyll *b* (BASSI, unpublished results).

This CD study of chlorophyll-proteins emphasizes the role of carotenoids since a strong optical activity was present in the 450-500 nm wavelength range. This is particularly evident in the light-harvesting type chlorophyll-proteins ($Chl_{a/b}$-P2, $Chl_{a/b}$-P2**, LHCI-680, and LHCI-730), while these contributions were less pronounced for those chlorophyll-proteins which have been demonstrated or inferred to belong to PSI or PSII reaction centres. On the other hand, the presence of 503 and 482 positive signals in both antenna-depleted PSI preparations, indicates the involvement of carotenoids in the PSI reaction centre. Similar considerations apply for Chl_a-P2 and -P3.

There are no clear explanations for the differences in the CD spectra of chloroplasts and single chlorophyll-proteins or solubilized membranes. Unsolubilized thylakoids not only have a more intense CD signal but they are also qualitatively different in their shape (19, 34). Tentative explanations propose that differential light scattering (34) or specific chlorophyll-chlorophyll interactions (19) are responsible for the observed CD pattern of the chloroplasts. Whatever the cause of these differences, CD spectra from different isolated chlorophyll-proteins show unique patterns and can be useful for their identification and characterization.

In Chlamydomonas (41), the double mutant F54-14 lacking Chl_a-P1 and ATPase has been exploited to isolate a PSI light-harvesting complex (CP0), consisting of 5 polypeptides (27.5, 27, 25, 23 and 19 kD). It fluoresces at 707 nm at 77 K and could be equivalent to a combination of LHCI-680 and LHCI-730 in maize. Two of these polypeptides (27.5, 27 kD) comigrate with $Chl_{a/b}$-P2 when thylakoids are anaylyzed

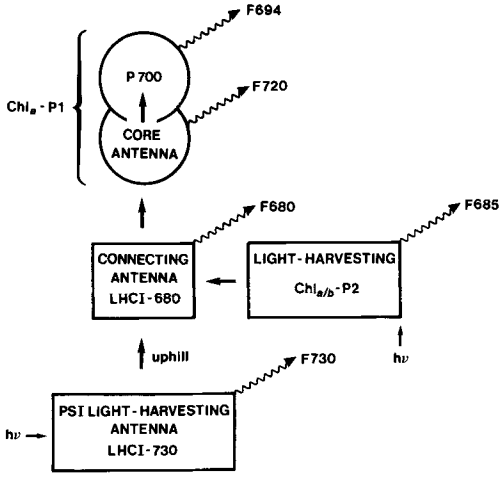

Figure 13. Proposed scheme for energy transfer to photosystem I in higher plants. Two light-harvesting antennae are specifically associated with the PSI reaction centre, and the phosphorylated "monomeric" form of $Chl_{a/b}$-P2 can also transfer energy to *P*-700 via the LHCI-680 connecting antenna. In the absence of Chl_a-P1, fluorescence is emitted in increased yield as a result of downhill energy transfer to LHCI-730. In the absence of LHCI-730, a low yield of fluorescence emission at 720 nm is predicted. If LHCI-680 is missing, we predict an increase in fluorescence yield at 685 nm from $Chl_{a/b}$-P2, and at 720 nm from Chl_a-P1. If both LHCI-680 and LHCI-730 are missing, there should be a low fluorescence yield at 720, as from the thylakoids of the chlorophyll *b*-less barley mutant, *chlorina f2* (38).

by lithium SDS-PAGE (18), as is the case for LHCI-680 in maize. The mutant F-14 lacks Chl_a-P1 and fluoresces at 707 nm. The mutant AC40 lacks the antenna CP0 and emits fluorescence with a lower yield at 717 nm, i.e., from Chl_a-P1. The double mutant has no long wavelength emission and fluoresces at 686 nm. Thus CP0 fluoresces at 707 nm in vivo unless the energy can be transferred to the PSI reaction centre Chl_a-P1. In the mutant y-1p, which lacks 4 of the polypeptides of CP0, the PSI antenna size is decreased, leading to an increase in fluorescence yield at 686 nm from $Chl_{a/b}$-P2. This is interpreted to mean that the four missing polypeptides form a connecting antennae between the core antenna of Chl_a-P1 and the light-harvesting LHCI-707 antenna, as well as $Chl_{a/b}$-P2 (22).

Using this model and the apparent homology of the photosystem I components in Chlamydomonas and maize, LHCI-680 would correspond to the connecting antenna, and LHCI-730 to the peripheral light-harvesting antenna (Figure 13). With this model, we can interpret the fluorescence emission properties of two types of barley mutants. Mutant *viridis-zb*^{63} (38) lacks Chl_a-P1 but has $Chl_{a/b}$-P2, LHCI-680 and LHCI-730, and has an increased 732 nm fluorescence emission from LHCI-730 which, in the absence of the reaction centre is no longer quenched. The thylakoids of *chlorina f2* mutants (29) lack $Chl_{a/b}$-P2, LHCI-680, and LHCI-730. They emit fluorescence at 77 K from the Chl_a-P1 core antenna at 720 nm.

We thus propose that in higher plants, the following sequence of energy transfer to PSI occurs: LHCI-730 → LHCI-680 → Chl_a-P1 (Figure 13). This model is consistent with the sequential removal of first LHCI-730 and then LHCI-680 from Chl_a-P1*, which implies that LHCI-680 is closer to the reaction centre and more tightly bound to it. In addition, the phosphorylated "monomeric" form of $Chl_{a/b}$-P2 can also transfer energy to *P*-700 via the LHCI-680 connecting antenna (11). LHCI-730 contains a long wavelength chlorophyll *a* species (Ca690), as shown by the CD spectrum (Figure 10) and acts as a sink for PSI, since energy transfer to LHCI-680 is uphill (see references 13, 30 and 35 for discussion about the occurrence of uphill energy transfer). This could function to concentrate energy for PSI and to protect the reaction centre from photo-oxidation.

The existence of a connecting antenna is also consistent with the efficient energy transfer from $Chl_{a/b}$-P2 to PSI in bundle sheath membranes of maize (11). In agranal chloroplasts $Chl_{a/b}$-P2 was seen mainly in its monomeric form after SDS-PAGE. It is possible that $Chl_{a/b}$-P2 monomers localized in stroma lamellae transfer energy to PSI via LHCI-680 whereas $Chl_{a/b}$-P2 oligomers found in mesophyll chloroplasts, transfer energy to PSII in appressed grana regions. The polypeptide of 7.8 kD found in $Chl_{a/b}$-P2 oligomer may be involved in the interconversion of $Chl_{a/b}$-P2 forms (11).

A regulatory mechanism for the distribution of the excitation energy between PSI and PSII during state 1-state 2 transitions (17, 31) has been proposed which involves phosphorylation of $Chl_{a/b}$-P2 in grana, its disconnection from PSII reaction centres, and subsequent migration into stroma lamella (1, 8, 10, 20, 25, 32, 40), where it can transfer energy to PSI (11, 27) via the LHCI-680 connecting antenna. We expect from the scheme in Figure 13, that phosphorylation of $Chl_{a/b}$-P2 in a mutant lacking LHCI-680 would result in an increased fluorescence yield at 685 nm at 77 K, in contrast to the decrease at 685 nm in wild type.

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