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The chlorophyll-a/b proteins of photosystem II in *Chlamydomonas reinhardtii*

Isolation, characterization and immunological cross-reactivity to higher-plant polypeptides

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Abstract. We have adapted the procedure for the isolation of PSII membranes from higher plants (D.A. Berthold et al., 1981, FEBS Lett. **134,** 231–234) to the green algae *Chlamydomonas reinhardtii.* The chlorophyll (Chl)-binding proteins from this PSII preparation have been further separated into single Chl-binding polypeptides and characterized spectroscopically. Seven single polypeptides were shown to bind Chl a and Chl b. In particular, we demonstrate that polypeptides p9, p10 and p22, which had not been previously shown to bind Chl a and b, have characteristics similar to those of CP29, CP26 and CP24 from higher plants. We note, however, that p9 and p10 are phosphorylatable in *C. reinhardtii,* at variance with CP29 and CP26 from higher plants. Our data support the notion that the PSII antenna systems in *C. reinhardtii* and in higher plants are very similar. Therefore, studies on the organization and regulation of light-harvesting processes in *C. reinhardtii* may provide information of general relevance for both green algae and higher plants.

Key words: *Chlamydomonas* (light-harvesting complexes) – Chlorophyll a/b protein (chlamydomonas) – Light-harvesting complex

Introduction

The PSII antenna system of higher plants has been extensively studied and is known to contain several Chl-a/b binding proteins in addition to the Chl-a-binding core complex. These are the major light-harvesting complex, LHCII, and three minor proteins named CP24, CP26 and CP29 after their apparent molecular weights (MW) in green gels (reviewed in Bassi et al. 1987; Green 1988). The role of the various Chl-binding polypeptides in light harvesting and light energy distribution to the reaction centers is not yet fully understood despite the recent progress in our understanding of their organization and participation in regulation mechanisms, such as the phosphorylation-mediated state transitions (Larsson et al. 1987; Bassi et al. 1988a, b).

The unicellular green alga *Chlamydomonas reinhardtii* is a model system well suited for studies of the photosynthetic apparatus because of the possibility of performing spectroscopic measurements in vivo, of obtaining mutants altered in single thylakoid proteins and of transforming cells with foreign DNA. However, whereas LHCI and LHCII complexes have been identified in *C. reinhardtii* (Delepelaire and Chua 1981; Wollman and Bennoun 1982), there has, as yet, been no report on the existence of the minor chlorophyll-protein complexes corresponding to higher-plant CP24, CP26 and CP29. We have therefore studied *C. reinhardtii* Chl-a/b proteins with the aim of understanding to what extent they are comparable to those which have been identified in higher plants.

Material and methods

C. reinhardtii wild type cells were grown in Tris-acetate-phosphate medium at a light flux of 200 lx and used in the mid-exponential phase ($3 \cdot 10^6$ cell $\cdot$ ml^{-1}) of growth.

Membrane fractionation. For the preparation of PSII membranes, cells were harvested from a 20-l culture with a continuous-flow centrifuge and resuspended in 150 ml of 20 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine) pH 8.0, 0.4 M sorbitol, 10 mM $MgCl_2$, 0.2% bovine serum albumin (BSA), and passed through a chilled Yeda press (Yeda Research and Development Co., Rehovot, Israel) at a pressure of 10^7 Pa. Broken cells were centrifuged at 6000 $\cdot$ *g* and resuspended in 400 ml of 20 mM Tricine pH 8.0, 0.1 M NaCl, 5 mM $MgCl_2$ and 0.2% BSA. The suspension was incubated for 15 min in ice, centrifuged at 8000 $\cdot$ *g* and resuspended in 20 mM (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (Hepes) pH 7.6, 15 mM NaCl, 5 mM $MgCl_2$ at

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Abbreviations: Chl = chlorophyll; IEF = isoelectrofocusing; LHC = light harvesting complex; MW = molecular weight; PAGE = polyacrylamide gel electrophoresis; PS = photosystem; RC = reaction centre; SDS = sodium dodecylsulfate

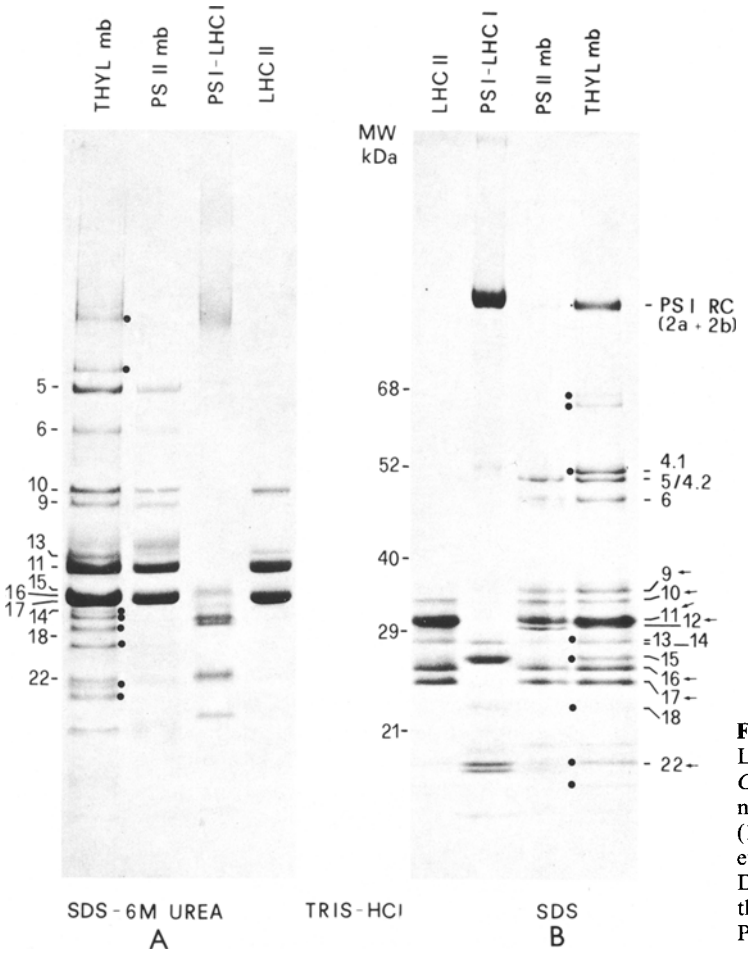

Fig. 1A, B. Polypeptide analysis of the PSI, PSII and LHCII preparations resulting from fractionation of *C. reinhardtii* thylakoid membranes. Polypeptides are named after Chua and Bennoun (1975) and Piccioni et al. (1981). **A** Gel Tris-chloride/SDS/8 M urea as in Piccioni et al. (1981). **B** Gel Tris-chloride/SDS as in Wollman and Delepelaire (1984). *Dots* indicate polypeptides present in thylakoid membranes *(THYL mb)* but deficient in the PSII membrane *(PSII mb)* preparation

2.5 mg Chl · ml^{-1}. Triton X–100 was then added from a 20% stock solution to 4%, and the solubilization was carried on at 4° C for 20 min. Undissolved material was pelleted by centrifugation at 3500 · *g* for 10 min. Photosystem-II membranes were then pelleted at 40000 · *g* (30 min), rinsed once in the above buffer and frozen in aliquots in 20 mM Hepes pH 7.6, 5 mM $MgCl_2$, 10 mM NaCl, 20% sorbitol. All solutions contained the protease inhibitors phenylmethylsulphonyl fluoride (PMSF), benzamidine and aminocaproic acid at concentrations of 0.2 mM, 1 mM and 5 mM, respectively.

The PSI–LHCI complex was obtained as described by Mullet et al. (1980), LHCII was obtained as in Burke et al. (1978), and the PSII reaction centre (RC) complex was obtained as in Diner and Wollman (1980).

Electrophoresis. For polypeptide analysis, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed in slab gels (25 · 20 · 0.1 cm^3) using three different SDS–PAGE gel systems: without urea as in Wollman and Delepelaire (1984), with 8 M urea as in Piccioni et al. (1981), with 6 M urea as in Bassi and Simpson (1987). Gels were stained with Coomassie blue. Non-denaturing octylglucoside/glycerol green gels were run at 4° C as in Bassi et al. (1987b). Non-denaturing isoelectrofocusing (IEF) in granulated gels was performed as previously described in (Bassi et al. 1988b; Dainese et al. 1990).

Spectroscopy. Absorption spectra of isolated chlorophyll-proteins were recorded with a model lambda 5 spectrophotometer (Perkin Elmer Co., Norwalk, Conn., USA) using a 1-nm slit width. Before recording the spectra the samples were centrifuged in a 0.1 to 1 M sucrose gradient containing 10 mM Hepes pH 7.6 and 0.06% dodecylmaltoside to make sure that free pigments and others contaminants such as carrier ampholytes were removed. Fourth-derivative analysis of the spectra was obtained using a built-in microcomputer using a wavelength range of 4 nm. Low-temperature fluorescence emission spectra were recorded with an instrument built by Professor R. Strasser (University of Geneva, Switzerland). Excitation was at 633 nm from an He-Ne laser source. Chlorophyll concentration and Chl-a/b ratios were determined as in Porra et al. (1989).

Electron microscopy. Membranes were prepared for electron microscopy as in Olive et al. (1979).

Results

Fractionation of membranes. As a preliminary approach to the study of PSII Chl-a/b proteins from *C. reinhardtii*, we modified the procedure originally described by Berthold et al. (1981) for the isolation of PSII membranes. This procedure is routine for higher plants but not, up to now, for *C. reinhardtii*. We also fractionated Triton X–100-solubilized thylakoids by sucrose-gradient ultracentrifugation to obtain PSI–LHCI and LHCII complexes.

The three preparations – PSII membranes, PSI–LHCI and LHCII – were analyzed by SDS–PAGE using two gel systems previously employed in the analysis of *C. reinhardtii* thylakoid proteins (Piccioni et al. 1981; Wollman and Delepelaire 1984). This is shown in

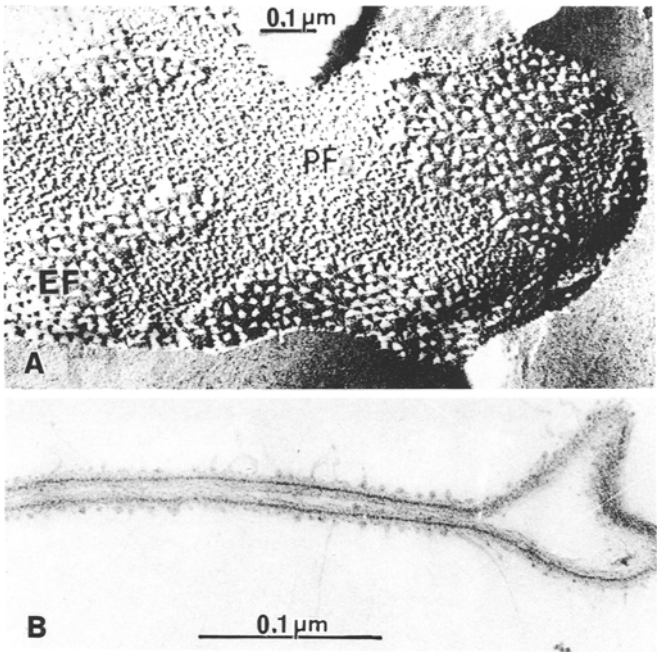

Fig. 2A, B. Characterization by electron microscopy of the *C. reinhardtii* PSII membrane preparation. (**A**) Freeze-fractured membranes showing exoplasmic fracture face (EF) and protoplasmic fracture face (PF) particles; × 100000. (**B**) Thin-section micrography showing the preparation composed of paired membranes; × 330000

Fig. 1A, B. The LHCII complex contains five polypeptides: p10, p11, p13, p16 and p17. The PSI–LHCI complex contains a large band in the 70-kDa region which corresponds to the apoproteins of CP1 (p2a and p2b), several polypeptides in the 18 to 30-kDa range that have been assigned in part to the LHCI (= CP0 in Wollman and Bennoun 1982) and in part to the PSI reaction centre as p20 and p21 (Girard et al. 1986), and several weakly stained bands with lower MWs. Here we should note that, although most of the LHCI (CP0) polypeptides have an apparent MW lower than LHCII polypeptides, there is an overlap between some LHCI and LHCII subunits in both gel systems. When compared to unfractionated thylakoids, PSII membranes lack several polypeptides of the thylakoid membranes (spots in thylakoid lane), most of which belong to the PSI–LHCI lane, and are enriched in LHCII polypeptides as well as in PSII RC polypeptides p5, p6 and D2. When observed by freeze-fracture and transmission electron microscopy, the PSII preparation appears to consist of pairs of appressed membranes (Fig. 2B) containing exoplasmic fracture face (EF) and protoplasmic fracture face (PF) particles (Fig. 2A), similar to those described for PSII membranes from higher plants (Dunahay et al. 1984). We then fractionated PSII membranes by non-denaturing SDS–PAGE (Fig. 3A, B) and analyzed the polypeptide composition of the green bands (Fig. 3C). The upper and third bands (a and c in Fig. 3A) contained only Chl a bound to a complex composed of polypeptides 5, D1, D2 and cytochrome b559 (band a) and polypeptide 6 (band c) (data not shown). The Chl-a/b-containing green bands (b, d, e, f, g) were excised from the gel, run on a similar gel with different acrylamide concentration to avoid the comigration of uncolored polypeptides (Fig. 3B), and finally loaded onto denaturing gels (Fig. 3C). Only polypeptides p11, p13, p16 and p17 are components of band b which corresponds to the high-MW oligomeric LHCII form also present in *Z. mays* (Fig. 3A). Polypeptides p9, p10 are found in the low-MW green bands (d–f) in addi-

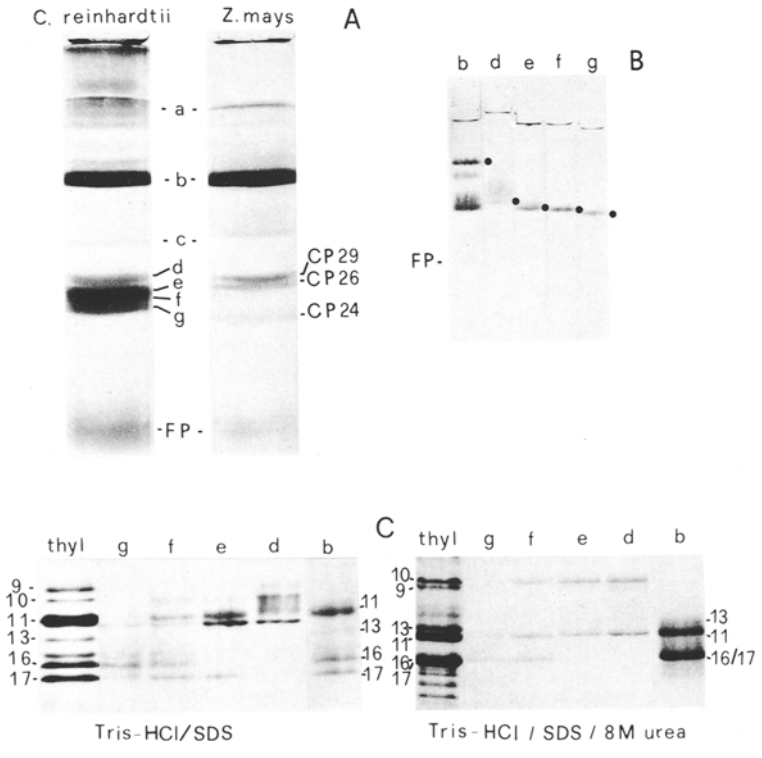

Fig. 3A–C. Non-denaturing "green" gels (11% acrylamide) of *Z. mays* and *C. reinhardtii* PSII membranes (**B**). Re-electrophoresis (14% acrylamide) of bands containing Chl-a and b. Gels in **A** and **B** were not stained. **C** Denaturing SDS–PAGE of green bands excised from gel in **B** in gel system Tris-chloride/SDS *(left)* and Tris-chloride/SDS 8 M urea *(right)*. *FP*, free pigments. *a–g*, various green bands isolated from gel in **A**. *Dots* in **B** indicate the green bands denatured in **C**

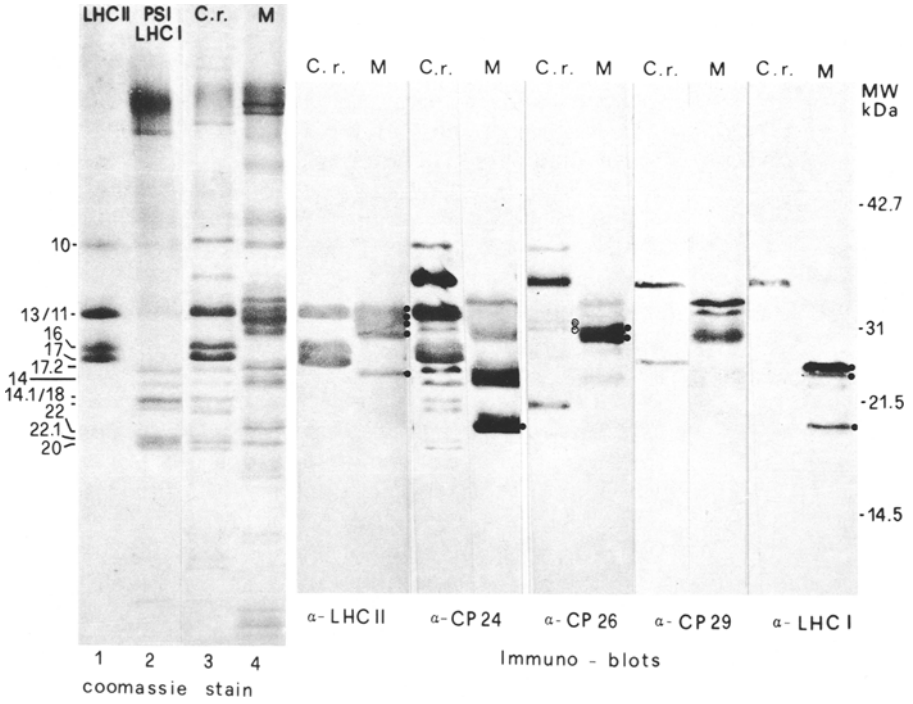

Fig. 4. Immuno-blot analysis of *C. reinhardtii (C.r.)* and *Z. mays (M)* thylakoid membranes. The gel system used was Tris-sulphate/SDS/6 M urea that minimizes comigration of LHCI and LHCII polypeptides. *C. reinhardtii* membranes were Tris-washed to remove oxygen-evolving-enhancer polypeptides. *Dots* indicate the *Z. mays* polypeptides which were used as antigens. *Open circles* indicate immuno-detected polypeptides which were not detected by Coomassie-blue staining

Table 1. Reaction of antisera raised against *Zea mays* Chl-a/b proteins with *C. reinhardtii* thylakoid-membrane polypeptides

	Polyclonals					Monoclonals			
Polypeptide	LHCII	CP24	CP26	CP29	LHCI	MLH1	MLH2	CMCP29[a]	CMLHCI
p10		+ +	+ +					+[b]	
p9		+ + +	+ + +	+	+		+		
p11/p13	+ +	+ +							
p11.1			+						
p11.2		+	+						
p16	+ +	+							
p17	+ +	+ +				+			
p17.2		+		+					
p14.1/p18		+	+ +						
P15		+							
p22		+							+
p22.1		+							

[a] The complete denotation of CMCP29 is CMpchl a/b P1:1 and of CMLHCI is: CMpLHCI:1 (Hoyer-Hansen et al. 1988)
[b] +, + +, + + + denote increasing strength in the immunoreaction; blank spaces indicate the absence of immunoreaction

tion to p11, p13, p16 and p17. In some experiments, using SDS–PAGE run in the absence of urea, we distinguished below p11 an additional band which comigrated with p11 in the presence of urea. Owing to its electrophoretic properties this band probably reflects a contamination by p12, the oxygen-evolving-enhancer subunit known as the 33-kDa polypeptide in higher plants.

Immunological analysis of Chl-a/b proteins. In a complementary approach to establish a correlation between Chl-a/b proteins in *Zea mays* and *C. reinhardtii*, we assayed thylakoid polypeptides with polyclonal and monoclonal antibodies raised against Chl-a/b proteins as described previously (Hoyer-Hansen et al. 1988; Di Paolo et al. 1990). In these experiments we used an alternative gel system in which p13 and p11 comigrate but which ensures a better separation of LHCI from LHCII polypeptides, thus avoiding comigration of Chl-a/b proteins that may have common epitopes. This is shown in Fig. 4, lanes 1–3. *Zea mays* thylakoids were included as a reference (lane 4). Polyclonal antibodies raised against the oligomeric LHCII of maize, recognized the three major bands of *C. reinhardtii* thylakoids (p11 + p13, p16 and p17) that were shown above to form the oligomeric LHCII in *C. reinhardtii*, while p10, present in the LHCII from sucrose gradient, was not recognized. The antiserum raised against maize CP24 had a broad spectrum thus recognizing most maize Chl-a/b proteins. Similarly, at least 12 *C. reinhardtii* polypeptides belonging either to PSII membranes (p9, p10, p11/p13, p16, p17) or to PSI–LHCI complexes (p17.2, p14, p14.1, p18, p15, p22 and p22.1) were recognized. This indicates that

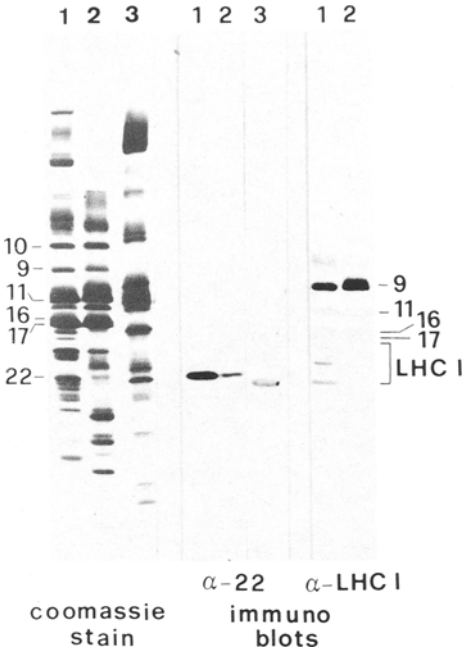

Fig. 5. Immuno-blot analysis of thylakoid membranes from *C. reinhardtii (1)*, PSII membranes from *C. reinhardtii (2)* and thylakoid membranes from *Z. mays (3)*. *a-22*, anti-p22 antibody; *a-LHCI*, anti-LHCI antibody

there are a minimum of 12 Chl-a/b proteins in *C. reinhardtii* thylakoids but does not give any indication as to which complex in *C. reinhardtii* corresponded to higher, plant CP24. The antiserum against CP26 did not recognize the oligomeric LHCII (p11–13 and p16–17); rather, p9 and p10 were recognized as well as p15, belonging to LHCI. Two additional faint bands were recognized; these had an apparent MW of about 30 kDa and were hard to detect with Coomassie blue (circles in Fig. 4). The upper band of the doublet was also recognized by anti CP24. The polyclonal raised against CP29 recognized p9 and p17.2. The antiserum raised against maize LHCI, surprisingly, recognized only *C. reinhardtii* p9 which is enriched in PSII membranes rather than LHCI polypeptides (which can be seen lane 2 on Fig. 4, left).

Monoclonal antibodies raised against higher-plant Chl-a/b proteins reacted very weakly although specifically with *C. reinhardtii* polypeptides. The results are summarized in Table 1 and compared with the recognition patterns of the polyclonal antibodies used in Fig. 4. The MLH1 monoclonal, which was seen to react with a subset of the oligomeric LHCII polypeptides (Bassi et al. 1988a), recognized p17 in *C. reinhardtii*; MLH2, selective for CP29 (Hoyer-Hansen et al. 1988) recognized p9 while CMCP29, directed against both CP26 and CP29, recognized p10. The CMLHCI monoclonal, which recognizes an epitope shared by an LHCI subunit and CP24 in higher plants (Bassi et al. 1987), specifically recognized p22, in *C. reinhardtii*. In addition, p22, which has been previously reported to be part of LHCI (Wollman and Bennoun 1982), was also present in PSII membranes while the other LHCI subunits were absent. This is illustrated in Fig. 5 by the immunoblots in which are compared the recognition patterns of an anti-p22 and an anti-LHCI antibody in thylakoid and PSII membranes (lanes 1 and 2 Fig. 5). Figure 5 also shows that the anti-p22 antibody specifically recognized CP24 in maize thylakoid membranes (lane 3).

Isolation and biochemical characterization of Chl-a/b proteins. In the attempt to further characterize the Chl-a/b proteins from *C. reinhardtii*, each of these was purified and further analyzed by absorption, low-temperature fluorescence-emission spectroscopy and by raising polyclonal antibodies. The purification was performed by solubilizing the membranes with 2% octylglucoside and 0.2% dodecylmaltoside – a treatment previously shown to dissociate multicomponent complexes in single chlorophyll proteins – followed by fractionation in a flat-bed IEF gel (pH 3.5–6) at 4° C (Dainese et al. 1990). Several green bands were eluted from the gel and analyzed by denaturing SDS–PAGE. The results of the fractionation are shown in Fig. 6A, B: LHCI polypeptides comigrated with p16 in fractions 3–5 but they were

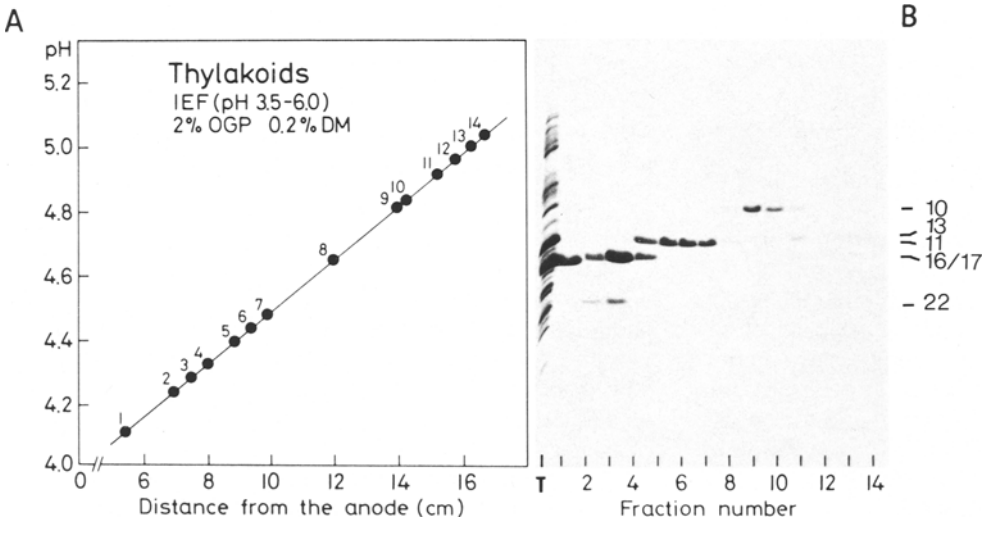

Fig. 6A, B. Purification of Chl-a/b proteins by non-denaturing isoelectrofocusing. Schematic plot of the migration of the green bands versus the pH gradient. Fourteen green bands were resolved; these are numbered starting from the anode.
B Analysis by SDS–PAGE of the green bands resolved in **A**. Polypeptides are numbered *(right)* as in Fig. 1. *T*, thylakoid membranes

Table 2. Characteristics of Chl-a/b proteins of *C. reinhardtii* PSII membranes

Polypeptide	Chl-a/b ratio	Absorption maximum	pI[a]	Apparent MW (kDa)[b]		
				Gel 1	Gel 2	Gel 3
p9	3.0	676.5	4.9	35	34	35
p10	2.4	676.5	4.8	38	33	39
p11	0.96	671	4.3–4.5	33.5	30.5	27.5
p13	1.9	671	4.8–5.0	33.5	26.5	28.5
p16	0.96	670	4.2–4.3	30.5	25.5	25
p17	0.76	670	4.1	30	25	25
p22	2.2	672	4.15	18	18.5	19
LHCII[c]	1.45	673	/	/	/	/

[a] When multiple bands were observed the extreme values were given
[b] Gel 1: Tris-sulphate/SDS/6 M urea; gel 2: Tris-chloride/SDS; gel 3: Tris-chloride/SDS/8 M urea
[c] LHCII, isolated by the procedure of Burke et al. 1978, contained polypeptides p10, p11, p13, p16, p17

easily separated by ultracentrifugation on sucrose gradients because of the higher mobility of LHCI. The P22 polypeptide was recovered from fraction 2 as an upper green band in a sucrose gradient loaded with fraction 2 treated with 20 mM NaCl and 5 mM $MgCl_2$. In these conditions, p16 was found as a pellet after 20 h centrifugation at 39000 rpm in a Beckman SW 41 rotor. Polypeptide 9 was better resolved in a similar gel but in the pH range 5–8. The characteristics of the individual Chl-a/b-binding polypeptides are summarized in Table 2.

It is well known from previous studies that p11, p16 and p17 show extensive immunological cross-reactions. We therefore raised polyclonals against p9, p10, p13 and p22. The results are shown in Fig. 7. The antibodies against p9 or p10 both cross-reacted with p9 and p10 and recognized several other Chl-a/b polypeptides: p11, p16, p17. On the other hand, polyclonal antibodies raised against p13 only recognized p11, p16 and p17, and the one against p22 was very specific for this latter polypeptide. This anti-p22 antibody cross-reacted specificaly with CP24 in maize (Fig. 5).

Spectral characterization of Chl-a/b proteins. The absorption spectra are shown in Fig. 8A, B: P11, 16 and 17 have similar absorption features and low Chl-a/b ratios while p9, p10 and p13 have a lower Chl-b content (Table 2). Polypeptides p9 and p10 also exhibited red-shifted absorption maxima at 676–677 nm and a characteristic Chl-b absorption component at 641 nm (Fig. 8A).

The absorption characteristics of the isolated complexes were further analyzed by their fourth-derivative spectra (Fig. 8C, D). Two major Chl-a and one or two Chl-b absorption components were obtained from each Chl-a/b-binding protein. The Chl-b absorption contribution was at 649–650 nm in the spectra from all the Chl-a/b proteins but p9 and p10 also exhibited a 639-nm component that was the major one in the case of p9 (Fig. 8C). All the Chl-a/b proteins showed two major Chl-a components, the more red-shifted of these peaking at around 679 nm. The second component was more variable, peaking at 664 nm in p9 and p10; at 666 nm in p17; at 668 nm in p11, p13 and p16; and 672 nm in p22. These data are summarized in Table 3.

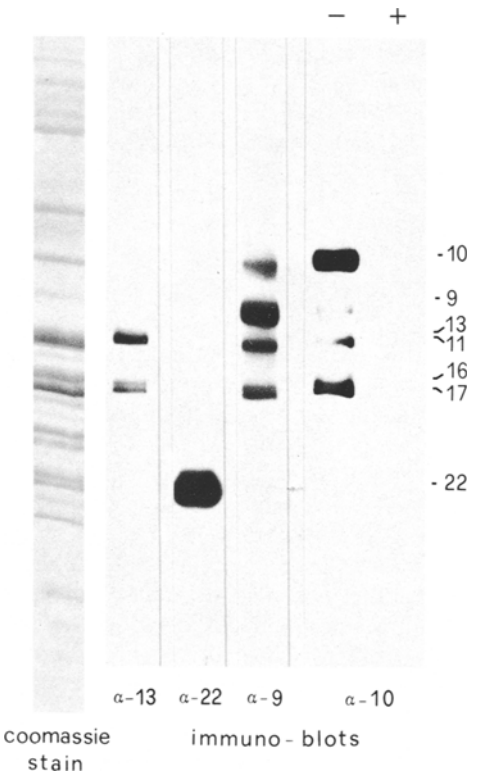

Fig. 7. Immunological cross-reactions between Chl-a/b proteins of *C. reinhardtii*. Polyclonal antibodies were raised in rabbits against polypeptides p9, p10, p13 and p22 and used to assay thylakoid membranes fractionated by SDS–PAGE. For p10, filters were assayed with the untreated antibody *(−)* or antiserum at a dilution of 1:200 was preabsorbed with purified p11 equivalent to 60 micrograms Chl *(+)*

Low-temperature emission spectra (Fig. 8E) showed a single major peak at 678 or 679 nm in all cases. A small shoulder was present in the case of p11 and p17 at 740 nm. As an interesting feature we note that the emission peak of p16 was particularly sharp since the width at half height was only 6 nm versus 12 nm in the case of p11.

For comparison are shown in Fig. 9 the absorption spectra of the PSII core complex (PSII RC), an LHCII

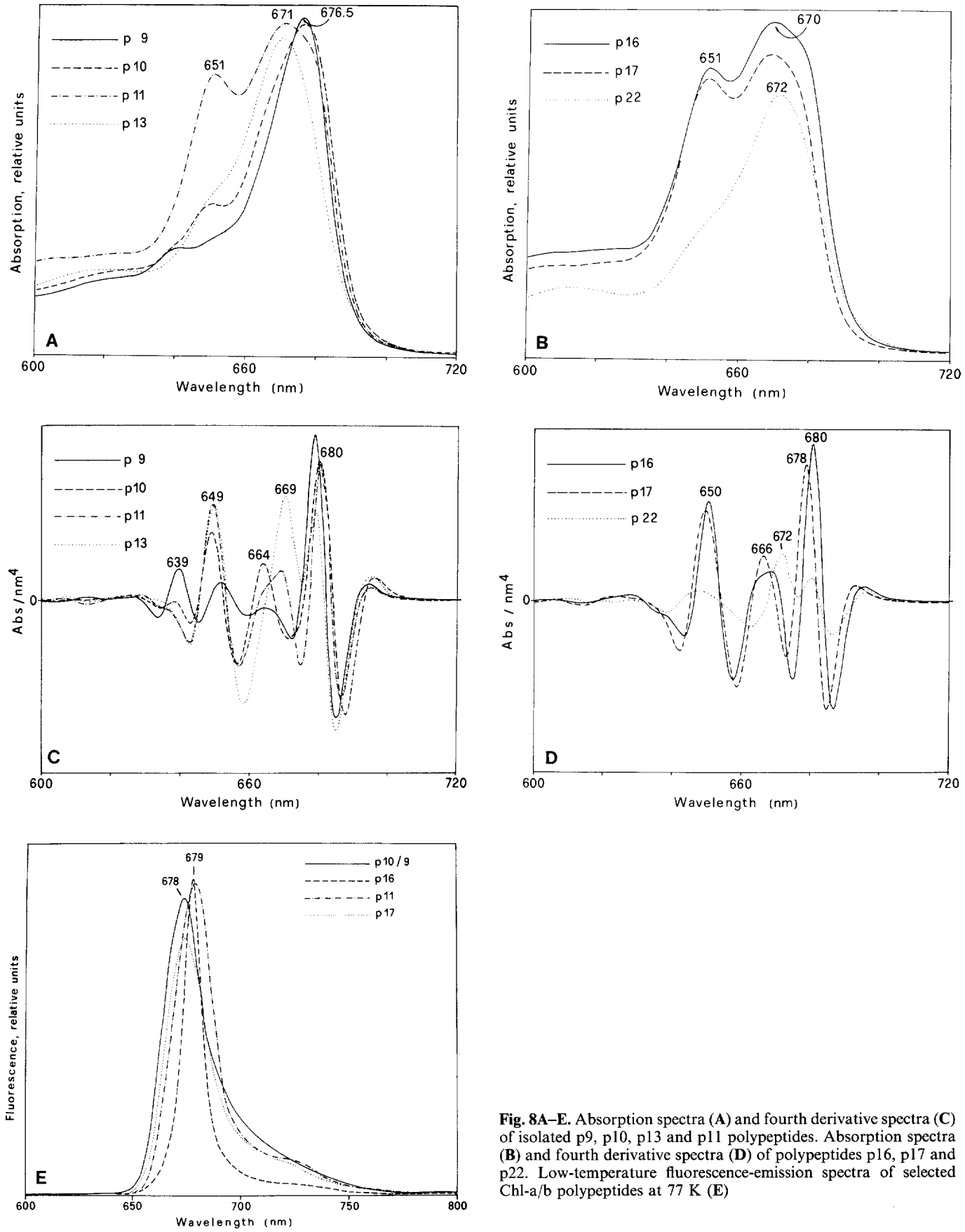

Fig. 8A–E. Absorption spectra (**A**) and fourth derivative spectra (**C**) of isolated p9, p10, p13 and p11 polypeptides. Absorption spectra (**B**) and fourth derivative spectra (**D**) of polypeptides p16, p17 and p22. Low-temperature fluorescence-emission spectra of selected Chl-a/b polypeptides at 77 K (**E**)

Table 3. Absorption components from the fourth-derivative absorption spectra of Chl-a/b proteins from *C. reinhardtii* PSII membranes. Derivation was calculated over 4-nm window

Polypeptide	Chl-a 1	Chl-a 2	Chl-b 1	Chl-b 2
p9	678.5	663.9	651.6	6 9.4
p10	679.9	664.1	648.8	639.0
p11	679.9	668.6	649.4	/
p13	678.9	669.6	649.3	/
p16	680.5	668.2	650.1	/
p17	678.4	665.9	649.8	/
p22	680	672	648	/
PSII RC	681.6	670.8	644.8	/

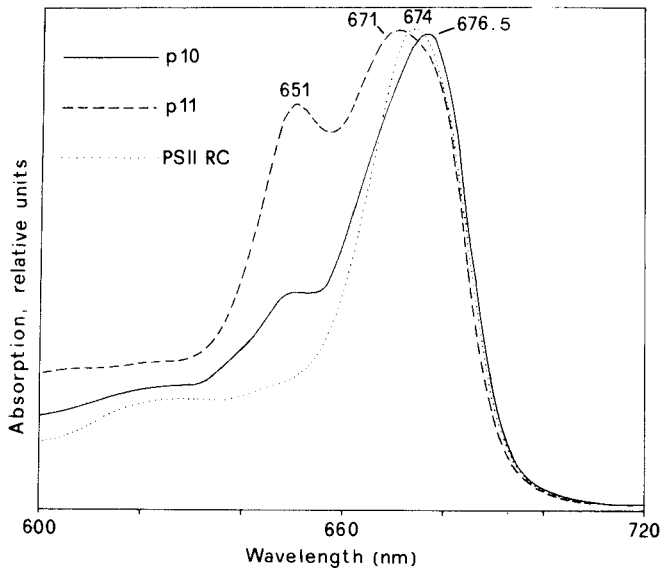

Fig. 9. Absorption spectrum of PSII reaction centre *(PSII RC)* compared with those of a component of the major LHCII *(p11)* and of a component of the long-wavelength absorbing antenna *(p10)*

subunit (p11) and a minor antenna component (p10 = CP26). It can be seen that the LHCII component absorbed at a shorter wavelength than the PSII core. In contrast, the absorption spectrum of p10 overlapped extensively with that of the PSII core, showing the most red-shifted absorption maximum. Thus, the minor PSII Chl-binding proteins accommodate Chl-a holochromes absorbing at a particularly long wavelength when compared with the various components of the PSII antenna system.

Discussion

Previous studies on the Chl-binding proteins of the peripheral antenna in *C. reinhardtii* have led to the identification of five LHCI (CP0) subunits, p14, p15, p17.1, p18, p22 (Wollman and Bennoun 1982; Ish-Shalom and Ohad 1983), and four immunologically related LHCII subunits, namely p11, p13, p16, p17 (Chua and Blomberg 1979; Delepelaire and Chua 1981). Moreover, a pigment-deficient mutant, the BF4 mutant, was shown to lack both LHCI and LHCII subunits as well as two other polypeptides p9 and p10 (de Vitry and Wollman 1988) which were therefore considered as possible Chl-binding polypeptides.

In the present work we have used a combined biochemical and immunological approach to further identify Chl-a/b, binding proteins belonging to the PSII antenna system. To this end we have prepared PSII membrane fractions from *C. reinhardtii* which compared well with PSII preparations from higher plants since they were depleted of PSI RC, LHCI and ATPase polypeptides while enriched in PSII RC intrinsic and extrinsic proteins as well as in polypeptides belonging to LHCII (Fig. 1). Electron-microscopy analysis confirmed that the PSII preparation was composed of paired membranes derived from grana partitions (Fig. 2). These data demonstrate that the various Chl-a/b proteins, whose characteristics are discussed below, are *bona fide* components of the PSII antenna system.

Diversity of Chl-binding proteins in PSII. A major difference stems from the comparison of reports on Chl proteins in *C. reinhardtii* and in higher plants: most of the Chl detected on green gels loaded with higher-plant thylakoids is associated with an oligomeric LHCII band migrating at about 64 kDa whereas this band is lacking in the case of *C. reinhardtii* (Delepelaire and Chua 1981). In our hands, the reason for this discrepancy is only methodological. Indeed Fig. 3 shows that, when analyzed by the same method (octylglucoside plus glycerol), separation patterns are almost identical in *Z. mays* and *C. reinhardtii*. Interestingly, this solubilization procedure could also resolve two components of the Chl-a-binding core complex, an upper green band containing subunits p5, D1, D2 and cytochrome *b*559 and a 43-kDa band containing subunit p6, as was previously described in maize (Bassi et al. 1987). This organization of PSII RC core complexes in two building blocks is consistent with the assembly pathway recently described for *C. reinhardtii*, in which p6 was proposed to assemble with a preformed p5, D1, D2, cytochrome *b*559 complex (de Vitry et al. 1989).

The Chl-a/b-binding complexes obtained after PSII-membrane solubilization were resolved into seven individual Chl-binding polypeptides (Figs. 3–6). From the absorption characteristics of their pigment complement as well as from their immunological relatedness to Chl-binding polypeptides from higher plants we could establish that, besides the oligomeric form of LHCII, the peripheral PSII antenna in *C. reinhardtii* also contained three as yet unidentified minor peripheral antenna proteins.

The oligomeric LHCII. The oligomeric form of LHCII comprises three major subunits p11, p16 and p17 and a minor component, polypeptide p13. These polypeptides should be considered equivalent to the five closely migrating polypeptides of maize LHCII (Bassi et al. 1987) since they were the only polypeptides of *C. reinhardtii* recognized by the polyclonal antibody raised against the

maize complex. However, whereas p11, p16, p17 were present in equimolar amounts in the membranes (de Vitry et al. 1983) and had similar pI and Chl-a/Chl-b ratios, p13 was present in minor amounts, was enriched in Chl-a and displayed a less acidic pI (Table 2). No counterpart to polypeptide p13 has yet been identified in the oligomeric form of LHCII from higher plants. Still another difference with respect to the higher-plant complex is that LHCII subunits from *C. reinhardtii* were found in both an oligomeric and a monomeric green band while the compositions of the two green bands in maize are mutually exclusive. In the latter case, polypeptides belonging to the major LHCII complex migrate in the oligomeric band while those belonging to the minor Chl-a/b proteins CP29, CP26 and CP24 migrate at the position of the monomeric band. We therefore conclude that the oligomeric form of LHCII is probably less stable in *C. reinhardtii* and partly dissociates into a monomeric form upon treatment with detergents.

Polypeptides p9 and p10 are the apoproteins of CP29 and CP26. When prepared by the procedure of Burke et al. (1978), LHCII was found to contain the four subunits described above (p11, p13, p16, p17) together with a minor component, p10. In higher plants, minor Chl-a/b proteins, CP26 and CP29, copurify with LHCII obtained by the same procedure (Hoyer-Hansen et al. 1988). Upon electrophoresis in non-denaturing conditions, these two complexes give rise to what has been improperly called the monomeric form of LHCII in higher plants. Similarly, p9 and p10 were found in green gels with monomeric LHCII but not with the genuine oligomeric LHCII. In the present work, we were able to demonstrate that p9 and p10 are genuine Chl-binding proteins since they still retained bound chlorophylls after purification on IEF gels. When isolated in their native form, they displayed higher Chl-a/b ratios (2.4 and 3.0 respectively) than the individual LHCII subunits (p11, p16 and p17) whose Chl-a/b ratios were consistently < 1. Moreover, p9 and p10 had red-shifted absorption peaks (676.5 nm). These characteristics are very similar to those of CP26 and CP29 in maize (Dainese et al. 1990). In fact p9 exhibits a small Chl-b absorption peak at 641 nm identical to that observed in CP29 (Spangfort and Andersson 1989; Dainese et al. 1990), while in p10 both the 641 and 650-nm shoulders are apparent as in maize CP26 (Dainese et al. 1990). In addition to these spectroscopic similarities, polyclonal antibodies raised against the minor Chl-a/b-protein complexes from maize (CP29, CP26 and CP24) strongly recognized p9 and p10 (Table 1). Furthermore, a one-to-one correlation between *C. reinhardtii* and maize minor complexes p9/CP29 and p10/CP26 could be made on the basis of the MLH2 monoclonal recognition of maize CP29 and p9 from *C. reinhardtii*, and of the CMpChl-a/b P1:1 monoclonal recognition of maize CP26 and p10 from *C. reinhardtii*.

Polypeptide p22 is the apoprotein of CP24. The Chl-protein complex CP24 from higher plants contains a single 20-kDa protein. This MW is significantly lower than that of the other maize Chl-a/b proteins (Bassi et al. 1987). CP24 has been also related to LHCI-680, a LHCI complex with similar function (Dunahay and Staehelin 1985; Bassi et al. 1987). The counterpart of CP24 in *C. reinhardtii* is p22: its apparent MW is 18 kDa and, like CP24, it is present both in PSII membranes and in the PSI–LHCI complex or isolated LHCI (Wollman and Bennoun 1982). In addition, the spectral characteristics of the isolated p22 closely resemble those of CP24 from higher plants. Last, as shown on Fig. 5, the two polypeptides are immunologically related. It is then likely that two forms of p22 exist, one partitioning in the grana and the other in the stroma membranes, similar to CP24 in higher plants (Bassi et al. 1987; Hoyer-Hansen et al. 1988).

Participation of the minor PSII antenna proteins in state transitions. Although the basic components of the PSII peripheral antenna appear to be very similar in higher plants and in *C. reinhardtii*, there is a major difference in their phosphorylation properties. Whereas, CP26 and CP29 are not phosphorylatable in higher plants, their counterparts in *C. reinhardtii*, p10 and p9, are heavily phosphorylated upon transition to state 2 (Wollman and Delepelaire 1984; Delepelaire and Wollman 1985). This indicates that there might be some differences in the molecular mechanism of state 1-state 2 transitions between algal cells and higher plants. In particular, this difference in phosphorylation status of CP26 and CP29 in higher plants and in *C. reinhardtii* may provide a clue for the interpretation of the striking difference in amplitude of the fluorescence quenching in state 2 – and therefore in the proportion of mobile antenna – which is about two to three times larger in *C. reinhardtii* than in higher plants (Wollman and Delepelaire 1984). Therefore it is tempting to suggest that either electrostatic repulsion between phosphorylated CP29 and CP26 and phosphorylated LHCII would increase the proportion of mobile LHCII in *C. reinhardtii*, or that CP26 and CP29 may themselves participate in the mobile antenna fraction when phosphorylated.

Two sets of observations support the notion that there are some differences in the phosphorylation processes which operate in *C. reinhardtii* and in higher plants. On the one hand, a maize mutant lacking cytochrome *b6/f* complexes displayed no phosphorylation of any Chl-a/b proteins (Bennett et al. 1988), whereas similar mutants from *C. reinhardtii* still underwent some phosphorylation of p9 and p10 (Wollman and Lemaire 1988). On the other hand, the thylakoid-bound kinase from *C. reinhardtii* was reported not to be recognized by polyclonal antibodies raised against the LHC-kinase isolated from spinach (Coughlan 1988). Therefore, the differences in the phosphorylation properties of CP29 and CP26 between *C. reinhardtii* and higher plants may originate from a change in substrate specificity of the LHC-kinases themselves, as well as from a change in the organization of CP29 and CP26 within the light-harvesting antenna. These hypotheses are currently under study.

Function of the minor Chl-a/b proteins. The present data are consistent with a model that has been recently

proposed for the organization of the PSII antenna system in higher plants (Bassi and Dainese 1990). In fact there are at least two types of Chl-a/b proteins. In the first group are the LHCII-type polypeptides, which have a low Chl-a/b ratio, are closely related immunologically and are present in bulk quantity in the thylakoid membranes. In *C. reinhardtii*, this group includes p11, p13, p16 and p17, and in *Z. mays*, five polypeptides with MWs between 26 and 30 kDa. In the two organisms, all of these polypeptides but one can be phosphorylated at an N-terminal, trypsin-cleavable, peptide. In both *C. reinhardtii* and maize, one of these polypeptides (p16 and 26 kDa, respectively) lacks this N-terminal segment and therefore can neither be cleaved nor be phosphorylated. The function of this component in *Z. mays* may be that of a tightly bound antenna since it was shown to be excluded from the LHCII migrating to stroma membranes upon phosphorylation (Bassi et al. 1988a, b).

A second group of Chl-a/b proteins includes p9 and p10 (CP26 and CP29 in higher plants) which have an higher Chl-a/b ratio and absorption peaks shifted to higher wavelengths than those of LHCII. These proteins have weak immunological cross-reactions with either the major LHCII or the LHCI polypeptides. In higher plants these proteins are closely associated with RCII and are not phosphorylated during state transition in contrast with the situation in *C. reinhardtii*. On the other hand, the red shift in the absorption peak is larger in maize or spinach than in *C. reinhardtii*. These data may therefore indicate that a diversification occurred among Chl-a/b proteins at an early stage of phylogenesis: the divergence between the two groups of Chl-a/b proteins described above would be larger in higher plants than in *C. reinhardtii*, resulting in the loss of phosphorylation sites on the minor Chl-a/b proteins only in higher plants. Polypeptide 22 corresponds to CP24 and therefore to the related complex LHCI-680 (Ish-Shalom and Ohad 1983; Bassi et al. 1985, 1987). These two proteins have been shown to act as a connecting antenna between LHCII and CP29 and between LHCII and LHCI-730 in PSII and PSI, respectively. Polypeptide P22 may well have a similar function in *C. reinhardtii*.

When the absorption spectra of the chlorophyll-binding components of PSII are compared, the longer-wavelength absorptions, due to the minor antenna complexes p9 and p10, overlap the absorptions of the Chl-a-binding core complex (Fig. 9). Similar absorption patterns for Chl-proteins have been previously observed in barley PSI (Bassi and Simpson 1987), maize PSI (Bassi et al. 1985), maize PSII (Bassi and Dainese 1989), and more recently in *C. reinhardtii* PSI (Bassi, data not shown). In addition, maximum efficiency in energy transfer from Chl-a/b antenna to PSII RC was observed at 684 nm in thylakoids and PSII membranes (Jennings et al. 1990). Thus a general scheme can be suggested whereby pericentral Chl-a/b proteins absorbing at higher wavelength with respect to both the major LHCII and the Chl-a core complex act as a connecting antenna. The significance of such an organization is not clear. As a working hypothesis, the long-wavelength-absorbing (pericentral) antenna may be understood as a sink for the excitation energy delivered by the major LHCII, such that it could be used by the PSII RC when traps are open and absorb at 680 nm. Interestingly, it has been reported that fluorescence can be emitted when traps are closed by specially oriented long-wavelength pigments (Tapie et al. 1984). Alternatively, the pericentral antenna may be aside from the main energy-transfer pathway and thus may not be essential for excitation transfer toward photochemically active reaction centres. It would then act rather as a protection against photoinhibition when traps are closed. While previous studies on the PSI antenna system support the first hypothesis (Ortiz et al. 1985; Bassi and Simpson 1987), recent fluorescence measurements on PSI rather tend to support the second hypothesis (Stahl et al. 1989). However, as no similar data are available on the PSII antenna system, further biochemical and functional studies are required in order to assign a functional role to the various Chl-a/b proteins of the photosyntetic antenna system.

Owing to the similar light-harvesting properties it shares with higher plants, the green alga *C. reinhardtii*, which is well suited for both molecular genetics and in-vivo spectroscopic studies, appears to be a promissing organism for the investigation of the functional organization of light-harvesting antennae in the thylakoid membranes.

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