Evidence for a chlorophyll *a/b*–protein complex associated with Photosystem II

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Received 6 March 1984

A new chlorophyll *a/b*–protein complex designated as LHCPx was separated by SDS–PAGE. This complex differs from the light-harvesting chl *a/b*–protein of PS II, by its chl *a/b* ratio, its fluorescence properties and its apoprotein composition. Evidence is provided that this complex corresponds to a part of PS I antenna.

Photosystem I *Chlorophyll–protein complex* *Chlorophyll antenna* *Chlorophyll* b *Electrophoresis* *Light-harvesting complex*

1. INTRODUCTION

It is now well established that SDS–PAGE resolves up to 7 chlorophyll-containing zones from thylakoids of higher plants and green algae (review [1–3]). From the nomenclature in [4], these zones are designated in order of their increasing electrophoretic mobilities: CP1a, CP1, LHCP1, LHCP2, CPa, LHCP3 and free pigments. CP1a and CP1 both show the long wavelength fluorescence of PS I and P700 photooxidation. They represent the PS I reaction centre associated, respectively, in CP1a and CP1 with more or less chl *a* [5,6]. CPa probably represents the PS II reaction centre complex while LHCP1 and LHCP2 should be, respectively, the trimeric and dimeric forms of the monomeric LHCP3 which corresponds to the light-harvesting antenna of PS II.

Recently, we have shown that it was possible in vitro, to reconstitute the oligomeric form LHCP1 from the monomeric one (LHCP3) by mixing the latter with liposomes of different lipids and especially liposomes of phosphatidyldiacylglycerol [7,8]. Surprisingly, no LHCP2 formation was observed in these experiments. Moreover, as reported in [6], the low amount of chl generally found associated with LHCP2 suggests that LHCP2 is a labile complex, but its relationship to LHCP1 and LHCP3 is not clear. This is particularly evident in studies of the cation-induced transformation of the oligomeric to monomeric forms of LHCP [9]. On the other hand, it has been reported that LHCP2 differs from LHCP1 and LHCP3 by its chl *a/b* ratio and its carotenoid content [10]. For these reasons, a precise reinvestigation of the chl *b*-containing band separated by our SDS–PAGE technique and migrating between LHCP1 and LHCP3 was undertaken. Here, we describe a new chl protein complex designated LHCPx. This complex which shows the same apparent electrophoretic mobility as the LHCP2 in [4] differs from LHCP1 and LHCP3 by its chl *b* content, its fluorescence properties and its apoprotein compositon. The results presented provide evidence that LHCPx represents part of the chl antenna of PS I. Thus, using a different approach, we were able to corroborate the recent findings in [11–14] and extend our knowledge of the PS I antenna.

Abbreviations: CF 1, chloroplast coupling factor; chl, chlorophyll; CP, chlorophyll–protein complex; LHCP, light-harvesting chlorophyll *a/b*–protein complex; L1, L3, LHCP1, LHCP3; Lx, LHCPx; PS II, Photosystem I; PS II, Photosystem II; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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00145793/84/$3.00

2. MATERIALS AND METHODS

Chloroplasts and thylakoid membranes were isolated from 2-week-old pea (*Pisum sativum*) seedlings as in [15]. Thylakoid membranes were suspended in 50 mM Tricine buffer (pH 8.0) at about 2 mg chl/ml and solubilized at 4°C in the same buffer containing 10% sucrose and 2% SDS (SDS/chl weight ratio, 8–10). SDS–PAGE was immediately carried out at 100 V in gel tubes containing 8% acrylamide, 0.21% *N,N'*-methylenebisacrylamide, 0.1% SDS and 0.1 M Tris–borate buffer (pH 8.2) as in [5,15]. No stacking gel was used. The electrophoresis buffer was the same in the gels and in the electrode chambers.

When the solubilization was performed with a mixture of Triton–SDS, Triton X-100 was first added to the thylakoid suspension using the same Triton/chl ratio as for SDS, and homogenized. Then, SDS was added as before and the solubilized sample was either loaded immediately onto the gel, or after a lag time of about 20 min. In any case, a previous centrifugation of the sample was done, the whole sample entered in the gel. Densitometric tracings at 670 nm and spectra of individual chl-containing bands were obtained in situ in the gel, using the gel scanning attachment of a Unicam SP 1800 spectrophotometer. Fluorescence emission and excitation spectra were performed at 77 K in gel slices using a laboratory-built device by Dubertret consisting of two monochromators connected through the sample chamber by a two-arm light pipe. A xenon light source was used as actinic light. The band pass was 9 nm for the excitation light and 3 nm for the analysis. Excitation spectra were corrected for the emission ray of the xenon lamp and normalized by computer.

When required for polypeptide determination, entire gel or excised chl-containing bands were analyzed after denaturation on a second SDS–PAGE (12% acrylamide gel) as in [16]. Gels were stained for 30 min, with 0.2% Coomassie brilliant blue in a mixture of acetic acid–H_2O–methanol (1:5:5, by vol.) and destained overnight in 25% methanol, 7% acetic acid.

For staphylococcal V8 protease digestion, polypeptides of interest were first separated on 12% polyacrylamide gels with a 5% stacker, using the discontinuous buffer system in [17]. These polypeptides were located, excised, and treated as in [18]. The concentration of *Staphylococcus aureus* V8 protease (Miles Research Products) was given in fig.6. Digested polypeptides were separated on a 15% gel, and silver-stained as in [19].

As a control, a PS I fraction was prepared by Triton X-100 solubilization of pea chloroplasts as in [11]. A spectrophotometric measurement of P700 was made on an Aminco DW-2a dual wavelength, double beam spectrophotometer by the reversible light-induced bleaching at 698 nm, using 725 nm as an isobestic point. This PS I fraction was found to possess 1 P700/100 chl.

3. RESULTS

3.1. *Electrophoretic separation of chl–protein complexes*

Fig.1A depicts the classical densitometric pattern which is obtained when thylakoid membranes are subjected to SDS–PAGE after a mild solubilization with SDS at 4°C. Seven chl–protein complexes are resolved and designated as in [4], except that an additional CP1 complex is termed CP1b. The CP1 bands have been previously

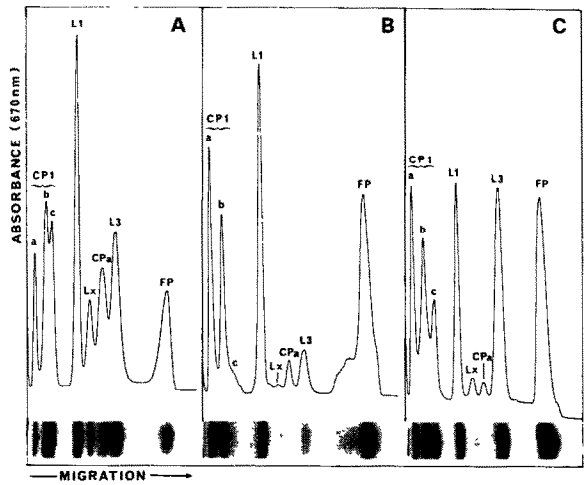

Fig.1. Densitometric tracings at 670 nm of chlorophyll-containing bands separated by SDS–PAGE-solubilized pea thylakoids and corresponding gels. (A) SDS-solubilization; SDS/chl ratio = 8 (w/w). Run immediately after detergent addition. (B) Triton/SDS solubilization. Run immediately after detergent addition. (C) As in B, but run 20 min after the addition of detergents.

characterized on the basis of their P700/chl ratios which increase from CP1a to CP1c [5] duc to a progressive depletion of the chl bound to these complexes.

LHCP1 (L1) and LHCP3 (L3) are, respectively, oligomeric and monomeric forms of the chl antenna of PS II of which CPa probably represents the reaction centre [5,15]. The complex earlier designated as IIb1 [5] has been termed LHCPx (Lx) and more precisely characterized below.

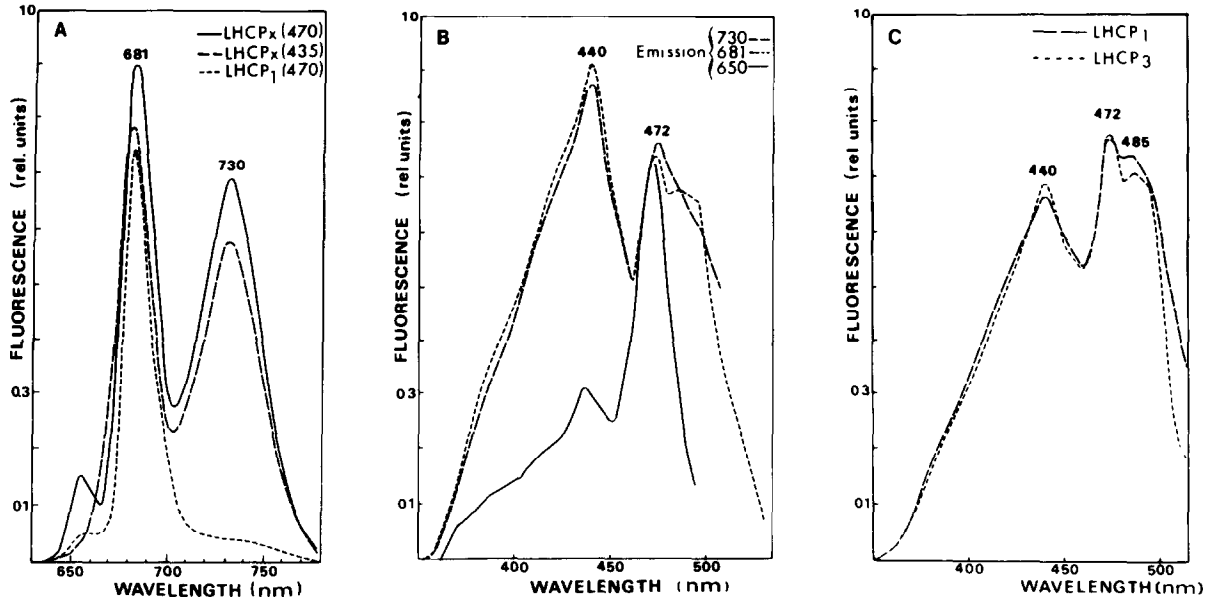

Fig.2. (A,B) Room temperature absorption spectra of chl–protein complexes in situ on gel slices. (C) Fluorescence emission spectra of CP1a, CP1b and CP1c on gel slices at 77 K. Asterisk: CP1c eluted from gel slices and dialyzed.

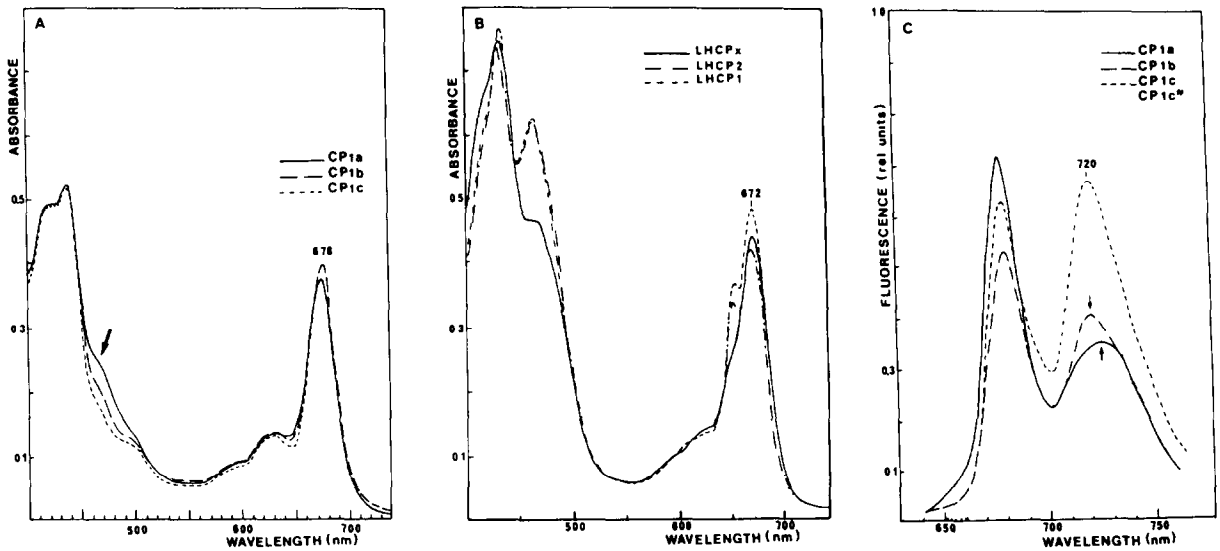

Fig.3. (A) Fluorescence emission spectra of LHCPx in situ on gel slices at 77 K with excitation at 470 and 435 nm. Idem for LHCP1 with excitation at 470 nm. (C) Fluorescence excitation of LHCPx in situ on gel slices at 77 K for the emissions at 730, 681 and 650 nm. (D) Fluorescence excitation spectra of LHCP1 and LHCP3 in situ on gel slices at 77 K for the emission at 681 nm.

Fig.1B represents the patterns obtained when the solubilization is performed with a mixture of Triton–SDS which allows a better preservation of the aggregated forms CP1a and CP1b, as well as of the oligomeric LHCP1. Both here and in fig.1A, the run was begun immediately after adding detergent. When the sample is incubated for at least 20 min in presence of the two detergents before electrophoresis, the oligomeric forms decrease in favor of the monomeric ones (fig.1C). Moreover, LHCPx which is scarcely detectable in fig.1B, becomes more apparent.

3.2. *Spectrophotometric characterization*

Fig.2A compares the absorption spectra of CP1a, CP1b and CP1c. It is obvious that in the blue region of these spectra, at 470 nm, the absorption decreases from CP1a to CP1c. This may be attributed to a loss of chl *b*. The comparison of the fluorescence emission spectra at 77 K (fig.2C) shows that the 720 nm fluorescence emission observed with CP1c gel slices, is progressively shifted to longer wavelengths as indicated by arrows in CP1b and CP1a. The strong emission at 681 nm is probably due to the detergent effect because if CP1c is eluted from the gel and extensively dialysed, there is only the 720 nm emission (CP1c with asterisk in fig.2C). On the other hand, the excitation spectra for the emission at 720 nm show a decrease in the participation of chl *b* from CP1a to CP1c (not shown). In fig.2B, the absorption spectra at room temperature of LHCP1, LHCP3 and LHCPx are compared. These spectra are very similar, except that LHCPx contains less chl *b* than LHCP1 and LHCP3 as shown by the decrease in absorbance at 652 and 470 nm. When measured on eluted LHCPx, the chl *a*/*b* ratio varies between 2.5 and 3, whilst it is in the order of 1.2–1.3 for LHCP1 and LHCP3.

The fluorescence emission spectra of LHCPx differ from those of LHCP1 and LHCP3 in that a strong emission at 730 nm is observed when the excitation is performed at 470 or 435 nm (fig.3A). In contrast to what is observed with the CP1s (fig.2c, CP1c*), the emission at 681 nm is not suppressed by elution and dialysis of LHCPx (not shown). The excitation spectra of LHCPx for the 730 and 681 nm emissions are quite similar. The contribution of chl *b* and carotenoids is less in LHCPx (fig.3B) when compared to the excitation spectra for emission at 681 nm of LHCP1 and LHCP3 (fig.3C).

3.3. *Polypeptide composition of LHCPx and oligomeric CP1s*

To determine the polypeptide composition of

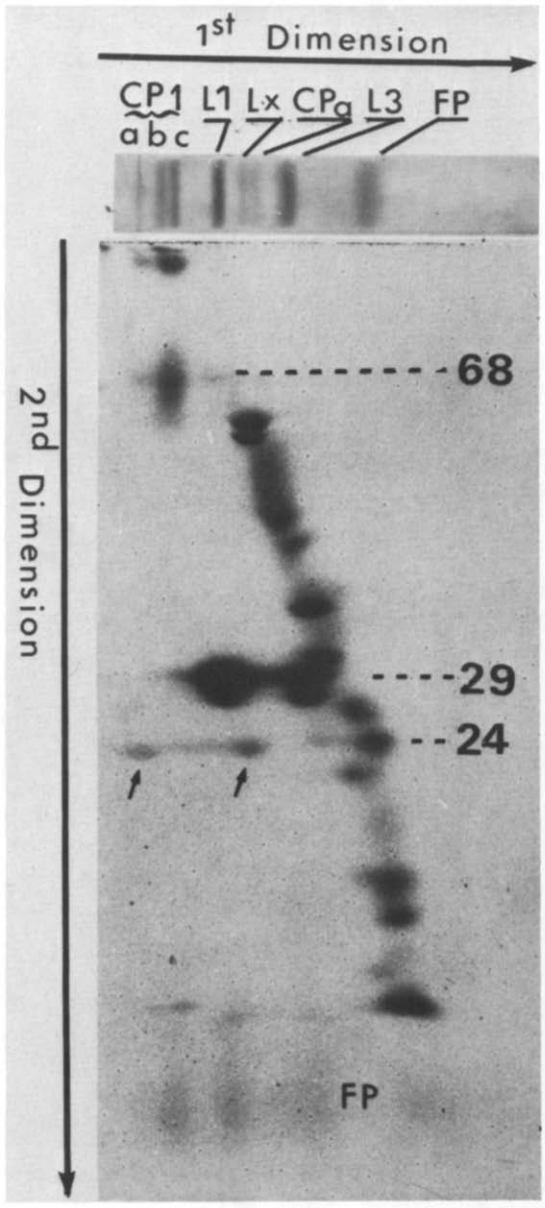

Fig.4. Two-dimensional electrophoretic map of the chl–protein complexes and polypeptides of pea thylakoids. The arrows indicate a 24 kDa polypeptide present in CP1b and LHCPx. First dimension: 8% acrylamide, second dimension: 12% acrylamide.

the chl–protein complexes a 2-dimensional electrophoresis was performed with a gel similar to that presented in fig.1A. It is shown in fig.4 that:

(i) LHCPx possesses only one major polypeptide of about 24 kDa and thus differs from LHCP1 and LHCP3 which both contain two polypeptides of 29 and 27 kDa;

(ii) In addition to the 68–66 kDa polypeptides of CP1c, the CP1b contains a 24 kDa polypeptide apparently similar to that found in LHCPx. Peptides belonging to CP1a are not detected as their concentration is too low.

In order to be more conclusive about the polypeptide composition of CP1a,b,c and LHCPx, gel slices corresponding to these complexes were excised from the tube gels. After denaturation, these were subjected to further electrophoresis in a second dimension. As shown in fig.5, the 24 kDa polypeptide is found in CP1a, CP1b, LHCPx and in the PS I fraction prepared as in [11]. In addition, a 29 kDa polypeptide, migrating in the same way as the 29 kDa polypeptide of LHCP1 (or 3) is also found in CP1a, CP1b and LHCPx. Re-electrophoresis of LHCPx gel slices also gives a major polypeptide at about 56 kDa. This corresponds to the α- and β-subunits of CF1, which comigrate with LHCPx in the first dimension, and which appear as spots displayed diagonally in the second dimension (fig.4). Using overloaded slices (right side of fig.5), additional polypeptides of 26, 18, 12 kDa appear in CP1a, whilst in CP1b only the 12 kDa polypeptide is detectable. Thus, all the polypeptides found in CP1a seem to be constituents of the PS I fraction.

3.4. *Partial peptide mapping*

To test critically the possibility that the 24 kDa polypeptides of LHCPx and CP1a and PS I are related protein components, partial proteolyses of these polypeptides using *S. aureus* protease were performed. The same comparison was done with the 29 kDa of LHCPx and LHCP1. In fig.6, it is shown that the digestion products of the 24 kDa polypeptides are similar for LHCPx, PS I and CP1a (not reported). In the same way, LHCP1 and LHCPx's 29 kDa appear similar.

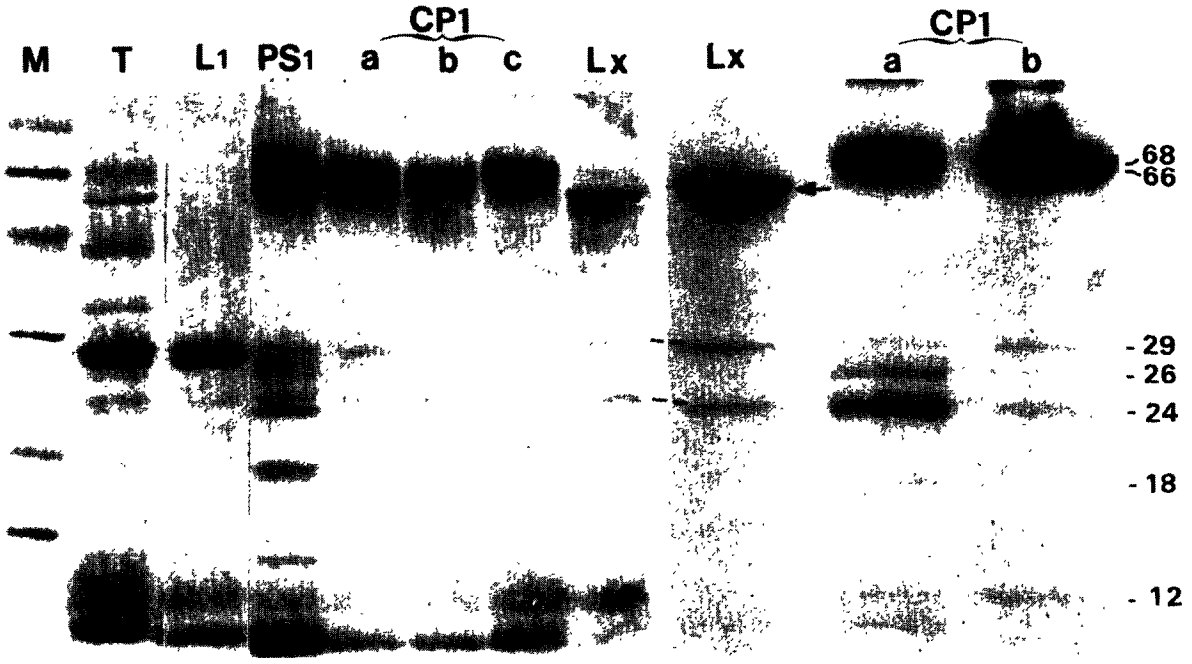

Fig.5. Gel slices of chl–protein complex re-electrophoresed in a second dimension. M, markers; phosphorylase *b* 94 kDa; bovine serum albumin 67 kDa; ovalbumin 43 kDa; carbonic anhydrase 30 kDa; soybean trypsin inhibitor 20 kDa; α-lactalbumin 14 kDa. T, thylakoids as control. PS I, a fraction containing 1 P700/100 chl prepared as in [11]. Loads with 5 mm diameter gel slices of L1 (LHCP1); CP1a, CP1b, CP1c and Lx (LHCPx). On the right side overloading with 12 mm gel slices of Lx (LHCPx). CP1a and CP1b. The arrow indicates the polypeptides of CF1 which comigrate in the first dimension with LHCPx.

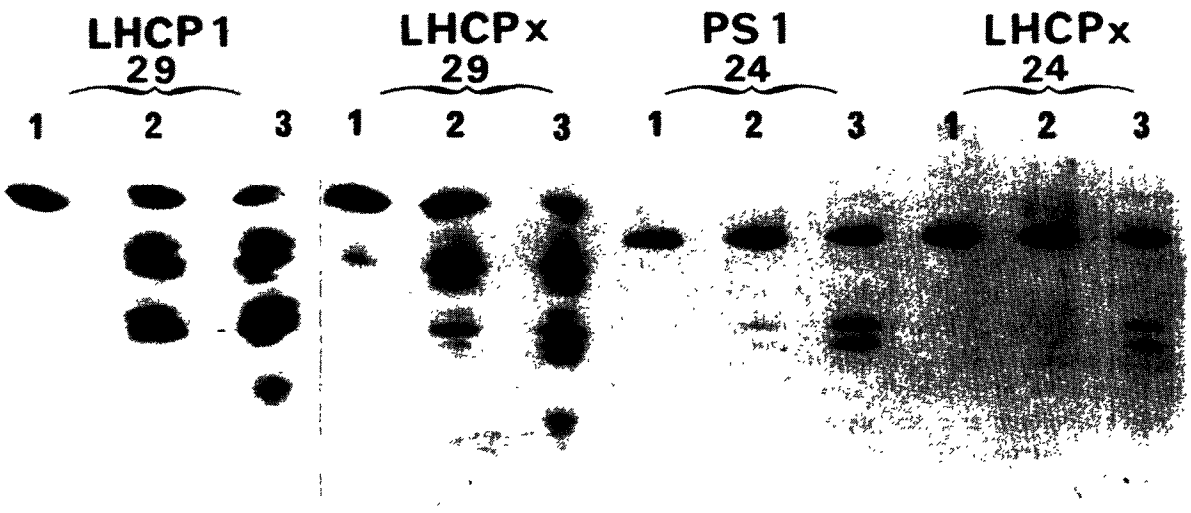

Fig.6. Partial proteolytic digest of the 29 kDa polypeptides of LHCP1 and LHCPx and of the 24 kDa polypeptides of a PS I fraction and LHCPx: (1) undigested; (2) 0.1 μg V8 protease; (3) 0.5 μg V8 protease. Gel was stained with silver.

4. DISCUSSION AND CONCLUSION

It has been demonstrated that some of the chl-containing bands separated by SDS–PAGE represent either aggregated or oligomeric forms of individual chl–protein complexes [1–3]. These bands are thought to represent the in situ supramolecular organization of these complexes. An example of an oligomer is given by LHCP1 which is probably a dimer of LHCP3 [15,20]. A different situation is observed with the CP1 complexes. In agreement with [6], we do not think that CP1a or CP1b are oligomers of CP1, but are likely to represent a more complicated molecular organization including, in addition to the 58–56 kDa apoproteins of CP1, some other low molecular polypeptides. Many preparations, differing in the physical methods of membrane fractionation, have shown such additional polypeptides [6,11–14,21,22]. Moreover, the presence of chl *b* in these supramolecular forms of CP1 was previously suspected [15,22]. The existence of a specific antenna complex associated with PS I was first proposed on a theoretical basis [23], this antenna being structurally independent from PS II [24,25]. Up to now, the only evidence of the presence of a light-harvesting complex related to PS I was reported in *Chlamydomonas* [26]. In higher plants, it has been shown by greening experiments [12] and fractionation studies [11] that in addition to the 58–56 kDa polypeptides of CP1, some low-M_r polypeptides are involved in the organization of the PS I antenna. Very recently, it has been proposed [14] that 10% of the chl associated with a PS I fraction is chl *b*. Moreover, this PS I fraction can be split into a core complex fraction and an antenna termed LHC1. Chl *b* remains associated with the antenna fraction. This fraction shows a long wavelength fluorescence emission (735 nm) and is composed of a group of polypeptides of 19–24 kDa. The core fraction exhibits a shorter wavelength fluorescence emission (722 nm). Our results demonstrate for the first time that using higher plant thylakoids it is possible to obtain, by electrophoretic separation, either the chl *b*-containing complex alone or associated with supramolecular forms of CP1 (CP1a and CP1b). Furthermore, as we have also found the polypeptides of this complex present in PS I fractions, it is likely that the LHCPx we describe is the essential part of the PS I antenna termed LHC1 in [14].

A final proof that LHCPx is a part of PS I antenna is provided by the fact that we have found traces of P700 in this complex: about 1 P700/1000 chl.

A point remaining to be clarified is related to the presence of the 29 kDa polypeptide in LHCPx. It has been reported [27] that polypeptides of LHC1 are synthesized in the cytoplasm and matured in

the chloroplast as in the case of PS II antenna (apoproteins of LHCP1 and LHCP3). For that reason, authors in [14] mention that it would be interesting to see if any homologies exist in the primary structure of the apoproteins of these two antennae. If the 29 kDa polypeptide of LHCPx is not a contaminant, results presented in fig.6 may provide evidence that the two antennae are synthesized via partially common pathways. However, it is necessary to remain wary of this interpretation as the 29 kDa polypeptide of LHCPx might come from the degradation of LHCP1 during the course of electrophoresis. If we consider the 2-dimensional analysis presented in fig.4, it is clear that this possibility must be taken into account. Nonetheless, it is reasonable to maintain this hypothesis until proof to the contrary, as the 29 kDa polypeptide is also present in CP1a.

Two chl *a/b*–proteins associated with PS I were recently described in [30]. This finding appears in good agreement with the presence of two different polypeptides in LHCPx described above.

ACKNOWLEDGEMENTS

We are grateful to Dr G. Dubertret who facilitated spectrofluorimeter experiments and to Mrs B. Maison for English corrections. This work received financial support from the CNRS, France (ATP: Conversion de l'énergie dans les membranes biologiques).

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