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Identification, isolation and partial characterisation of a 14–15 kDa pigment binding protein complex of PS II from spinach

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We described recently a new Chl *a/b* binding polypeptide in PS II from spinach (Irrgang, K.-D., Bechtel, C., Vater, J. and Renger, G. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 375–378, Kluwer, Dordrecht). This 14–15 kDa chlorophyll *a/b* binding polypeptide complex was isolated and purified from intrinsic photosystem II membrane polypeptides. About 3 Chl *a* and 1 Chl *b* were bound per protein molecule. A polyclonal antiserum was induced against this pigment-protein complex. SDS polyacrylamide gradient gel electrophoresis in combination with immunoblotting revealed reactivities with two polypeptides of very similar relative molecular masses of 14 and 15 kDa that are clearly identified in thylakoids, PS II membrane fragments, ISO and RSO thylakoids. Their N-termini were blocked by an as yet unidentified modifying group. Using analytical isoelectric focusing under denaturing conditions their isoelectric points were determined to be 5.2–5.3 and around 6.0–6.5. The isolated polypeptides of the pigment-protein complex tend to self-associate into oligomeric forms of about 66–70 kDa. Furthermore, under mild solubilisation conditions an oligomeric pigment-protein complex of 120 kDa was observed. This oligomer was shown to be heterogeneously composed of the 14–15 kDa proteins and at least another pigment-binding polypeptide with an M_r of 22–24 kDa. The low molecular mass pigment-protein complex *(CP14–15)* is proposed to act as an additional antenna complex within PS II.

Introduction

Light-induced photosynthetic water oxidation and plastoquinone reduction take place in a supramolecular thylakoid membrane protein complex termed Photosystem II [1,2]. The polypeptide composition of the PS II is still a matter of debate. To our present knowledge, the smallest functionally intact O_2-evolving complex, the so-called PS II core complex, consists of at least seven polypeptides with molecular masses of 47, 43, 34, 33, 31, 9 and 4 kDa [1,2]. The photochemically active reaction centre of the PS II is located within a subcomplex of the 34, 31, 9 and 4 kDa proteins namely, the D1-D2-Cyt*b*559 complex [3,4]. Recently, the presence of some low-molecular-mass proteins ($M_r < 10000$) was reported [5]. Some of them were purified and partially characterised by immunoblotting and N-terminal sequence analysis [5–12]. However, the function of these small subunits is not yet known. Besides the core complex and small subunits, several Chl *a/b* binding polypeptides in the M_r range of 24000–30000 have been identified and isolated forming the core – and peripheral antenna apparatus of PS II (see Refs. 13,14 for reviews). In this contribution we report on the identification, purification and partial characterisation of a 14–15 kDa Chl *a/b*-binding protein complex, probably containing two different polypeptides, and discuss its possible role.

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Abbreviations: Chl, chlorophyll; CP, chlorophyll protein complex; ISO, inside out; LHC I and II, light-harvesting complexes of Photosystems I and II; Mes, *N*-morpholinoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PS I and II, Photosystems I and II; RSO, right-side-out.

Materials and Methods

Isolation of PS II. PS II membrane fragments were isolated from spinach chloroplasts according to Berthold et al. [15] with modifications as described in Ref. 16 and addition of proteinase inhibitors [17].

Thylakoid membrane fractionation. Using the aqueous two-phase technique developed by Albertsson [18]

thylakoid membranes were fractionated by Yeda press treatment as described recently by Andersson et al. [19] and Akerlund et al. [20]. Dextran 500 and Poly(ethylene glycol) 3350 were applicated as polymers. The ISO and RSO vesicles were kindly provided by Dr. W.P. Schröder.

Preparation of intrinsic PS II membrane proteins. Purified PS II membrane fragments were depleted of extrinsic polypeptides by the high salt wash procedure reported in Ref. 17.

Density gradient experiments. Solubilisation of the intrinsic membrane proteins with β-dodecyl maltoside and separation via detergent-containing linear sucrose gradients were performed as described recently [17]. Gradients were analyzed for protein [21] and Chl distributions [22]. Sucrose content was determined refractometrically.

AcA 54 gel-filtration chromatography. The intrinsic membrane proteins were resuspended in 10 mM Mes-NaOH (pH 6.5), 10 mM NaCl, 2 mM benzamidine, 1 mM PMSF and SDS added to a detergent to Chl ratio of 16:1. Solubilisation was allowed for 30 min. Aggregates were removed by low-speed centrifugation using an Eppendorf minifuge (10 min, 12000 rpm). Solubilised proteins equivalent up to approx. 25 mg Chl (approx. 62.5 mg protein) were loaded onto an AcA 54 gel-filtration column (87 cm × 1.8 cm). The equilibration and running buffer was 10 mM Mes-NaOH (pH 6.5), 10 mM NaCl, 2 mM benzamidine, 1 mM PMSF, 0.4% (w/v) SDS. The flow rate was 30 ml/h and fractions of 0.5 ml were collected. All the manipulations were carried out at room temperature in the dark. The fractions were analysed for protein [21] and Chl content [22].

Analytical electrophoresis. One-dimensional SDS/urea/PAGE was performed essentially as in Ref. 23. SDS-containing polyacrylamide gradient gels (13–20 w/v% acrylamide; dimensions: 125 × 165 × 2 mm) were run according to Ljungberg et al. [24]. For two-dimensional PAGE we used a slightly modified version of O'Farrell's method [25]. Using samples of intrinsic PS II membrane polypeptides 50–100 μg total protein were separated within 9000–9500 V h after solubilisation in lysis buffer [25]. After electrophoresis the one-dimensional gels have been equilibrated up to 1 h in Laemmli sample buffer (0.5% (v/v) β-mercaptoethanol) [26] and then polymerised in 2% (w/v) agarose in stacking gel buffer prior to running in the second dimension. The two-dimensional gels had the same composition as described recently [23]. Running was carried out overnight at 10 mA/slab gel and staining as recommended by Oakley et al. [27].

Preparative SDS / urea / PAGE. Preparative gel electrophoresis was performed using the procedure reported recently [23]. Gel and tank buffers were the same as recommended by Laemmli [26]. We used a separating gel with dimensions of 85 × 140 × 3 mm and a stacking gel of 15 mm in length, which was poured without slots. After preelectrophoresis for 20 min at 20 mA, the gels were run at the same amperage at 4°C in the dark until the marker Bromophenol blue reached the bottom of the gel. The protein was indirectly localised by staining two marginal strips of the preparative gel with either 0.1% (w/v) Coomassie R-250 dissolved in 50% (v/v) methanol, 7.5% (v/v) acetic acid, 10% (w/v) trichloroacetic acid or silver as in Ref. 27. Additionally, the pigment-protein complex was directly identified by its red fluorescence after excitation with UV-light (λ = 365 nm). The protein band was cut out of the unstained part of the gel and finally electroeluted using the method described previously [28]. Usually, electroelution was completed within 24 h at 8–10 mA/gel tube.

Electrophoretical identification of a 120 kDa pigment-protein complex. PS II membrane fragments (corresponding to 1 mg Chl) were solubilised with β-dodecyl maltoside for 1 h in the cold and dark (detergent/Chl ratio 10:1 (w/w)). Non-solubilised polypeptides were eliminated by centrifugation (10 min, 12000 rpm) using a Beckman minifuge. The supernatant was further treated with SDS for 5 min at room temperature using SDS/Chl ratios varying from 2:1 to 16:1 (w/w). Subsequently, the samples were electrophoretically separated as described previously [23] with the buffer systems developed by Laemmli [26]. Control samples were merely solubilised with SDS for 30 min at room temperature in the dark with detergent/Chl ratios of 2:1–16:1 (w/w) and run in coelectrophoresis.

Immunological experiments. 300 μg of the electrophoretically purified pigment-protein complex (solubilised in 62.5 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS) were injected into a New Zealand rabbit using standard procedures. We titrated the antiserum on nitrocellulose filters applying a dot blot ELISA system with an alkaline phosphatase-conjugated goat anti rabbit antibody (Medac, dilution 1:2000) in combination with Fast Red and Naphtol AS MX phosphate (Sigma) as substrates. Immunoblotting was carried out either on nitrocellulose or PVDF membranes with the buffer system developed by Towbin et al. [29]. The membranes were usually saturated with 5% (w/v) skimmed milk powder (Uelzena) in PBS (pH 7.4) for 1 h at 45 °C prior to exposing them to the appropriate antiserum dilution. Anti-pigment antibodies have been separated from the total antiserum by the following experimental protocol: Approx. 10 mg Chl were extracted with 80% (v/v) acetone and filtered. Aggregated polypeptides were removed by low-speed centrifugation (8000 rpm, 10 min, 4°C) using either an SS 34 or an HB 4 swinging bucket rotor in a Sorvall centrifuge. The pigment extract was concentrated by vacuum centrifugation at room temperature in the dark. The dried pigment was

resolubilised in PBS (pH 7.4), 1% (v/v) Tween 20, aggregates eliminated by centrifugation and then preincubated with the antiserum at dilutions as indicated above for 30–120 min at 8°C. Polyclonal antisera directed against the extrinsic 10 kDa polypeptide [30] and a peptide of the 9 kDa α-subunit of the heterooligomeric cytochrome *b*-559 [31] were kindly provided by the groups of Profs. B. Andersson and W. Cramer. These antisera were used to identify possible contaminations by these low molecular mass polypeptides in the isolated CP 14–15. We could not detect any α-subunit of the cytochrome *b*-559 and extrinsic 10 kDa polypeptide in our preparations.

Spectroscopic measurements. Absorption spectra were recorded at room temperature using a Perkin-Elmer double-beam spectrophotometer (model 556) in combination with a baseline corrector (Hitachi). We used a slit width of 1 nm. Derivative spectra were calculated applying a home-made computer program (developed by B. Hanssum) on the basis of a spline algorithmus.

Results

Identification and purification of the 14–15 kDa pigment protein complex

PS II membrane fragments depleted of extrinsic proteins have been solubilised with β-dodecyl maltoside and separated via detergent-containing linear sucrose gradients (Fig. 1a). Using this technique a PS II core complex could be isolated being capable of electron transfer from 1,5-diphenylcarbazide to 2,6-dichlorophenolindophenol [17]. In fractions containing the polypeptides of the Chl *a*/*b*-binding LHC II several low-molecular-mass proteins of until now unknown function were observed. Among them a polypeptide of approx. 14–15 kDa was identified (see Fig. 1b, lanes 3–5). On the other hand, the PS II core complex was completely devoid of this protein (Fig. 1b, lanes 6–9). The intrinsic PS II membrane proteins have also been separated by gel-filtration chromatography using an AcA 54 column in the presence of SDS (see Fig. 2.1). The fractions were collected and analysed by SDS/urea/PAGE. A typical electrophoretogram shows an enrichment of a 14–15 kDa polypeptide together with other low molecular mass proteins (see Fig. 2.2, lanes 5, 9 and 10). The bands focusing near 30 kDa are due to contaminating polypeptides of the LHC II.

Gel electrophoretic analyses of PS II membrane fragments and intrinsic membrane proteins run in the cold and dark indicated at least one pigment-containing band resolved in the low molecular mass region ($M_r < 20$ kDa, see Fig. 3). Its identification was either achieved by transillumination of the gels with VIS- or after excitation with UV-light ($\lambda = 365$ nm) and localising the pigment-containing bands by their red fluorescence. We isolated, purified and characterised it with special emphasis on the following questions:

(*1*) is the 14–15 kDa pigment-protein complex an independent component of PS II? (*2*) does the complex specifically bind pigments and, if so, does is it act within PS II as a single light-harvesting unit or as a subunit of a larger Chl-protein complex? (*3*) is there any structural homology with respect to its polypeptide backbone or pigment composition to the other so far known Chl *a* and/or Chl *a*/*b* binding proteins of PS II? (*4*) what could be its functional role within PS II?

Fractions containing the 14–15 kDa polypeptide obtained either by density gradient centrifugation or gel-filtration chromatography experiments were concentrated as described above, dialysed against electrophoresis sample buffer and separated by preparative gel electrophoresis. After localisation, the pigment-protein complex was excised and recovered by electroelution [28]. In 90–95% of our experiments ($n = 20$) it was homogeneous as was judged by analytical one-dimensional electrophoresis (see Fig. 4, lane 2).

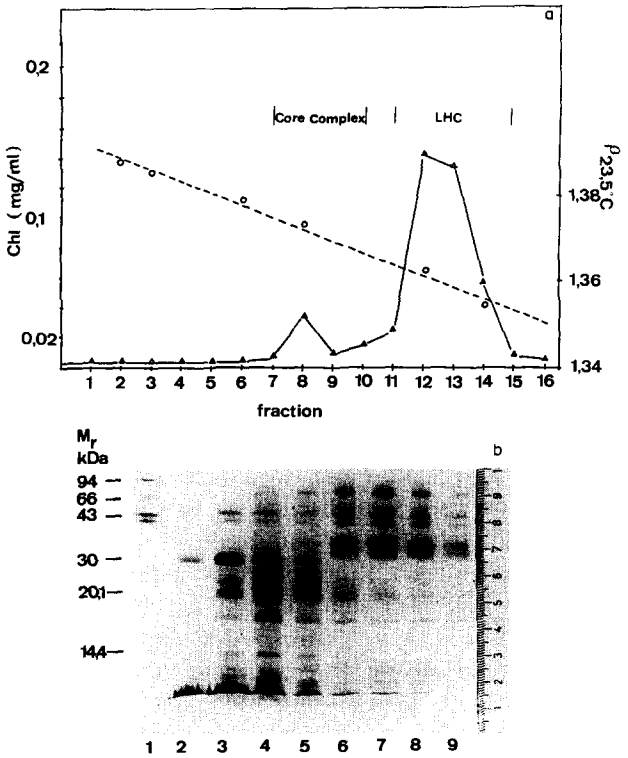

Fig. 1. Sucrose density gradient centrifugation in the presence of β-dodecyl maltoside. (a) Chlorophyll distribution of the fractionated gradient (△ ——— △), refractometrical determination of sucrose (○---○). (b) SDS/urea/PAGE of density gradient fractions. Lane 1: molecular mass marker proteins; lanes 2–5: LHC II and CP 14–15 containing fractions; lanes 6–9: PS II core complex containing fractions. Staining was carried out with silver according to Ref. 27.

Biochemical and spectroscopic characterisation of the isolated pigment-protein complex

The purified monomeric SDS-solubilised pigment-protein complex has a relative molecular mass of 14–15 kDa as has been estimated from uniform one-dimensional SDS/urea/polyacrylamide gels (Fig. 4, lane 2). Frequently, however, we observed an heterogeneous oligomerisation of the isolated complex to aggregates of relative molecular masses of 66–70 kDa (see below). This heterogeneity could be confirmed by two-dimensional PAGE and immunoblotting experiments both in the monomeric and oligomeric states (vide infra). Starting, for instance, with PS II membrane fragments or intrinsic polypeptides a 14–15 kDa protein was observed mainly in one- and two-dimensional PAGE, if the proteins were solubilised prior to running as follows: (1) solubilisation by sonication in sample buffer of the Laemmli gel electrophoresis system [26] using 2% (w/v) SDS; (2) solubilisation for 1–2 h in the presence of 2% (v/v) Triton X-100 and 9.5 M urea under vigorous mixing. In contrast to that, we did not detect any 14–15 kDa protein after mild presolubilisation with β-dodecyl maltoside (detergent/Chl ratio 10:1 (w/w)) and subsequent incubation with Laemmli-sample buffer with increasing amounts of SDS (SDS/Chl ratio 2:1–16:1 (w/w)). Under these experimental conditions a polypeptide of 22–24 kDa, probably belonging to the minor Chl protein complex, concomitantly diminished with the disappearance of the 14–15 kDa protein. At the same time, a 120 kDa green band appeared on SDS-urea polyacrylamide gels that was clearly separated from CP 110, the reaction centre complex of PS I (Fig. 5, lanes 8–12). On the other hand, the 14–15 kDa and 22–24 kDa polypeptides were immediately visualised without presolubilisation with the glycosidic detergent after exposure to SDS at all SDS/Chl (w/w) ratios and the large pigment-containing complex of 120 kDa was scarcely observable (Fig. 5, lanes 2–6). From these results it follows that

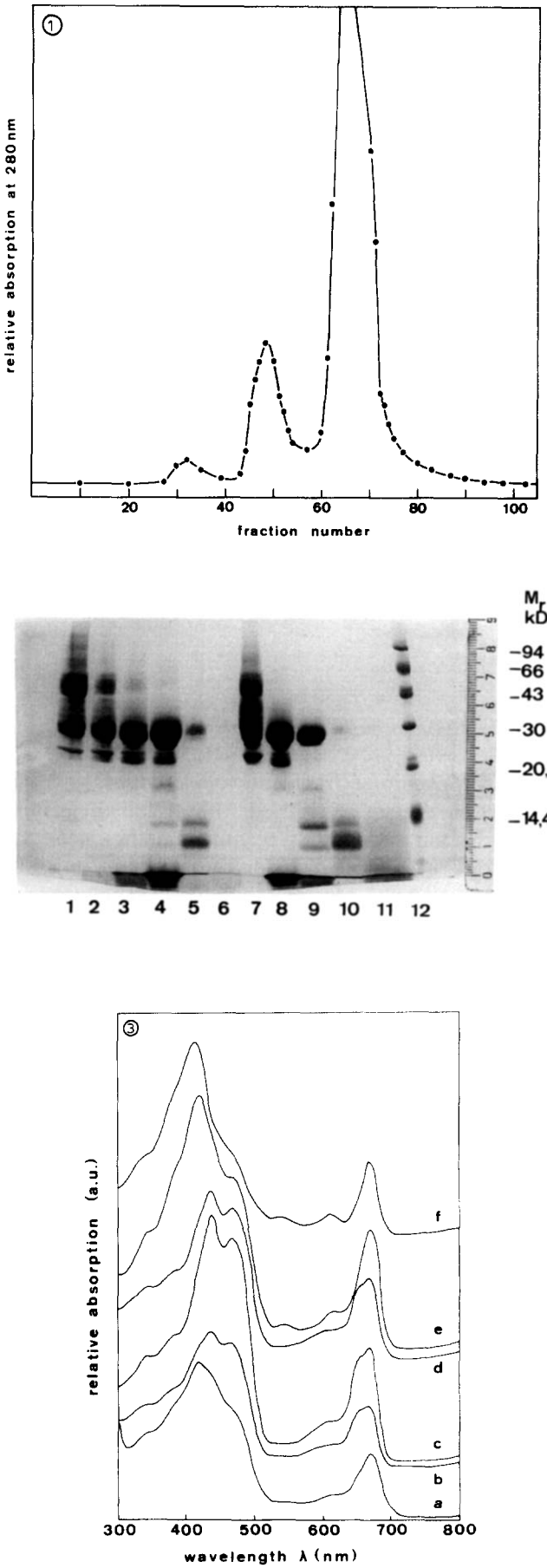

Fig. 2. Gel-filtration chromatography on an AcA 54 column. 2.1. Elution profile of an AcA 54 column chromatography 10.32 mg Chl were loaded in 6.8 ml equilibration buffer (for experimental details see Materials and Methods). 2.2. SDS/urea/PAGE of intrinsic PS II membrane protein fractions. Separation was achieved by AcA 54 gel-filtration chromatography using 0.4% (w/v) SDS in the eluent. Staining: 0.1% (w/v) Coomassie R 250 Lane 1 and 7: fraction 45; lane 2 and 8: fraction 47; lanes 3 and 9: fraction 50; lane 4: fraction 55; lanes 5 and 10: fraction 65 (low molecular mass polypeptides including the 14–15 kDa protein); lanes 6 and 11: fraction 75 (free Chl *a* and SDS monomers); lane 12: marker polypeptides. 2.3. Room temperature absorption spectra of different fractions of the AcA 54 gel filtration chromatography experiment demonstrated in 2.2. (a) fraction 45; (b) fraction 47 (dilution: 1:5); (c) fraction 50 (dilution 1:10); (d) fraction 55 (dilution: 1:20); (e) fraction 65 (dilution: 1:2); (f) fraction 75 (dilution: 1:2).

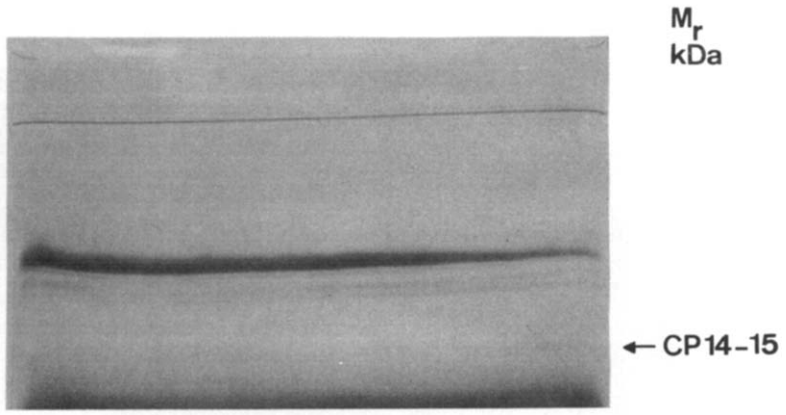

Fig. 3. Preparative SDS/urea/PAGE of intrinsic PS II membrane proteins (14% and 6% acrylamide in the separating and stacking gel; 5 M urea and 0.1% (w/v) SDS). Electrophoresis was carried out in the dark at 4°C.

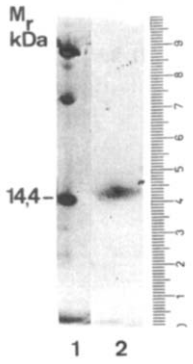

Fig. 4. SDS/urea/PAGE of the purified pigment-protein complex of 14–15 kDa. Lane 1: 5 μg molecular mass marker proteins; lane 2: 2 μg of the monomeric pigment-protein complex of 14–15 kDa purified by preparative PAGE and subsequent electroelution; staining: silver [27].

the 14–15 kDa protein and at least a part of the minor pigment-protein complexes tend to associate to a large Chl-protein complex of about 120 kDa. This complex seems to be very stable after β-dodecyl maltoside presolubilisation, since a clearly focusing pigment-containing band was obtained in all of our experiments ($n = 6$). Being surrounded by a bulky β-dodecyl maltoside layer, SDS is obviously unable to denature this complex.

In Fig. 2.3 the absorption spectra of the peak fractions of the gel-filtration column chromatographies are shown. The 14–15 kDa pigment-protein complex eluted normally within the decrease of the slope of the third peak (see Fig. 2.3, absorption spectrum e). The room temperature absorption spectrum of the electroeluted SDS-solubilised pigment-protein complex is demonstrated in Fig. 6. It is characterised by a maximum in the red at 670 nm due to Chl *a* and a small asymmetry near to 650 nm, indicating the presence of Chl *b*. The small shoulders at 625 and 470 nm are probably due to pheophytin and carotenoids. Presumably, the former was artificially induced by a loss of Mg^{2+} during the preparation and therefore has no functional importance. The dashed curve of Fig. 6 depicts the calculated second derivative spectrum. Three main pigment components were resolved, peaking at 672, 664 and 645 nm. The first and second peaks probably represent two Chl *a* populations differing in their conformational states and that at 645 nm is attributed to Chl *b*. The Chl *a* molecules absorbing at 664 nm could belong to a pigment population either loosely bound to the protein backbone or being disconnected and merely enclosed within SDS micelles. But this observation requires further investigations. In addition, a detergent-induced artificial alteration of functionally active pigment molecules cannot be excluded. First evidence came from results of studies on the state of chlorophyll in vivo (see Ref. 32 for a review, and references therein). Deconvolution of absorption spectra of different plants clearly revealed discrete pigment forms at 664–666 nm [32].

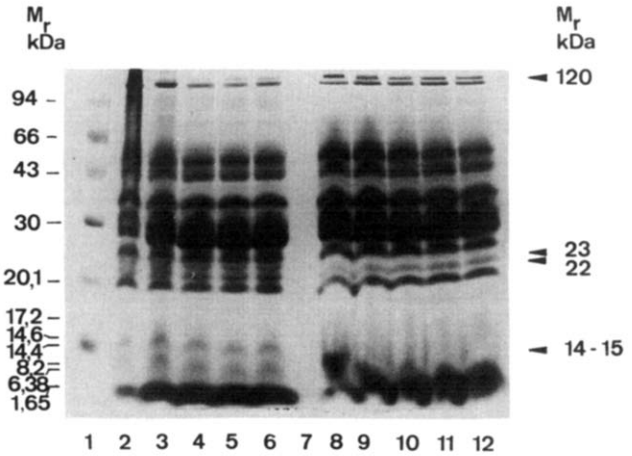

Fig. 5. SDS/urea/PAGE of polypeptides from PS II membrane fragments either after solubilisation with SDS/Chl ratios (w/w) of 2:1 (lane 2), 5:1 (lane 3); 8:1 (lane 4); 10:1 (lane 5); 16:1 (lane 6) or after presolubilisation with β-dodecyl maltoside followed by SDS treatment with SDS/Chl ratios (w/w) of 2:1 (lane 8); 5:1 (lane 9); 8:1 (lane 10); 10:1 (lane 11) and 16:1 (lane 12). 10 μg molecular mass marker proteins (lane 1). Samples: 60 μg Chl/slot. Staining: 0.1% (w/v) Coomassie R-250.

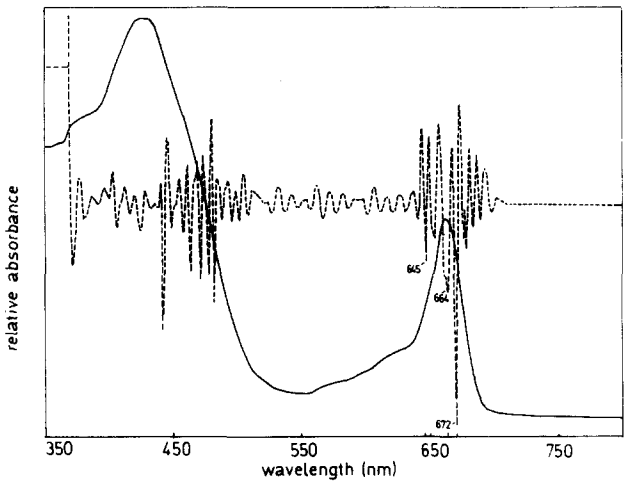

Fig. 6. Room temperature absorption spectrum of the isolated CP 14–15. The calculated second derivative spectrum is shown in dotted lines.

Using the methods developed by Arnon [22] and Bradford [21], quantitative estimations of the pigment content and protein concentration yielded about 3 Chl *a* and 1 Chl *b* per protein molecule. A small shoulder at approx. 470 nm gives some evidence for the presence of carotenoids within the detergent-solubilised complex; however, their number has not yet been determined. In relationship to the total amount of pigment of the original material employed, approx. 2% was recovered in the isolated and purified pigment-protein complex.

For a better electrophoretical resolution of the PS II core and LHC II polypeptides in general and especially for determining the isoelectric point of the 14–15 kDa polypeptide two-dimensional gels were run according to O'Farrell's procedure [25] with some modifications as described above. Although two-dimensional gel electrophoresis is the most powerful technique to resolve complex protein compositions, it has not been widely used for the separation of thylakoid membrane polypeptides. Only a few groups apply this method routinely [33–40]. Using intrinsic PS II membrane polypeptides we obtained the following results by our two-dimensional gel electrophoretic analyses: in all experiments performed, a 14–15 kDa protein with a p*I* of 5.2–5.3 was resolved (see the arrows in Fig. 7a and b). We sometimes found one to two additional spots of approximately the same M_r, but with isoelectric points of 6.0–6.5 (see Fig. 7a and b). This inhomogeneity of the protein was a further explanation for the different oligomeric forms with relative molecular masses of 66–70 kDa in one-dimensional SDS-polyacrylamide gels (vide infra). A posttranslational modification of the protein (perhaps a phosphorylation) or a polymorphism may be the reasons for multiple spots, but further investigations are required to corroborate this idea. At the moment, an artificial modification during the preparation or the electrophoresis procedure cannot be totally excluded. This point will be discussed in the next paragraph. Under short equilibration conditions (< 30 min) we were able to separate the proteins in the low molecular mass region into at least ten spots (gels not demonstrated). The number of proteins detected within the M_r of 3–14000 fits very well with the recently obtained data from electrophoretical analyses [11], N-terminal sequencing of isolated polypeptides and the identification of the loci of the corresponding genes within the chloroplast genome [5–12]. Those amino acid sequences which do not correspond to any of the open reading frames of chloroplast genomes [41,42] are probably encoded within the nucleus [7,9,11]. Using longer equilibration periods for the one-dimensional gels the polypeptides of relative molecular masses smaller than 10 kDa were often lost due to diffusion (Fig. 7a and b). With respect to the proteins with a $M_r > 14000$, highly reproducible two-dimensional gel electrophoretic patterns were obtained. We found multiple spots as well as a tendency to streaking for the proteins of the LHC II, its oligomeric forms and the 47 and 43 kDa Chl *a* binding polypeptides. This is in agreement with the data reported recently [36–40]. Streaking of the spots was preferentially observed after prolonged equilibration of the one-dimensional gels. It seems worth mentioning that, under denaturing conditions, the bulk of the pigment-binding

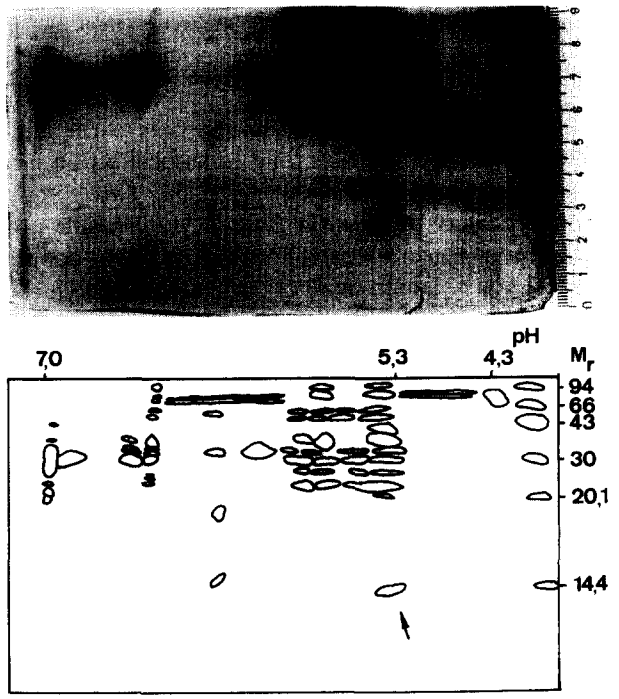

Fig. 7. (a) Two-dimensional PAGE (first dimension IEF; second dimension SDS/urea/PAGE) of intrinsic PS II membrane proteins. (b) schematic diagram of the spot pattern obtained immediately after silver staining of the gel by drawing on a transparency. Sample concentration: 100 μg/100 μl.

polypeptides are focusing within a narrow range of 5.2–5.6 in all of our IEF-experiments.

To unambiguously identify the 14–15 kDa pigment-protein complex as an independent component of PS II and to completely exclude a degradation product, we attempted to sequence the polypeptide by solid phase and gas phase sequencing. Unfortunately, our N-terminal sequence analysis was not successful, indicating that this pigment-protein complex is blocked at its N-terminus.

Immunological characterisation of the pigment-protein complex

To investigate the possibility of the purification of a degradation product of one of the so far known pigment-protein complexes, to localise the CP 14–15 at different experimental stages of the isolation procedure and to probe eventual homologies with other cab-polypeptides the electrophoretically purified pigment-protein complex was used for the induction of a polyclonal antiserum. Applying a solid-phase-ELISA (vide supra), we measured a fairly high titer of anti CP 14–15 within the antiserum showing immune responses up to dilutions of 1:8000. The specificity of the antiserum was tested by immunoblotting after separating the polypeptides on polyacrylamide gradient gels. In Fig. 8 a typical result of such an experiment is demonstrated. We found very strong immunoreactivities of approximately the same intensity with two proteins of relative molecular masses of 14 and 15 kDa in PS II membrane fragments (Fig. 8, lane 5), in right-side- and inside-out-thylakoids (Fig. 8, lanes 3 and 4). The isolated SDS-solubilised pigment-protein complex, however, was merely identified in its oligomeric forms of about 66–70 kDa. These aggregates were scarcely observed in the thylakoid fractions as well as in the PS II membrane fragments (see Fig. 8, lanes 3–5). The corresponding blots with the non-immune serum revealed no significant colouration. To further confirm our hypothesis that the above described oligomeric pigment-protein complex of 120 kDa consists of the CP 14–15 and at least one other Chl-protein with an M_r of 22–24 kDa we excised the high molecular mass complex and electroeluted the polypeptides from the gel slices. Then, the samples were sonicated in the presence or absence of a chaotropic agent (50 mM guanidinium-HCl) in order to dissociate the oligomer. A subsequent reelectrophoresis and immunoblotting with α-14 again revealed a double band of 66–70 kDa (see Fig. 9., lanes 2–5). Although we extensively prolonged the immunostaining time, under no experimental conditions was a reversal into their monomeric forms observed. This result unequivocally demonstrates the participation of both the polypeptides of 14 and 15 kDa in the oligomerisation into aggregates of 66–70 kDa as well as 120 kDa.

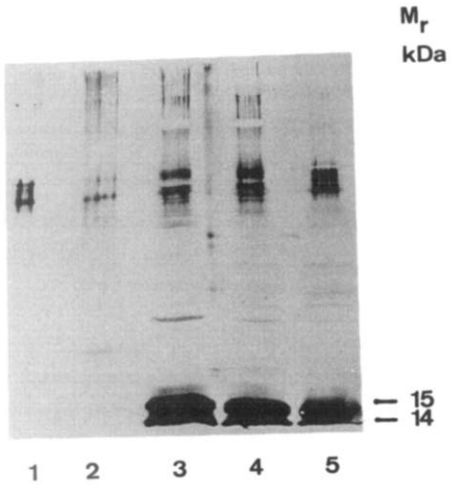

Fig. 8. Immunoblotting with α-14 (dilution: 1:2000) of different thylakoid membrane fractions, PS II membrane fragments and the PS I containing solubilised stroma thylakoids. Lane 1: CP14–15 (oligomeric forms) equivalent to 1 μg protein; lane 2: PS I containing supernatant after preparation of PS II; lane 3: right-side-out thylakoids; lane 4: inside-out thylakoids; lane 5: PS II membrane fragments; samples in lanes 2–5 were loaded equivalent to 20 μg Chl.

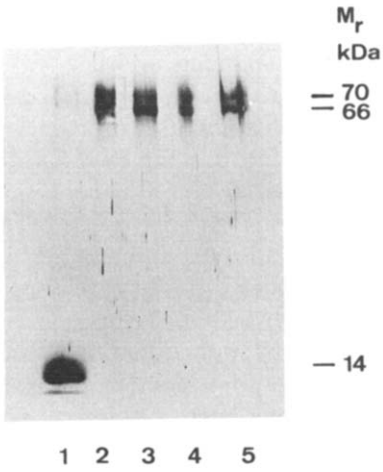

Fig. 9. Immunoblotting of CP 120 challenged with α-14 (dilution: 1:2000) after preparative electrophoresis, subsequent electroelution and reelectrophoresis. Prior reelectrophoresis the samples were treated by ultrasonication in the presence or absence of guanidinium-HCl to dissociate the oligomeric form. lane 1: sucrose gradient fraction containing CP14–15, control; lane 2: electroeluted fraction treated with ultrasonication (15 min); lane 3: electroeluted fraction treated with ultrasonication (30 min); lane 4: electroeluted fraction incubated with 50 mM guanidinium-HCl (30 min); lane 5: electroeluted fraction first incubated with 50 mM guanidinium-HCl (30 min) followed by an ultrasonication period of 30 min.

Discussion

We have described above the identification, isolation and partial characterisation of a so far unknown

pigment-binding protein complex from PS II of spinach consisting of two components with M_r of 14 and 15 kDa. With respect to the questions raised above, the following conclusions can be drawn:

(*1*) The pigment-protein complex is an independent component of PS II membrane fragments and intrinsic PS II membrane proteins. This clearly demonstrates that both its polypeptides are integral membrane proteins as would be expected for a pigment-protein complex of higher plant thylakoids. Using one- and two-dimensional PAGE they were identified in all of our preparations ($\Sigma = 40$). If a contaminating proteinase would degrade a larger pigment-binding polypeptide, this enzyme would always produce a splitting product of the same size and isoelectric point indicating a site-specific digestion. Furthermore, this proteinase should be insensitive against the proteinase inhibitors applied. Besides those described in Materials and Methods *p*-hydroxy mercuribenzoate and EDTA were also tested without any alterations of the protein patterns (data not demonstrated). Immunoblotting experiments gave no evidence for any proteolysis fragments of a larger pigment-protein complex. All these findings show that a degradation process is rather unlikely. In two-dimensional gel electrophoretic analyses of other groups polypeptides in the M_r range of 13–15 kDa were detected in grana and PS II preparations from higher plants [33,37–40]. Masojidek et al. reported the identification of several polypeptides in this range, but they did not analyse possible pigment-binding proteins [39,40]. Interestingly, they ascribed two proteins with isoelectric points of 5.22 and 5.51 only to PS II. These data very nicely match with our results for the determination of the relative molecular mass and the IEP of the polypeptide reported here. Hierholzer et al. [43] also identified a 14 kDa protein in PS II preparations. Using stroma-specific antibodies they presented experimental evidence for a stroma exposition of this protein. Possibly this polypeptide is not identical to the cytochrome *b*-559 as was suggested by the authors in Ref. 43. The strongest evidence for the occurrence of a low-molecular-mass pigment-protein complex in higher plant thylakoids is delivered by the report of Krishnan et al. [44]. The authors isolated thylakoid membranes from mesophyll chloroplasts of *Sorghum vulgare* and found a 15 kDa pigment-binding polypeptide with a room-temperature absorption spectrum showing maxima of 669 and 648 nm. Interestingly, this complex revealed likewise a strong tendency to self-associate into oligomers of different sizes [44]. Therefore, we believe that both the Chl-protein complexes from *Sorghum vulgare* and spinach are homologous. After separating thylakoid membrane proteins of chloroplasts from *Chlamydobotrys stellata* by PAGE in the presence of Deriphate 160 a low molecular mass pigment-polypeptide complex was found. This complex of 9 kDa, however, merely contained Chl *a* [45]. Recently, other laboratories also succeeded in the identification of pigment-protein complexes with molecular masses of < 20 kDa. Vainstein et al. and Anandan et al. [46,47] reported on a 17 kDa pigment-protein complex belonging to LHC I (now designated LHC Ic) from barley and maize thylakoids. G.F. Peter et al. [48] fractionated the LHC of barley PS II by Deriphat-PAGE into five different pigmented complexes. One of these, designated LHC IIe, was shown to be composed of a 12–13 kDa apoprotein and several pigments including Chl *a* and *b* as well as carotenoids (neoxanthin, violaxanthin, lutein). Although differences between both pigment-polypeptide complexes from barley and spinach are evident, e.g., with respect to their relative molecular masses, the Chl *a*/*b* ratios and absorption spectra, they could be related to each other, but comparative studies are required to confirm this assumption. Distinctions could be explained by use of different isolation procedures and electrophoretical systems. During the preparation of CP 14–15 losses of pigment molecules (Chl *b*, carotenoids) and pheophytinisation especially of Chl *b* cannot completely be excluded. Both pigment-protein complexes show similarities with respect to their relative pigment content (2% in comparison to 2–3% [48]) and the lack of any cross-reactivities with antibodies directed against other pigment-containing polypeptides. Our polyclonal antiserum furthermore did not cross-react with any other component of the cab-protein family. We are now working out experimental conditions for stabilising the pigment-protein complex and furthermore to substitute the preparative SDS/urea/PAGE used in the final purication step by a chromatographic procedure. As far as we know from the literature, this is the first report on a low-molecular-mass pigment-binding polypeptide complex observed in spinach.

(*2*) From our data we conclude that the 14–15 kDa proteins belong to a real pigment-binding protein complex of PS II membrane fragments from spinach; however, it is not yet clear whether this new component acts as a single or a larger Chl-protein complex (see Fig. 5 lanes 2–6 and 8–12). Concerning the above described oligomeric forms, artificial aggregations of different Chl-protein complexes due to β-dodecyl maltoside- and SDS-induced hydrophobic interactions cannot be fully excluded at the moment. This behaviour explains the difficulties to detect the 14–15 kDa proteins within gel electrophoretic patterns of PS II membrane proteins. With respect to the oligomeric pigment-containing complexes of about 120 kDa and of about 66–70 kDa, an analysis of their exact polypeptide and pigment compositions, their stoichiometries as well as a detailed functional analysis are required. This could be performed by use of a combination of circular dichroism (see Garab et al. [49] and references therein),

linear dichroism and fluorescence emission spectroscopy (see Ref. 50). With these methods we are able to study the organisation and orientation of the pigments within the different Chl-protein subcomplexes and clarify whether an efficient energy transfer is mediated by pigment molecules being associated with distinct polypeptides. These experiments could especially help us to answer the question whether the large pigment-containing complex is representing rather a functional unit or an artificial aggregate. From different lines of evidence the oligomeric states of carotenoid-chlorophyll proteins seem to be the functionally active forms, e.g., the LHC IIb probably exists as a trimer (Refs. 51–52; Ref. 53 for a review), but this still remains a matter of debate. Furthermore, it has to be investigated whether we are faced with two distinct apoproteins of a common pigment-protein complex or modified forms of one polypeptide having different electrophoretical mobilities.

(*3*) The question of homology can currently not be answered adequately, because the sequence analysis of CP 14–15 was hindered by a N-terminal modification. This phenomenon has also been observed for other pigment-protein complexes, e.g., CP 29, CP 25, CP 27, CP 43, the PS II reaction centre polypeptides D1 and D2 or possible pigment binding proteins of PS I [54–58]. From the absorption spectrum in the red and the pigment composition, a relation to other Chl *a*/*b*-binding polypeptides could be deduced. However, the types of pigment (especially regarding the carotenoids) and their exact quantity have to be analysed.

(*4*) From the results obtained so far we propose that the CP 14–15 is acting as an additional antenna complex within PS II. Although its location is completely unknown, we clearly identified both polypeptides of this complex in RSO and ISO thylakoids. This demonstrates that both occur in the grana and the stroma-exposed PS II antenna complexes. On the other hand, the immunodecoration experiments indicated negligible amounts of these proteins in the Triton X-100-containing supernatant obtained after pelleting the PS II membrane fragments. Therefore, neither polypeptide can be attributed to PS I. Lately, Ikeuchi et al. [58] have identified 9 and 14 kDa protein components in PS I from spinach. The latter polypeptide was found to be tightly attached to the PS I core complex and also blocked at its N-terminus. However, the authors in Ref. 58 could not detect any pigment in these proteins. For that reason, a correspondence of both polypeptides of the CP 14–15 to the 14 kDa-subunit found in PS I preparations is not very likely. Affinity-column-purified IgG molecules of the polyclonal antiserum directed against the CP 14–15 should provide a tool for an immune electron microscopical study to localise the Chl-protein complex in its native environment.

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