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POLYPEPTIDE PROFILES OF CHLOROPHYLL · PROTEIN COMPLEXES AND THYLAKOID MEMBRANES OF SPINACH CHLOROPLASTS

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Summary

In addition to the major chlorophyll · protein complexes I and II, two minor chlorophyll proteins have been observed in sodium dodecyl sulfate (SDS)-polyacrylamide gels of spinach chloroplast membranes. These minor pigmented zones appeared to be derived from the light-harvesting chlorophyll *a/b* · protein and from the reaction centre complex of Photosystem II.

Data are presented on the polypeptide profiles of purified digitonin-subchloroplast particles, with special regard to the effect of solubilization temperature and extraction of lipids. The results are compared with the SDS-polypeptide pattern of spinach thylakoids obtained under exactly the same conditions with respect to electrophoresis technique, solubilization method and presence of lipid. In addition, the effects of temperature and lipid extraction on the distinct chlorophyll · protein complexes appearing in SDS gel electrophoretograms of chloroplast membranes were studied by slicing the chlorophyll-containing regions and subjecting them to a second run with or without heating or extraction with acetone. By supplementing these data with an examination of the polypeptide composition of cytochrome *f* and coupling factor, it has been possible to identify most of the major chloroplast membrane polypeptides.

Introduction

When chloroplast thylakoids are solubilized with SDS or sodium dodecyl benzene sulphonate, three chlorophyll-containing bands are usually obtained upon electrophoresis in SDS-polyacrylamide gels (for a review see ref. 1). The three pigmented zones were termed complex I, II and III, corresponding to the increasing order of their electrophoretic mobility. Complex III contains

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; SDS, sodium dodecyl sulphate.

no protein and consists of detergent-complexed free pigment. The complexes I and II were shown to be chlorophyll proteins characterized by a high and a low chlorophyll *a/b* ratio, respectively. Mainly for that reason it was originally suggested that the complexes I and II are related to PS I and PS II, respectively.

Recently more direct evidence has been obtained for the relation between complex I and PS I. Several PS I preparations from higher plants produced complex I upon SDS gel electrophoresis [2—4]. On the other hand, mutants lacking *P*-700 but having normal PS II activity did not contain complex I [1,5]. In contrast to complex I, no evidence has been obtained that a relation exists between complex II and PS II. PS II activity was observed in flashed bean leaves [6] and mutants lacking complex II [7], and to be absent in a mutant which still contained this chlorophyll · protein [8]. Therefore, Thornber and Highkin [7] have renamed this complex the light-harvesting chlorophyll *a/b* · protein.

Though some reports on the occurrence of minor pigmented zones in addition to complexes I and II have appeared, the nature of these components has not been established, nor has any evidence been presented so far as to the location of the PS II-reaction centre on SDS-polyacrylamide gels [1]. In this paper it is shown that between the two major chlorophyll proteins two minor chlorophyll proteins may be observed in SDS-polyacrylamide gels, which are derived from the light-harvesting chlorophyll *a/b* · protein and from the reaction centre complex of PS II.

Previously [9,10] we reported the preparation of three kinds of chlorophyll · protein complexes from digitonin-treated spinach chloroplasts: PS I particles (F_I), the reaction centre complex of PS II (F_{II}) and a photochemically inactive chlorophyll protein (F_{III}). The properties of F_{III} are similar to those of the light-harvesting chlorophyll *a/b* · protein described by Thornber and Highkin [7]. The SDS-polypeptide profiles of the lipid-extracted subchloroplast particles F_I, F_{II} and F_{III} were recently presented [10]. Continuation of this study has shown, however, that both lipid extraction and heating not only removed the pigment but also strongly influenced the polypeptide pattern of the chlorophyll · protein complexes.

Machold [11] reported that delipidation of thylakoid membranes of *Vicia faba* chloroplasts with 80% acetone alters the electrophoretic mobility of the protein moieties of the pigment · protein complexes. The protein moiety of complex I migrated significantly faster after extraction of the pigments, whereas the protein moiety of complex II had a somewhat lower mobility. Moreover it was shown that removal of the pigments by acetone, as well as heating of the detergent-protein extract, increased the tendency of complex I-protein to form oligomers, which were retained on the top of the gel. Similar results were obtained by Chua et al. [5]. These authors observed that complex I was dissociated by heat into free pigment and a constituent polypeptide of molecular weight 66 000. Anderson and Levine [12] found that removal of lipid from chlorophyll · protein complex I from *Chlamydomonas reinhardi* resulted in the appearance of two polypeptides of molecular weight around 60 000. They further showed that two polypeptides with molecular weight in the range of 25 000 were associated with the light-harvesting chlorophyll · protein complex, and that the main polypeptide of complex II had a somewhat

lower electrophoretic mobility after lipid extraction. Genge et al. [13], on the other hand, reported that loss of chlorophyll from complex II in bean chloroplasts resulted in a decrease in apparent molecular weight of the protein of about 3000. Henriques and Park [14] have recently shown that re-electrophoresis of the complexes I and II from lettuce revealed a major component of 67 kdaltons and two minor peaks at 61 and 58 kdaltons, and a major polypeptide of 25 kdaltons and two minor ones of 27.5 and 23 kdaltons, respectively.

In this paper data are presented on the polypeptide profiles of purified digitonin-subchloroplast particles, with special regard to the effects of solubilization temperature and extraction of lipids. The results are compared with the SDS-polypeptide pattern of spinach thylakoids obtained under exactly the same conditions with respect to electrophoresis technique, solubilization method and presence of lipid. In addition, the effects of solubilization temperature and lipid extraction on the distinct chlorophyll · protein complexes appearing in SDS gel electrophoretograms of chloroplast membranes were studied by slicing the chlorophyll-containing regions and subjecting them to a second run with or without heating or extraction with acetone. By supplementing these data with an examination of the polypeptide composition of other thylakoid proteins, such as cytochrome *f* and coupling factor, it has been possible to (tentatively) identify most of the major chloroplast membrane polypeptides.

Methods

Spinach chloroplast membranes were isolated and washed in buffer and 1 mM EDTA as described by Anderson and Levine [15]. When indicated, the procedure of Strotmann et al. [16] was used to remove ribulose-1,5-diphosphate carboxylase and coupling factor from the thylakoids. The isolation of subchloroplast particles and the determination of PS I and PS II activities were carried out as described previously [9,10]. Chlorophylls *a* and *b* were measured spectrophotometrically by the method of Arnon [17]. Absorption spectra were recorded with a Cary, model 14 R, spectrophotometer. *P*-700 was determined from the light-induced absorbance change at 698 nm and from the oxidized minus reduced difference spectrum as described by Yamamoto and Vernon [18], using an Aminco DW-2 dual-wavelength spectrophotometer fitted with a side-illumination attachment. An extinction coefficient of 64 000 $M^{-1} \cdot cm^{-1}$ was used for calculating the amount of *P*-700 [19]. *P*-680 was determined from the light-induced 435 minus 460 nm absorbance difference by the method of van Gorkom et al. [20].

The polypeptide composition was analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% SDS using the procedure of Hoober [21]. However, gel electrophoresis was routinely performed in slab gels, 2.4 mm thick, using a Pharmacia GE-4 electrophoresis apparatus. Gels were prerun for 90 min at 150 mA prior to the application of protein samples. Electrophoresis was carried out in dim light for 30 min at 50 mA and for 3—4 h at 150 mA, the temperature being held at 10—15°C. Occasionally, the 8% acrylamide gel was replaced by a gel concentration gradient as described by Chua

and Bennoun [22]. This method was more suitable to provide optimal resolution of polypeptides in the low molecular weight region. The gels were stained with Coomassie Brilliant Blue G-250 and destained by the method of Weber and Osborn [23], using a Pharmacia GD-4 gel destainer. Molecular weights were estimated from a standard plot as described by Weber and Osborn, using phosphorylase *A* (94 000), bovine serum albumin (68 000), fumarase (49 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), α-chymotrypsinogen A (25 700) and cytochrome *c* (12 400) as marker proteins. Though the calculated molecular weigths may show a variance of up to 6%, the individual bands consistently appeared in characteristic positions in relation to each other.

Chlorophyll · protein complexes and thylakoid membranes were usually solubilized in a mixture containing 0.05 M Tris buffer, pH 8.4, 1% SDS, 2% mercaptoethanol, 5% sucrose and 0.05% bromphenol blue. The final chlorophyll concentration and the ratio SDS to chlorophyll were about 1 mg per ml and 10 : 1 (w/w), respectively. Since other concentrations of SDS were also used in some experiments, we studied the effect of SDS concentration on the polypeptide profile by varying both the final concentration and the ratio SDS to chlorophyll. No significant differences were observed when the SDS to chlorophyll ratio was varied between 5 : 1 and 50 : 1 and the final SDS concentration between 0.1 and 2%. A little more chlorophyll may be solubilized, however, when the higher concentrations of SDS are used. The mixture was kept at 4°C for 30 min and the SDS-solubilized samples were either used directly or heated at various temperatures before electrophoresis. Unless otherwise specified, heating was carried out for 2 min. Lipids and photosynthetic pigments were either not removed or removed prior to solubilization with SDS by extraction with 90% acetone at 0°C. Removal of lipids with a 1 : 2 (v/v) mixture of chloroform/methanol, as described by Henriques and Park [24], gave results similar to those obtained by extraction with 90% acetone. Usually, various amounts of sample,ranging from 2 to 20 μl, were applied to the gel in order to permit resolution of both major and minor polypeptides.

For the large-scale isolation of pigmented bands 300—400 μl of thylakoid membranes (approx. 500 μg chlorophyll) solubilized in 1% SDS (SDS/chlorophyll, 10 : 1) were applied to 3.3-mm gels. The pigmented zones were cut out from the gels and the chlorophyll · protein complexes eluted by homogenization in 0.05 M Tris buffer, pH 8.4, containing 0.1% SDS, using a Bühler homogenizer. After centrifugation the extract was concentrated by ultrafiltration in an Amicon concentration apparatus or in a collodion bag, and used for analytical-scale electrophoresis either directly or after heat treatment or acetone extraction. In another series of experiments, gel strips containing the chlorophyll · protein bands were directly placed on a second gel and re-electrophoresed without further treatment or after incubation at higher temperatures. Two-dimensional electrophoresis was carried out by subjecting gel slices containing the resolved chloroplast membrane polypeptides, either directly or following heat denaturation, to a second, identical SDS-polyacrylamide gel electrophoresis.

Photographs were taken with a Polaroid camera. Gels were stored in the dark after immersion in 55% methanol for 48 h and subsequent drying by means of a Pharmacia GSD-4 gel-slab drier.

Ribulose-1,5-diphosphate carboxylase and coupling factor were released from spinach chloroplasts by the method described by Strotmann et al. [16]. The chloroplasts were first extracted with 10 mM pyrophosphate to remove ribulose-diphosphate carboxylase and then with an 0.3 M sucrose solution of low ionic strength to release the coupling factor. The 30 000 × *g* supernatants of these extracts were almost completely free of contaminating proteins. Cytochrome *f* was prepared by the method of Nelson and Racker [25]. Ferredoxin, plastocyanin and ferredoxin-$NADP^+$ reductase were isolated from spinach leaves and purified as described by Borchert and Wessels [26].

Results

(A) Effects of solubilization temperature and acetone extraction on the polypeptide profile of chloroplast membranes

Fig. 1 shows the polypeptide profiles of washed, EDTA-treated chloroplast lamellae dissolved in SDS at various temperatures (see also Fig. 6). It is seen that there are significant changes in the pattern depending on the solubilization temperature. At low temperature, polypeptide bands were generally observed at 95, 63, 57, 53, 50, 47, 45, 42, 39, 37, 32.5, 24, 22, 20.5, 19, 17.5, 15.5, 14.5, 13 and 11.5 kdaltons and at the front of the gel. The three major chlorophyll-containing zones, which correspond to the chlorophyll · protein com-

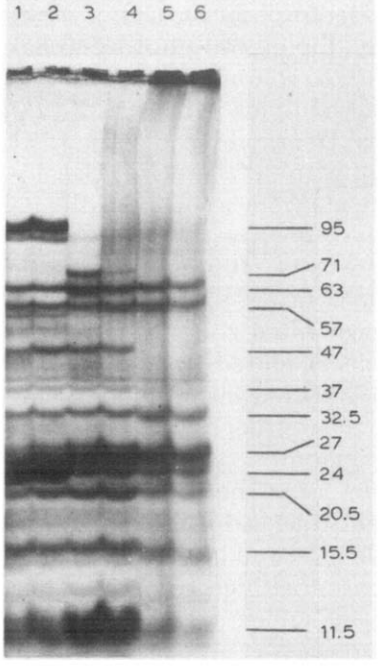


Fig. 1. Effect of solubilization temperature and lipid extraction on the SDS-polypeptide pattern of washed, EDTA-treated spinach chloroplast lamellae. The membranes were dissolved in 1% SDS at 4°C (1), 45°C (2), 70°C (3) and 100°C (4). Slots 5 and 6 contained a membrane sample which was extracted with 90% acetone prior to solubilization with SDS. Polypeptides are designated by their apparent molecular size in kdaltons.

plexes I and II and detergent-complexed free pigment, were located at 95, 24 and below the 11.5 kdalton band, respectively. The apparent molecular weight of chlorophyll · protein complex I is uncertain as it was found to depend on the acrylamide concentration (70 000, 80 000 and 95 000 for 4, 6 and 8% acrylamide gels, respectively). Ferguson plots of the relative mobilities of chlorophyll · protein complex I from *C. reinhardi* also demonstrated that this complex behaved anomalously in SDS gels [22].

When chloroplast membranes were solubilized in SDS at 60—70°C, chlorophyll was released from chlorophyll · protein complex II and several changes in the polypeptide pattern were observed. Chlorophyll · protein complex II was resolved into two distinct bands at 24 and 27 kdaltons, the latter being much more intense than the 24 kdalton band. In some experiments the faint 22 kdalton band was hidden under the intense 24 kdalton chlorophyll · protein band, but was then resolved upon heating. In addition, the band at 47 kdalton, and sometimes also the 20.5 kdalton band, were intensified, whereas the 53, 50, 45 and 42 kdalton bands diminished in intensity. Chlorophyll · protein complex I was partially converted into a band with an apparent molecular weight of 90 000. However, most of the chlorophyll was still associated with the 95 and 90 kdalton polypeptide bands. Sometimes this transformation had already started at lower temperature, as is shown, for instance, in Fig. 1. It was favoured, among other things, by a high SDS/chlorophyll ratio and the presence of 0.1 M $MgCl_2$ during the solubilization procedure, and by ageing of the preparation.

Solubilization at 70—80°C caused release of chlorophyll from chlorophyll · protein complex I; the bands at 95 and 90 kdaltons disappeared and a new intense band was observed at 71 kdaltons. When the membranes were dissolved in SDS by boiling for 2—5 min, the latter polypeptide disappeared and some protein was retained on the top of the gel. At the same time the intensity of the band at 47 kdaltons was strongly reduced, whereas the 53, 50, 45 and 42 kdalton bands were no longer present. The remaining polypeptide bands proved to be stable towards heating with SDS.

As shown in Fig. 1, delipidation of thylakoid membranes with 90% acetone or chloroform/methanol prior to SDS treatment had much the same effect on the polypeptide profile as prolonged heating. The bands at 95, 71, 53, 50, 47, 45 and 42 kdaltons were no longer observed, and the 24 kdalton band was resolved into two distinct bands at 24 and 27 kdaltons, the latter being more intense than the 24 kdalton band. As a result of the extraction with organic solvent some Coomassie Blue-stained material was retained at the origin. Apparently, heating or delipidation caused some polypeptides to aggregate, so that they became unable to penetrate the gel. On the other hand, it has been shown that some membrane proteins were soluble in chloroform/methanol [22,27].

(B) Chlorophyll · protein complexes obtained by electrophoresis of SDS-treated chloroplast membranes

Following electrophoresis of chloroplast membranes dissolved in 1% SDS at low temperature one may obtain three major and two minor chlorophyll-containing regions (Fig. 2). Except for the fastest moving zone, which was

Fig. 2. Electrophoretogram of SDS-solubilized thylakoid membranes before staining, showing the three major and two minor chlorophyll-containing regions at 95 (chlorophyll · protein complex I), 47, 39—42, 24 (chlorophyll · protein complex II) and about 10 kdaltons (free pigment).

found to consist of detergent-complexed pigment, all other green zones were shown to contain protein. The apparent molecular weights of the minor chlorophyll · protein complexes were 47 000 and 39 000—42 000. The chlorophyll-containing 47 kdalton band was not always found to be present, whereas the intensity of the green 39—42 kdalton band was variable and diminished upon prolonged electrophoresis. The chlorophyll in the latter pigment · protein complex appeared to be more loosely bound than in the major chlorophyll · protein complexes located at 95 and 24 kdaltons.

To compare the pigment composition, the pigmented zones were cut out from the gel and the absorption spectra of the eluted chlorophyll · protein complexes were measured (Fig. 3). The 47 and 24 kdalton bands were enriched in chlorophyll *b* and had an absorption maximum at 672 nm. The 95 and 39—42 kdalton bands, on the other hand, had a high chlorophyll *a/b* ratio and absorption maxima at 676 and 672 nm, respectively. The absorption maximum of the free-pigment zone was located at 668 nm. When the solubilization was performed with 0.1% SDS, the 95 kdalton band was found to contain *P*-700 (50—40 chlorophyll *a* molecules per *P*-700). The other bands did not show any *P*-700 activity. *P*-680 could not be found in any of these chlorophyll · protein complexes.

To identify the polypeptide composition of the complexes, the chlorophyll-

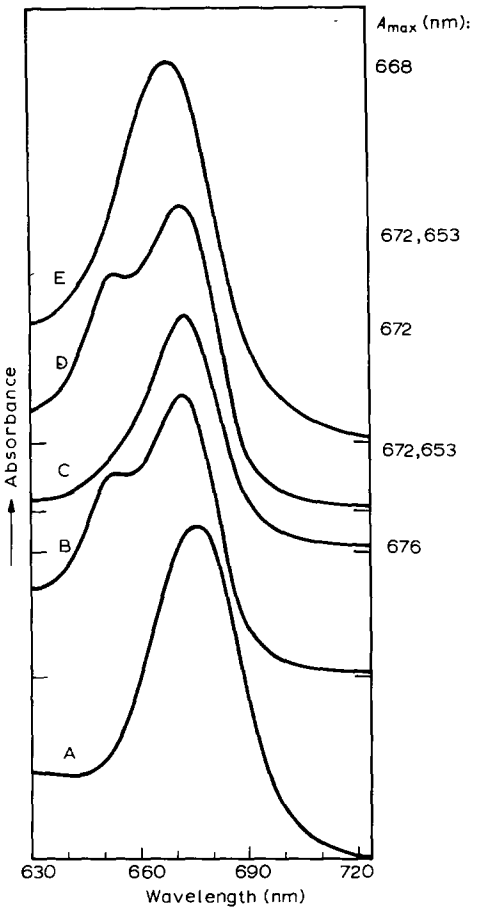


Fig. 3. Absorption spectra in the red of the chlorophyll-containing regions shown in Fig. 2 after elution from the gel. A, 95 kdalton band (chlorophyll · protein complex I); B, 47 kdalton band; C, 39—42 kdalton band; D, 24 kdalton band (chlorophyll · protein complex II); E, free pigment zone. λ_{max}, absorption maximum.

containing bands were cut out from the gels and rerun either directly or following heat denaturation. The 95 kdalton band migrated with the same mobility as in the first run, but after heating this complex was gradually transformed into a chlorophyll protein with a somewhat higher mobility (90 kdaltons). Subsequently, the chlorophyll was released and staining of the gel revealed one intense polypeptide band at 71 kdaltons. A slight smear of protein material was often found to move just in front of the 71 kdalton band and frequently a faint band at about 50 kdaltons was observed. After boiling with SDS or extraction with acetone aggregation occurred and all protein material was retained on top of the gel. It should be remarked here that re-electrophoresis of gel strips was found to be a milder procedure than re-electrophoresis of the chlorophyll proteins after elution from the preparative gel. In the latter case, for instance, partial transformation of chlorophyll · protein complex I into its 71 kdalton protein moiety occurred even without heating.

When the light-harvesting chlorophyll *a/b* · protein (24 kdaltons) was cut out from the gel and rerun without heating, one green band with the same mobility was generally observed. Following heating at 60—70° C, all the chlorophyll was released and two distinct polypeptide bands were revealed: a minor band at 24 and a major one at 27 kdaltons. Both polypeptides proved to be stable, as even after prolonged boiling or acetone extraction the bands maintained their intensity.

After re-electrophoresis, the minor green band at 47 kdaltons was partially converted to the 24 kdalton chlorophyll · protein complex and to pigment-free polypeptides with an apparent molecular weight of 53 000 and 27 000. Following heat denaturation, all the chlorophyll was released and staining revealed major polypeptide bands at 27 and 53 kdaltons. The 53 kdalton band disappeared after prolonged boiling. Since a second electrophoresis run of the 24 kdalton chlorophyll · protein complex occasionally produced a minor green band at 47 kdaltons, and the absorption spectra of the 24 and 47 kdalton complexes are similar, these experiments suggest that the chlorophyll-containing band at 47 kdaltons is a dimer of the light-harvesting chlorophyll *a/b* · protein. The 53 kdalton polypeptide could then represent the chlorophyll-free protein moiety of this dimer. After extraction with acetone the main polypeptide was located at 27 kdaltons.

When the chlorophyll-containing band with a molecular weight of 39 000—42 000 was rerun, the chlorophyll was lost and staining of the gel revealed a major polypeptide band at about 47 kdaltons. This indicated that this chlorophyll · protein complex was very unstable and was converted to a 47 kdalton polypeptide even without heating. After prolonged boiling or delipidation with acetone most of the 47 kdalton band was lost.

It was found that the pigment-free polypeptides of molecular weight 63 000, 57 000, 37 000, 32 500 and below 22 000 migrated in a second electrophoresis run with the same mobility as in the first one, irrespective of whether the samples were rerun directly or following heat denaturation.

In order to demonstrate more conclusively the conversion of individual polypeptides upon heating, and to avoid as much as possible changes due to the elution procedure as well as contamination with neighbouring polypeptides which might be cut along with the particular band, chloroplast membranes were subjected to two-dimensional SDS gel electrophoresis. Transformation of polypeptides by heat treatment was indicated by the appearance of spots located beyond the line connecting the top on the left with the bottom on the right of the gel. In addition to the conversion of the chlorophyll · protein complexes I and II, distinct changes were observed in the 40—50 kdalton region (Fig. 4). Polypeptides in the 50 kdalton range migrated significantly faster after heating, whereas the 40 kdalton region partially showed a somewhat lower mobility. Upon prolonged heating most of the polypeptide material in the 40—50 kdalton region disappeared.

(C) SDS gel electrophoresis of purified digitonin-subchloroplast particles and some other chloroplast membrane proteins

When purified PS I particles were subjected to SDS gel electrophoresis one chlorophyll · protein band was observed at 95 kdaltons. In addition to the

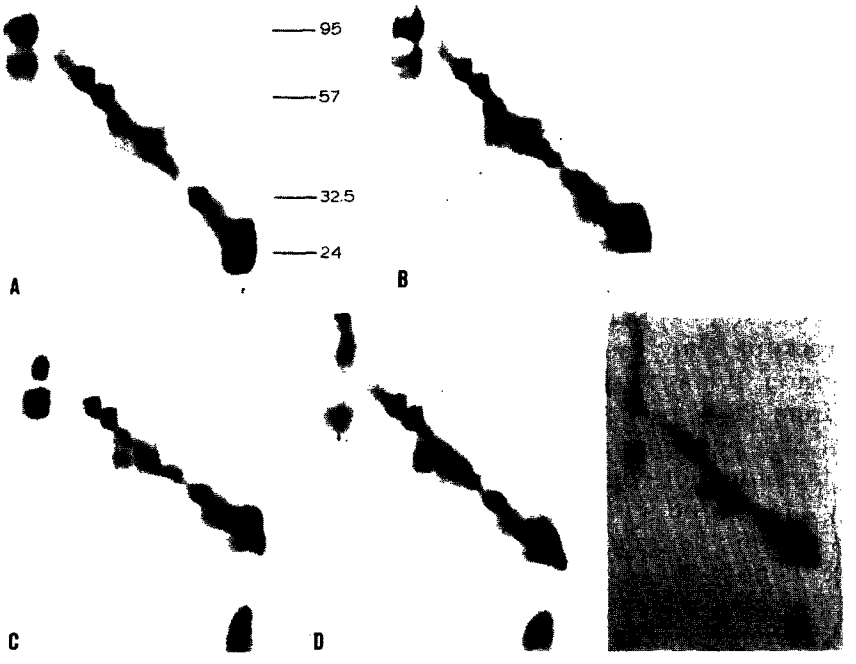


Fig. 4. Two-dimensional SDS gel electrophoresis of thylakoid membranes. Slices from the first gel containing all resolved polypeptides (membranes dissolved in SDS at 4°C) were placed directly (A) or after heat treatment (B, 45°C; C, 70°C; D, 100°C; E, 10 min 100°C) on a second gel, and electrophoresis was then performed as in the first run.

95 kdalton band, staining of the gel revealed some minor polypeptide bands in the low molecular weight range, viz. at about 20 and 15 kdaltons and at the front of the gel. Heating with SDS at 40—50°C resulted in a gradual transformation of the 95 kdalton chlorophyll · protein into a second, faster moving chlorophyll · protein complex at 90 kdaltons. The absorption spectra of the 95 and 90 kdalton bands were very similar and both chlorophyll · protein complexes were found to contain *P*-700. Solubilization at higher temperature caused release of chlorophyll, the appearance of faint bands at 71 and about 50 kdaltons and aggregation. Upon prolonged heating as well as extraction with acetone the protein material was retained at the origin. The minor polypeptides at 20, 15 and below 10 kdaltons, which might be constituent parts of the PS I particle or could be derived from a contaminating protein, proved to be resistant to heating and delipidation.

The light-harvesting chlorophyll *a/b* · protein F_{III} [9] produced a green band at 24 kdaltons on SDS gels. After heating, the chlorophyll was released and two distinct polypeptide bands were observed at 24 and 27 kdaltons, the latter being more intense than the 24 kdalton band. There was no complete conversion of the 24 kdalton band to the 27 kdalton polypeptide, nor any decrease in intensity of the bands upon further heat treatment. Lipid extraction

of this subchloroplast particle with acetone resulted in the same polypeptide profile as heating.

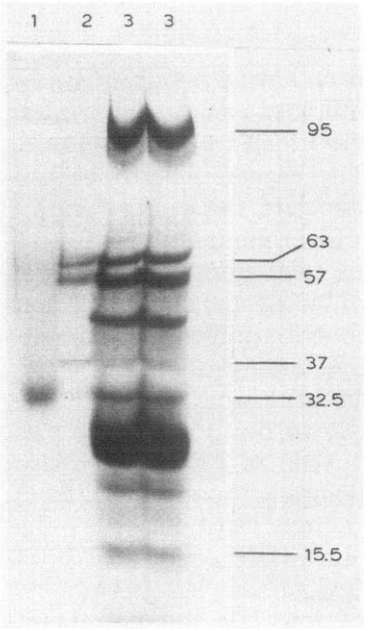
When purified PS II particles were subjected to SDS gel electrophoresis the chlorophyll was lost and two prominent polypeptide bands were observed at 50 and 42 kdaltons. Both bands disappeared after boiling, the 50 kdalton polypeptide being the most temperature-sensitive one. In several experiments a 46 kdalton band was observed instead of or in addition to the 42 kdalton band, which migrated slightly faster (42 kdaltons) upon heating to 60—70°C. Variable amounts of heat-resistant minor bands were found at 17.5 and 13.5 kdaltons and at the front of the gel. Attempts to detect a green band on the gel by the use of lower SDS concentrations, shorter incubation periods etc., were not successful. Only rarely a faint green band could be observed in the 40 kdalton region. It was found that even low concentrations of SDS removed chlorophyll from PS II particles and destroyed the *P*-680 activity. A definite identification of a particular polypeptide band with the PS II reaction centre protein was therefore not possible in this way. After lipid extraction of PS II particles only a faint band was observed at 42 kdaltons in addition to the minor polypeptides at 17.5 , 13.5 and below 10 kdaltons.

In order to identify the remaining polypeptides, the SDS gel electrophoresis pattern of chloroplast membranes was compared with that of well-characterized, purified thylakoid proteins. Fig. 5 shows that the coupling factor produced three prominent bands coinciding with the 63, 57 and 37 kdalton bands of chloroplast lamellae. No significant difference in polypeptide profile was observed when washed, EDTA-treated chloroplast lamellae were compared with the sucrose-washed thylakoid membranes as described by Strotmann et al. [16]. Previously [16,28] it has been shown, however, that EDTA extraction or washing with sucrose medium only partially removed the coupling factor from the chloroplast membranes.

Ribulose-1,5-diphosphate carboxylase showed two prominent bands at 56 and 12 kdaltons. Although the 56 kdalton band could be hidden under the 57 kdalton band of the coupling factor, the absence of a significant 12 kdalton band in the polypeptide pattern of chloroplast membranes confirmed the finding that this protein can be readily washed out. It may be assumed that ferredoxin and ferredoxin-$NADP^+$ reductase, which were found to produce polypeptide bands at 18 and 34.5 kdaltons, respectively, were also removed by the washing procedure.

The main band of cytochrome *f*, which cannot be released simply by EDTA washing, was found to coincide with the 32.5 kdalton polypeptide of chloroplast membranes (Fig. 5). Plastocyanin showed a major polypeptide band at 11.5 kdaltons and a minor one, probably the dimer, at 22 kdaltons. After heating, only one band at 11.5 kdaltons was obtained. The position of the polypeptide bands of coupling factor, ribulose-diphosphate carboxylase, ferredoxin, ferredoxin-$NADP^+$ reductase and cytochrome *f* was independent of the temperature of solubilization with SDS.

Previously [29] we have demonstrated that extraction of chloroplasts with 1.3% digitonin released the cytochromes *f* and b_6 as well as the coupling factor from the thylakoid membranes. In accordance with this result it was found that digitonin treatment of chloroplast lamellae resulted in a substantial loss of the



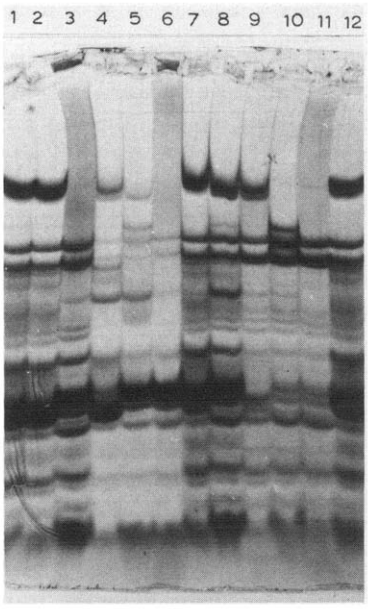


Fig. 5. SDS-polypeptide profiles of cytochrome *f* (1) and coupling factor (2) in comparison with those of washed, EDTA-treated chloroplast lamellae (3).

Fig. 6. Effect of digitonin extraction on the SDS-polypeptide pattern of thylakoid membranes, and the polypeptide composition of vesicles derived from the stroma lamellae. Slots 1—3, 7, 8 and 12 contained the unextracted preparation, slots 4—6 membranes extracted with 1.3% digitonin and slots 9—11 a sample of stroma vesicles. The membrane proteins were dissolved in SDS at 4°C (1, 2, 4, 7, 9 and 12), 70°C (5, 8 and 10) and 100°C (3, 6 and 11).

63, 57, 37 and 32.5 kdalton bands in the 10 000 × *g* sediment (Fig. 6). The main bands left were those derived from the Photosystems I and II and from the light-harvesting chlorophyll *a/b* · protein.

We also reported [30,31] that by treatment of spinach chloroplasts with a low concentration of digitonin, followed by centrifugation between 10 000 and 80 000 × *g*, vesicles were obtained which were primarily derived from the stroma lamellae and exhibited almost exclusively PS I activity, including cyclic photophosphorylation. In accordance with their relatively high chlorophyll *a/b* ratio (7.0), the stroma vesicles were found to lack the light-harvesting chlorophyll *a/b* · protein F_{III} [31]. For a more detailed discussion on the absence of complex II in stroma lamellae we refer to the review articles by Thornber [1] and Anderson [32]. It is shown in Fig. 6 that SDS gel electrophoresis of stroma vesicles produced as major bands those derived from PS I and the coupling factor. Only very small amounts of chlorophyll · protein complex II and its associated 24 and 27 kdalton polypeptides were observed, the latter no longer being more intense than the 24 kdalton band. Moreover, it is seen in Fig. 6 that stroma vesicles showed a reduction of the bands characteristic of the PS II reaction centre, which fits in with the low PS II activity of these preparations.

Discussion

It is shown in this paper that heating and delipidation have a strong influence on the SDS-polypeptide pattern of chloroplast membranes and subchloroplast particles. Several controversial results reported in the literature may therefore be due to the difference in procedures used. The regular electrophoresis procedure, which utilizes boiling with SDS to dissociate the sample and/or extraction of lipid material with acetone or chloroform/methanol, leads to a reduction or complete loss of the polypeptide bands characteristic of PS I and PS II. Previously published [10] polypeptide profiles of purified PS I and PS II particles therefore showed a relatively large contribution of contaminating proteins such as the light-harvesting chlorophyll *a/b* · protein and cytochrome *f*. Moreover, rather large amounts of protein were required to observe the distinct polypeptide bands. The same may apply to the polypeptide patterns of Triton-subchloroplast particles TSF 1 and TSF 2a [33], which also show a lot of polypeptide bands, presumably also including those derived from the coupling factor. The present systematic and comparative study of the effects of temperature and delipidation on the polypeptide profiles of both chloroplast lamellae and well-characterized, purified chloroplast membrane proteins allowed a more reliable identification of most of the prominent bands observed in SDS electrophoretograms of washed, EDTA-treated thylakoid membranes.

The absorption spectrum and the *P*-700 activity of chlorophyll · protein complex I, as well as the finding that purified PS I particles produce a green band with the same mobility on SDS gel electrophoretograms (95 kdaltons), confirm the view that this pigment protein contains the reaction centre of PS I. Upon moderate heat treatment a minor conformation change may occur, which is only reflected by a somewhat faster mobility (90 kdaltons) but does not give rise to a release of chlorophyll or a loss of the *P*-700 activity. However, the alternative possibility that a small polypeptide is dissociated from the complex, which migrates with the front, cannot be completely excluded. Heating at 70—80°C results in release of the chlorophyll and in the appearance of a polypeptide band at 71 kdaltons, which disappears, presumably due to aggregation, after heating at 100°C. It seems evident, therefore, that the 71 kdalton polypeptide is derived from PS I.

The finding that the subunits of the coupling factor migrate with the same mobility as the 63, 57 and 37 kdalton polypeptides, and the relative amounts of these bands are reduced by washing with EDTA, pyrophosphate and sucrose medium, or digitonin solution, indicates that these polypeptide bands are derived from the coupling factor and may be identified with the α, β and γ subunits of this protein. Recently, Henriques and Park [34] have also presented evidence that the two prominent high molecular weight polypeptides observed in SDS gel electrophoretograms of washed, lipid-extracted spinach chloroplast membranes may be identified with the α and β subunits of the coupling factor protein. Similarly, McEvoy and Lynn [35] and Süss [36] concluded that subunits of the coupling factor are present in the polypeptide profile of washed spinach and *V. faba* thylakoid membranes, respectively. Though the 63 and 57 kdalton bands were found to be particularly prominent in PS I-enriched

chloroplast fragments and have previously been considered as being derived from chlorophyll · protein complex I [12], more recent work [5,11] as well as the present results indicate that the polypeptide moiety derived from the *P*-700-bearing chlorophyll · protein complex is not located in the 60 kdalton but in the 70 kdalton region.

Experiments described in this paper show that the minor chlorophyll · protein complex located at 47 kdaltons and the 53 kdalton band are a dimer of the light-harvesting chlorophyll *a/b* · protein and its pigment-free protein moiety, respectively. This was concluded from the absorption spectrum and the partial conversion of the 47 kdalton band into a green 24 kdalton band and chlorophyll-free polypeptides at 53 and 27 kdaltons in a second electrophoresis run, and from the fact that sometimes a minor green band at 47 kdaltons could be observed upon re-electrophoresis of chlorophyll · protein complex II.

The polypeptide bands occurring in the 40—50 kdalton region are at least partially derived from the PS II reaction centre. After electrophoresis of chloroplast membranes a minor chlorophyll · protein complex has been observed at 39—42 kdaltons, which very easily loses chlorophyll and is mainly converted to a polypeptide with a molecular weight of about 47 000. The 50 kdalton polypeptide band was found to migrate faster after heat treatment. Both effects may lead to an intensification of the 47 kdalton band at moderate temperatures. Though purified PS II particles lose their chlorophyll upon SDS gel electrophoresis, polypeptide bands are obtained at 50, 46 and 42 kdaltons, the relative amounts of these bands depending on the solubilization conditions. Changes of the conformation of the protein molecules and of the content of lipid or detergent may be responsible for the heat modifiability of these proteins in SDS gel electrophoresis.

These results, as well as the high chlorophyll *a/b* ratio and the absence of *P*-700 support the view that the minor 39—42 kdalton chlorophyll · protein complex is derived from the PS II reaction centre. A more definitive proof of its identification with the PS II reaction centre complex is not possible, however, because the photochemical activity is no longer present after electrophoresis. Chua and Bennoun [22] have compared the polypeptide profile of thylakoid membranes of wild-type *C. reinhardi* with those of mutant strains lacking or deficient in PS II activity. It is interesting that their results showed a strong correlation between the presence of a membrane polypeptide of molecular weight 47 000 and the activity of the PS II reaction centre. This finding, and also our observation that stroma lamellae, which exhibit only a low PS II activity, contain reduced amounts of polypeptides in this region, support the assumption that polypeptides characteristic of PS II are found in the 40—50 kdalton range.

The 32.5 kdalton band was shown to migrate with the same mobility as cytochrome *f* and to be strongly reduced after digitonin extraction. This indicates that this polypeptide band may be identified with cytochrome *f*. A similar result was recently reported by Süss [36]. This author isolated cytochrome *f* from *V. faba* and found his preparation to consist of one polypeptide equivalent to the 31 kdalton band observed in SDS electrophoretograms of *V. faba* thylakoid membranes. By using SDS gel electrophoresis, Singh and Wasserman [37] and Klein and Vernon [33] determined a molecular weight

of 32 000 and 33 000, respectively, for the cytochrome *f* monomer.

Pigment · protein complex II (molecular weight 24 000) is well characterized now. Purified light-harvesting chlorophyll *a/b* · protein (F_{III}) shows the same electrophoretic mobility and is also extensively transformed into a 27 kdalton polypeptide upon heating or delipidation. Anderson and Levine [12] and Machold [11] also reported that the protein moiety of complex II after extraction of the pigments had a somewhat lower mobility compared with the chlorophyll-containing complex. The finding that even after prolonged boiling with SDS the polypeptide band at 24 kdaltons did not completely disappear suggests that the light-harvesting chlorophyll *a/b* · protein consists of two polypeptides with apparent molecular weights of 24 000 and 27 000, the latter being the major one. Association with chlorophyll may enhance the electrophoretic mobility of the 27 kdalton polypeptide, so that it comigrates with the 24 kdalton polypeptide.

Polypeptides in the lower molecular weight range are not definitely identified yet. Proteins which may contribute to bands in this region are the cytochromes b_{559} [38] and b_6 [39], and the hydrophobic membrane component of the ATPase complex [40]. The minor bands below 20 kdaltons, observed in SDS gel electrophoretograms of PS I and PS II particles, may be derived from contaminating proteins but could also represent constituent parts of the subchloroplast particles. In this connection it is of interest that Bengis and Nelson [41] have recently shown that the low molecular weight polypeptides present in a PS I reaction centre from Swiss chard could have a function in the photosynthetic electron transport between plastocyanin and ferredoxin. Further isolation and characterization of chloroplast membrane proteins is needed to allow an unequivocal assessment of the origin of polypeptide bands in the low molecular weight region.

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Addendum

Most of the results described in this paper were presented at the 4th International Congress on Photosynthesis, September 1977, Reading, U.K. (Congress Abstracts p. 405). At this Congress we learned that D.B. Hayden and W.G. Hopkins (Congress Abstracts p. 151) have also resolved two minor chlorophyll · protein complexes migrating between complexes I and II on SDS-polyacrylamide gels. The absorption spectra of these two complexes were very similar to those represented in this paper. At the same time, R. Remy, J. Hoarau and J.C. Leclerc (Congress Abstracts p. 314) presented evidence for the occurrence of a dimeric form of the light-harvesting chlorophyll *a/b* · protein in SDS-polyacrylamide gels. After completion of this manuscript, the above-mentioned results have been published in refs. 42 and 43, respectively.

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