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TWO-DIMENSIONAL SEPARATION OF CHLOROPLAST MEMBRANE PROTEINS BY ISOELECTRIC FOCUSING AND ELECTROPHORESIS IN SODIUM DODECYL SULPHATE

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Summary

Proteins of chloroplast subfragments enriched in Photosystem I and Photosystem II electron flow activity have been analyzed by two-dimensional polyacrylamide gel electrophoresis. In the first dimension, polyacrylamide gel isoelectric focusing (pH 5—7) was used in the presence of Triton X-100, followed at right angle by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Characteristic fingerprints were obtained for the Photosystem I and II fractions and a correlation between the major proteins separated by isoelectric focusing and the major polypeptides separated by undimensional SDS electrophoresis was established. Two dominant spots of 68 000 and 60 000 daltons appeared in the two-dimensional patterns of Photosystem I fractions (p*I* values about 5.6); two spots with molecular weights of 33 000 and 23 000 were characteristic for Photosystem II fractions (p*I* values about 5.3 and 6.3). Photosystem I fractions were furthermore characterized by a series of spots in the 44 000—33 000 range (p*I* values from about 5.9 to 6.8). The two-dimensional system revealed that (a) several SDS-polypeptides have multiple forms differing in charge only, (b) some proteins separated by isoelectric focusing are resolved in the second dimension into polypeptides of different size. The two-dimensional method combining Triton X-100 isoelectric focusing and SDS electrophoresis provides a higher degree of resolution than either of the unidimensional methods thus allowing a detailed analysis of chloroplast membrane proteins.

Introduction

Proteins are essential components of thylakoid membranes. Either weakly bound to the membrane (extrinsic proteins) or firmly embedded in the lipid

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

matrix (intrinsic proteins) they are responsible for functions such as light-driven electron transport and photophosphorylation and some of them are probably involved in the maintenance of the structure of the membrane. Although a number of extrinsic proteins have been attributed to known proteins of the chloroplast (coupling factor, carboxydismutase) the intrinsic proteins are not as well characterized because of their relative insolubility. So far the two chlorophyll-protein complexes are the only intrinsic proteins identified with certainty [1]. Progress in further identification of these proteins depends on the avilability of methods for their solubilization, separation and characterization.

Solubilization by the anionic detergent sodium dodecyl sulphate (SDS) and electrophoresis in SDS-containing polyacrylamide gels has been widely used to separate membrane proteins. SDS electrophoresis of lipid-containing or lipid-extracted thylakoid membranes resolves up to 20 polypeptides with molecular weights ranging from 100 000 to 10 000, approximately [1—6]. In subchloroplast fractions enriched in Photosystem I activity, polypeptides in the range of 70 000—50 000 daltons among them the chlorophyll-protein complex I predominate whereas Photosystem II fractions contain mainly polypeptides in the 30 000—23 000 daltons range, the most abundant polypeptide being the chlorophyll-protein complex II [2,3,5].

Although SDS electrophoresis has given much information on the intrinsic proteins of thylakoids it suffers from some inherent drawbacks: (a) SDS solubilizes proteins to their polypeptide subunits, thereby destroying their native form and biological activity; (b) SDS-polypeptides may represent aggregates or sets of polypeptides with very similar molecular weight.

Another possibility of separating membrane proteins is by polyacrylamide gel isoelectric focusing. For membrane solubilization prior to isoelectric focusing a non-ionic detergent and/or urea have to be used, since the charged SDS molecules interfere with isoelectric focusing. We have previously shown, that isoelectric focusing in the presence of Triton X-100 releases a great number of proteins from thylakoids, among them proteins characteristic for PS I and PS II fractions [7].

The purpose of this investigation was to subject thylakoid proteins which focused in the pH range of about 5—7 to a separation by SDS electrophoresis in the second dimension in order to identify the proteins separated by isoelectric focusing with the known SDS-polypeptides. The combination of isoelectric focusing with SDS electrophoresis in a two-dimensional system will allow a separation of membrane proteins according to two independent parameters i.e. their charge and their molecular weight. In our two-dimensional system we found the proteins of the chlorophyll-protein complexes as well as several other polypeptides. It appears that some of the SDS-polypeptides resolved by unidimensional electrophoresis are actually heterogeneous mixtures which can be separated by the two-dimensional system into components of similar size but different isoelectric point. On the other hand some of the proteins separated by isoelectric focusing were resolved in the second dimension into polypeptides of different size. Working with PS I and PS II subfractions of thylakoids in order to obtain a rough functional characterization, we thus obtained characteristic fingerprints for these fractions, allowing the correlation of the proteins

separated by isoelectric focusing with the SDS-polypeptides. Our approach should be useful for a more detailed analysis of membrane proteins.

Materials and Methods

Preparation of chloroplasts and chloroplast subfractions. Spinacia oleracea var. Nobel was grown in a phytotron as described previously [8] and leaves were harvested after 8 weeks of growth. Chloroplasts, Photosystem II- and Photosystem I-enriched fractions were prepared as described previously [7,9].

Sample preparation before isoelectric focusing and unidimensional SDS-electrophoresis. Before applying them on the isoelectric focusing gels PS I and PS II fractions were treated as described before [7]. The particle suspensions for SDS electrophoresis were suspended in 0.5 M sucrose and 0.05 M tricine buffer (pH 7.5) and the chlorophyll concentrations were adjusted to 2 mg/ml for PS II and 0.5 mg/ml for PS I fractions [10]. SDS (1% final concentration) was added to the samples which were stirred for 30 min at 4° C. Electrophoresis was then performed with samples containing about 30 μg of protein without further treatment.

Isoelectric focusing and measurement of pH gradient. The electrofocusing system was basically the system devised by Allen [11] with the following modification: 6% gels of acrylamide cross-linked with 3.4% *N,N'*-methylenebisacrylamide contained 1% Triton X-100 and 8 M urea as well as 2.5% carrier ampholines prepared by mixing the ampholines pH 3.5—10 and pH 4—6 in the ratio 2 : 1 (v/v). Electrofocusing was performed at 4° C with the Ortec Model 4200 electrophoresis system for 14 h. A LKB Model 2103 power supply was used with the initial settings of 50 V and 8 mA, the current declining to 2 mA and the voltage rising to 200 V during the experiment. For the measurement of the pH gradient gel strips were cut in 0.5-cm pieces, eluted in 1 ml of distilled water for 1 h and the pH was determined at 22° C subsequently. The pH values were reproducible but the pI values cannot be considered as absolute due to the fact that the pH was determined at 22° C, whereas isoelectric focusing was carried out at 4° C; other factors such as the presence of urea in the gel may also influence pI values.

Staining of isoelectric focusing gels. Before the staining operation, gels were fixed over night in 50% methanol, in order to remove most of the ampholines which stained diffusely with Coomassie Blue. Staining and destaining was performed as described before [7].

Experimental protocol for two-dimensional separation. The two-dimensional separation procedure was basically the same as described previously [12,13] and adapted for our use as mentioned below. For the first dimension with isoelectric focusing three sets of four identical samples were loaded on one slab gel containing 12 sample wells. After the run, gel strips corresponding to the samples were cut. One gel strip was used to determine the pH gradient, two gel strips were stained and one strip was equilibrated before loading it on the SDS gel for the second dimensional run. For equilibration a gel strip was placed in 50 ml of 0.05 M Tris (pH 7.5) containing 2% SDS and 1% mercaptoethanol which was gently stirred for 2 h. This treatment was necessary to solubilize the bands but may result in the loss of some of the minor bands. Gels were stored

frozen in this solution. After the incubation, gel strips were loaded on the SDS gel and fixed onto the gel in 1% agarose (dissolved in the equilibration buffer and heated to 80°C).

Sodium dodecyl sulphate gel electrophoresis. For one- and two-dimensional SDS electrophoresis we used the discontinuous system "Gel system I" from Maurer [11] with two modifications: (a) the separation gel contained 9% acrylamide supplemented with 2.5% cross-linking *N,N'*-methylenebisacrylamide and (b) 0.1% SDS was incorporated in the separation and concentration gels as well as in the electrode buffers. Electrophoresis was performed with the Ortec Model 4200 for about 4 h at 4°C with a constant current of 20 mA, the anode being connected to the bottom of the gel.

Staining of SDS electrophoresis gels. Gels from two-dimensional runs were soaked over night in 50% methanol and then stained according to Chrambach [11]. Gels from unidimensional runs were also stained with Coomassie Blue according to this method. Destaining was performed in 7% acetic acid for several days. Gels were scanned with a Zeiss Disk-ZK4 scanner at 600 nm.

Determination of molecular weights by SDS electrophoresis. Molecular weights were determined from a calibration curve obtained with the following proteins (from Boehringer): serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 500). Standards were incubated in 1% SDS, 1% mercaptoethanol at room temperature for 30 min before the run, about 20 μg of each standard was applied to the gels. Mobilities were calculated taking into account swelling of gels after the staining and destaining procedures.

Results

Isoelectric focusing

Previous experiments with pH gradients from 3.5 to 10 have shown that several major protein peaks are located in the pH 4.5—7 region (proteins Nos. 2—13 in ref. 7). In order to increase the resolution of this region, we expanded the pH gradient. Experimenting with different pH gradients we first verified that there was no retention of bands in the upper part (more acidic part) of the gels. We also incorporated 8 M urea in the gels in order to prevent aggregration effects which can appear in the more acidic region. Fig. 1 shows isoelectric focusing patterns of PS I and PS II fractions from spinach chloroplasts. Comparing these patterns with our previous results with a steeper pH gradient [7], we were now able to observe more bands and to distinguish more clearly the differences between the two types of particles. In the pH 6—7 region, where two strong double peaks were observed (Nos. 10—13) in the old system, the new gradient resolved up to 10 bands. For PS II fractions (Fig. 1B) we found a prominent band at about pH 5.3 corresponding to peak No. 2 focusing at about pH 4 with the steeper gradient [7]. This discrepancy in p*I* values is probably due to the presence of urea in the new system. The second characteristic protein of PS II fractions focused at about pH 6.3. Apart from these major peaks, a number of minor bands appeared in a reproducible manner which were characterized by their p*I* values as indicated in Fig. 1B. PS I fractions showed a prominent band at pH 5.6—5.7 which only appeared strongly in

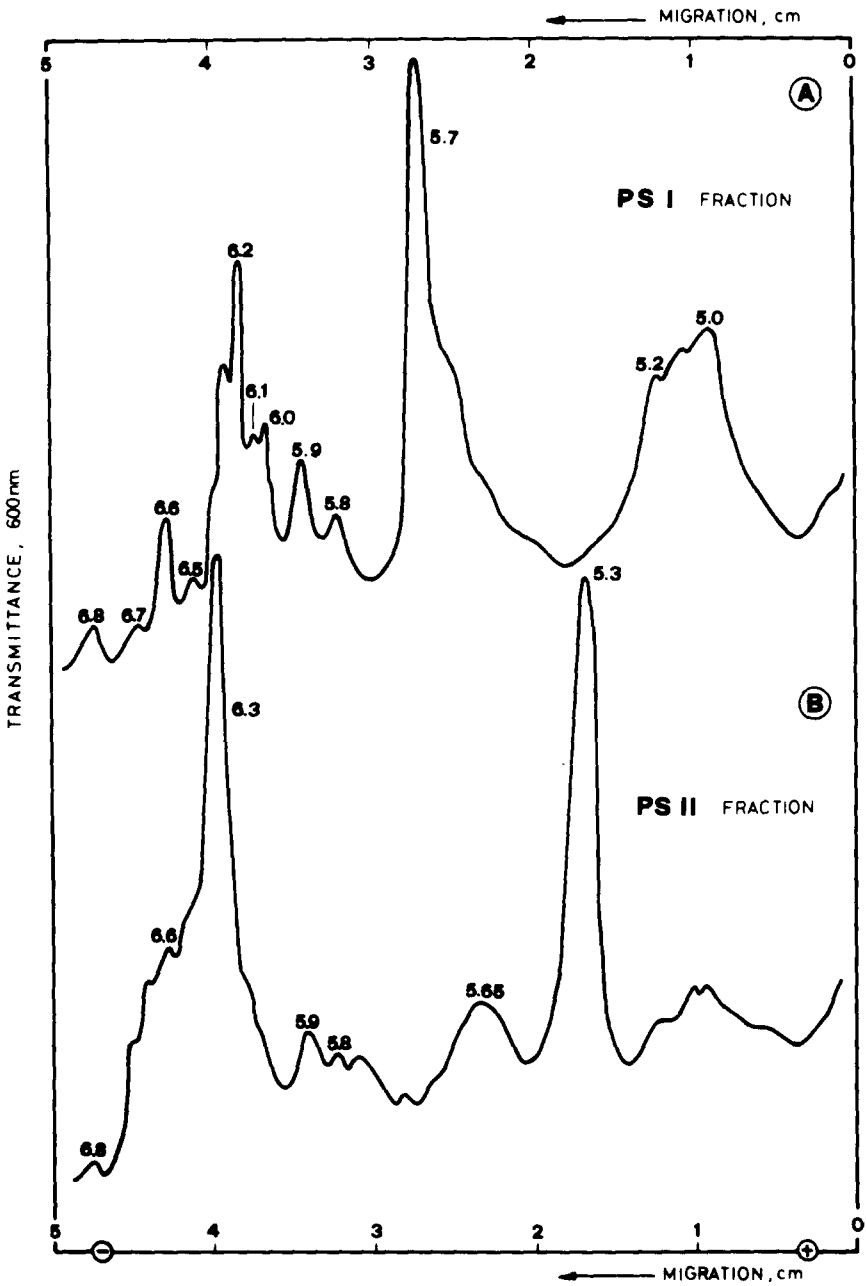

Fig. 1. Densitograms of PS I and PS II fractions of spinach chloroplasts. Fractions were solubilized in 1% Triton X-100 and separated by isoelectric focusing on polyacrylamide gels containing 1% Triton X-100 and 8 M urea. The pH gradient extended from pH 5 to 7. Proteins are designated by their p*I* values.

gels containing urea in addition to Triton. In the pH 6—7 region, more bands were present in this fraction than in the PS II fraction, which only showed one prominent peak at pH 6.3. Major proteins appeared at pH 5.8, 5.9, 6.0, 6.2, 6.6 and 6.8. Compared with our previous results [7] where the protein No. 11

seemed to be the same in both fractions PS I and PS II, the results with the expanded pH gradient allowed to distinguish clearly two closely spaced bands focusing at pH 6.2 (PS I) and 6.3 (PS II).

Sodium dodecyl sulphate electrophoresis

Fig. 2 shows scannings of SDS gels with polypeptide patterns of PS I (2A) and PS II (2B) fractions of spinach chloroplasts. PS I fractions were rich in polypeptides of 95 000, 70 000, 60 000 and 14 000 daltons. Some additional polypeptides in the 50 000—34 000 dalton range which were characteristic of PS I fractions were weakly stained but appeared consistently (44 000, 42 000, 40 000, 35 000 and 34 000). The peaks appearing in the 28 000—20 000 dalton range corresponded to the very strong group of polypeptides characteristic for PS II fractions. In the 19 000—14 000 dalton range, polypeptides of 19 000 and 17 000 daltons seemed to be characteristic for PS I fractions. Major components of PS II fractions were polypeptides of 60 000, 50 000, 34 000, 26 000, 21 000 and 14 000 daltons. In these experiments we used lipid containing samples, which are not drastically solubilized (1% SDS, 30 min, 4°C), in order to distinguish the two major chlorophyll-protein complexes. For complex I we found a molecular weight of about 95 000 (Fig. 2A) and for complex II a broad peak from 28 000 to 21 000 (Fig. 2B) which appeared to comprise at least three polypeptides of 28 000, 21 000 and possibly 20 000 daltons (see Fig. 2A as a reference). Table I shows the mean molecular weights of polypeptides separated by SDS electrophoresis in order to give an idea of the variability of molecular weights under our experimental conditions.

Two-dimensional electrophoresis

Fig. 3 shows typical patterns of PS I and PS II fractions obtained by Triton X-100 gel electrofocusing (pH 5–7) followed by SDS electrophoresis at right angle. The schematic drawing represents a typical experiment. Very strong spots appeared besides only weakly stained spots. Sometimes, a greater amount of spots has been found but these variable spots are not included here. The comparison of the molecular weights with those obtained by unidimensional SDS electrophoresis allows the correlation between the major isoelectric focusing bands and the major SDS-polypeptides. PS I patterns (Fig. 3A) show horizontal streakings in the high molecular weight range (78 000—60 000) over a pH range from about pH 4.5 to 5.9. The incorporation of urea into the isoelectric focusing gel reduced the diffuse streakings in the second dimension and led to the appearance of distinct spots at about pH 5.6 having a molecular weight of about 68 000 and 60 000. These spots were the strongest of the PS I patterns; they corresponded to the strong group of SDS-polypeptides of 70 000 and 60 000 daltons in Fig. 2A. No polypeptides with a higher molecular weight than 78 000 were found in the two-dimensional system in contrast to unidimensional SDS electrophoresis where a polypeptide of 95 000, and aggregates higher than 100 000 daltons were present.

A series of six spots between pH 5.8 and 6.8 having a molecular weight of 44 000, 42 000, 37 000 (a and b) and 33 000 (a and b) were also characteristic for PS I fractions; they correspond to polypeptides of the 50 000—34 000 dalton range in the unidimensional SDS electrophoresis (Fig. 2A). These poly-

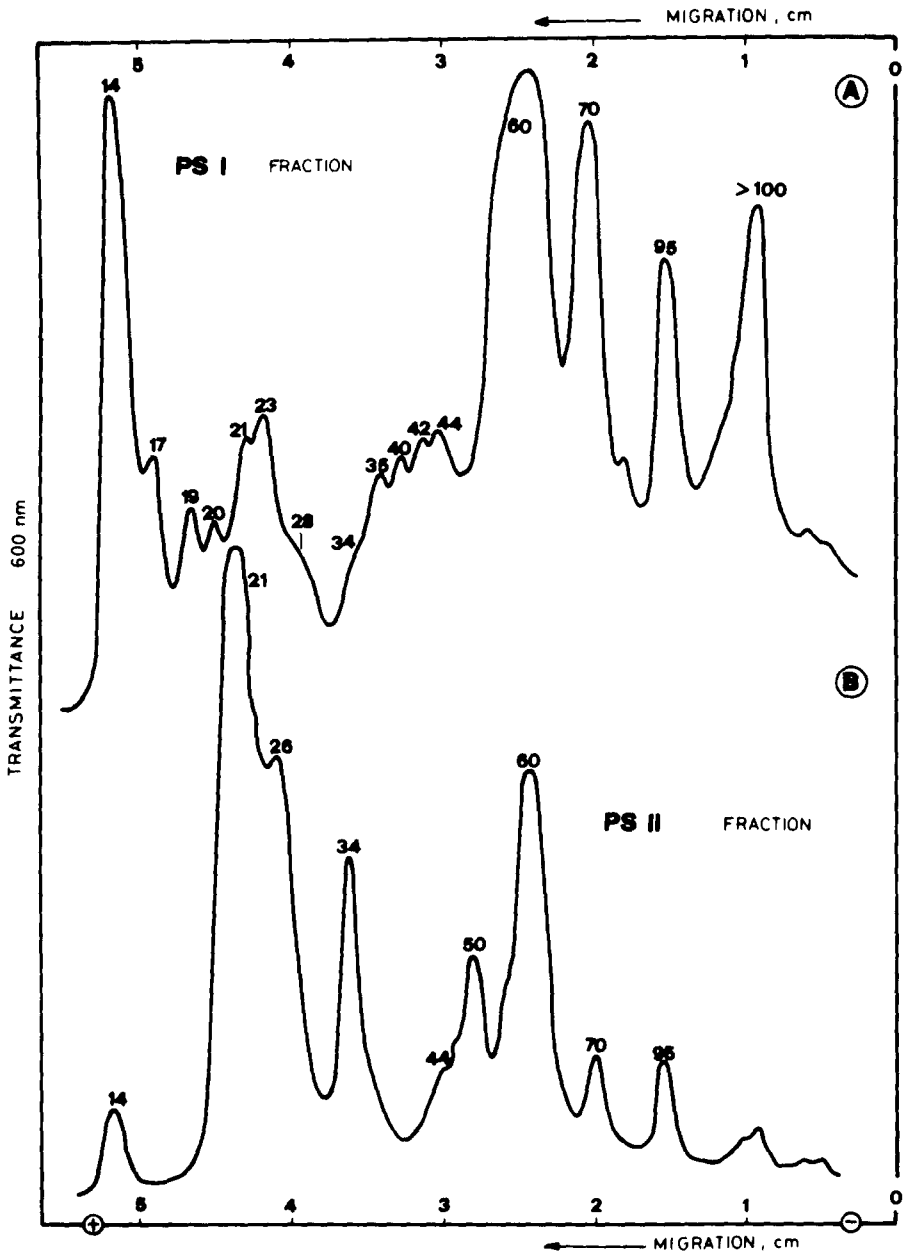

Fig. 2. Densitograms of PS I and PS II fractions of spinach chloroplasts. Fractions were solubilized in 1% SDS and separated by electrophoresis on polyacrylamide gels containing 0.1% SDS. Proteins are designated by their molecular weights in kilodaltons = 1000 daltons).

peptides appeared as rather weak bands, whereas in the two-dimensional pattern they were unexpectedly conspicuous and characteristic for the PS I fractions. In the same pH range appeared also some spots with lower molecular weights (25 000, 19 000 (a and b) and 12 000 daltons).

PS II patterns show two very strong spots at pH 5.3 (33 000 daltons) and 6.3

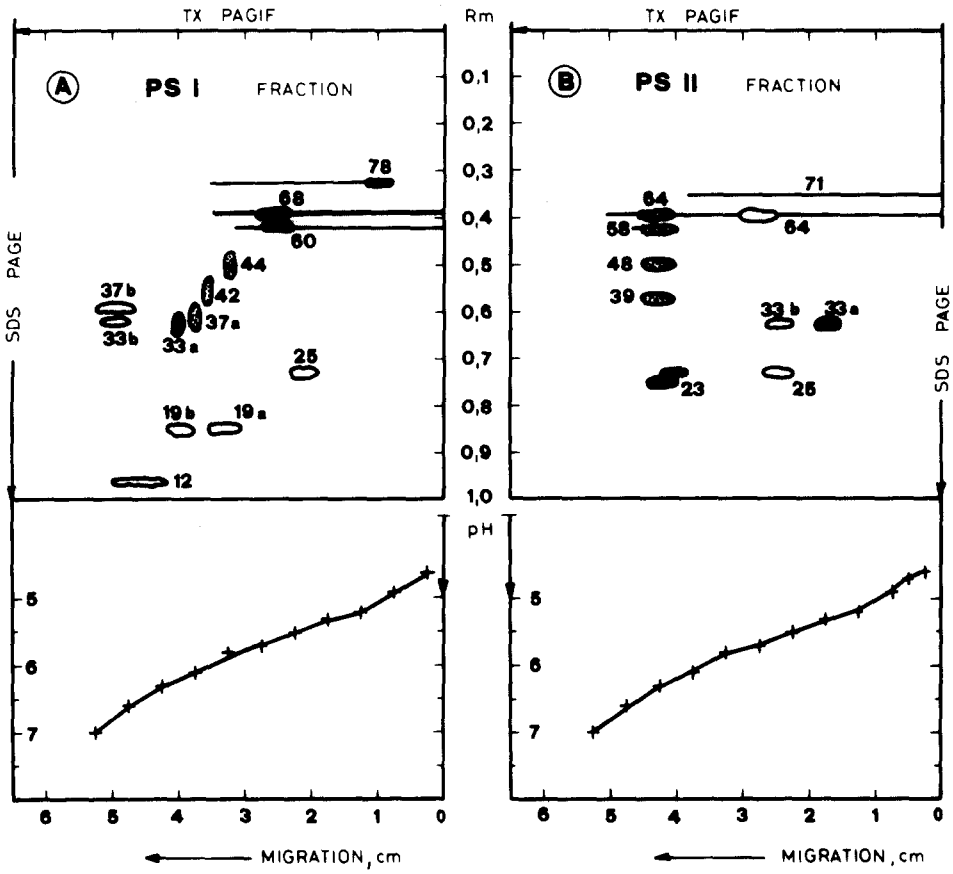

Fig. 3. Two-dimensional electrophoretic protein patterns of PS I and PS II fractions of spinach chloroplasts; schematic illustration. Triton X-100 isoelectic focusing (TX-PAGIF) from right to left. The corresponding pH gradients are shown in the lower part of the figure. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), from top to bottom. Spots are designated by their molecular weight in kilodaltons (≈ 1000 daltons); the corresponding p*I* values are found on the curves below. R_m (mobility) = distance of protein migration/length of gel after destaining X length of gel before destaining/distance of bromophenolblue migration. The variablility of molecular weights is comparable to the variability in the unidimensional system (cf. Table I).

(23 000). Another weaker spot at about pH 5.6 had a molecular weight of 25 000. These three spots corresponded to the "group II polypeptides" [14] characteristic for PS II fractions, in unidimensional SDS electrophoresis. The second characteristic feature of PS II fractions is a series of higher molecular weight spots at pH 6.3 (64 000, 58 000, 48 000, 39 000). These spots always apppeared one beneath the other, above the strong 23 000 daltons spot (Fig. 3B). In the unidimensional SDS electrophoresis (Fig. 2B) we found peaks at 60 000 and 50 000 daltons appearing in the PS II fractions, which may correspond to the 64 000, 58 000 and 48 000 daltons spots in the two-dimensional electrophoresis. Two-dimensional separations of unfractioned thylakoids showed essentially the polypeptides seen in PS I and PS II fractions.

So far we have analyzed by the two-dimensional method only a part of the isoelectric focusing gel where we knew many bands to be present. There are

TABLE I

MEAN MOLECULAR WEIGHT (AND DEVIATION) OF POLYPEPTIDES FROM PS I AND PS II FRACTIONS SEPARATED BY UNIDIMENSIONAL SDS-ELECTROPHORESIS

Data in kilodaltons (= 1000 daltons) represent the average of six experiments rounded to the nearest 0.5 kilodalton.

Photosystem I fraction		Photosystem II fraction	
100 ± 5			
95 ± 0	(not always visible)	95 ± 0	(not always visible)
82 ± 2	(not always visible)		
70 ± 2		70 ± 0	(not always visible)
64 ± 1	often appearing as one	64 ± 1	often appearing as one
58 ± 0	broad peak of 60 kD	58 ± 0	broad peak at 60 kD
54 ± 0	(not always visible)	53 ± 1	(not always visible)
48 ± 0	(not always visible)	49 ± 1	
44 ± 0		44 ± 2	
42 ± 0			
40 ± 0			
39 ± 0	(not always visible)		
35 ± 0			
33 ± 1		33 ± 1	
28 ± 0	(not always visible)	27 ± 1	
25 ± 1	(not always visible)	25 ± 0	(not always visible)
23 ± 0		23 ± 0	
21 ± 0		21 ± 0	
20 ± 0		20 ± 0	
18 ± 1		18 ± 1	
16 ± 1	(not always visible)	16 ± 1	(not always visible)
14 ± 0		14 ± 0	
12 ± 0		12 ± 0	

also several bands in the alkaline region of the isoelectric focusing gels. Preliminary results indicate that they give rise in the two-dimensional system to a series of spots of low molecular weight (20 000—12 000).

Discussion

We have shown recently that solubilization of thylakoid membranes by Triton X-100 followed by isoelectric focusing in Triton X-100 containing gels (a method already used with success in the study of erythrocyte membrane proteins [15]) permits many proteins to enter the polyacrylamide gel. Among these proteins several peaks were characteristic for PS I and PS II fractions [7]. In this investigation we have directed our interest on the pH gradient between pH 5 and 7 only and improved the resolution in this region. In this pH range we found about 12 major proteins in PS I fractions and seven proteins in PS II fractions (Fig. 1). In addition to these principal bands a number of very closely spaced bands can appear, especially in the more acidic part of the gradient, which may be due to an artifactual heterogeneity. Methodological artifacts producing multiple bands can be due to interactions of proteins with urea or other components of the system. Heterogeneity, not directly due to isoelectric focusing conditions, may arise by the interaction of proteins with different ligands such as lipids or carbohydrates.

In order to characterize further the major proteins separated by isoelectric

focusing it was important to find out which thylakoid membrane proteins were solubilized and separated by this new method. We therefore compared these proteins with the polypeptides separated by SDS electrophoresis. Indeed, almost all of our present information on chloroplast membrane proteins has been obtained by SDS electrophoresis [2,3,5,14]. With unidimensional SDS electrophoresis of PS I and PS II fractions we found patterns (Fig. 2) in many respects similar to those obtained by Klein and Vernon [3]. However, we did not use mercaptoethanol in addition to SDS for solubilization. Without mercaptoethanol, PS II patterns showed a series of higher molecular weight polypeptides (60 000—50 000) which do not appear in samples containing mercaptoethanol [3]. The two-dimensional method gave characteristic fingerprints for PS I and PS II fractions (Fig. 3). When comparing the spots of the two-dimensional fingerprints with the unidimensional SDS electrophoresis, one has to remember that these spots arise from the Triton X-100-extractable proteins. For PS I fractions (Fig. 3A) we found two strong spots with molecular weight of 68 000 and 60 000 characterized by p*I* values of about 5.6. These polypeptides need urea in addition to Triton X-100 in order to focuse distinctly. Polypeptides in the 70 000—50 000 daltons range have been shown to be characteristic of PS I fractions and were denoted "group I polypeptides" [14]. Among these polypeptides there are probably the apoprotein(s) of chlorophyll-protein complex I [3,4,6]. In the PS II fractions (Fig. 3B) the two strongest spots corresponded to polypeptides of 33 000 (33a) and 23 000 daltons, focusing at about pH 5.3 and 6.3, respectively. These two spots, together with a third, weaker spot of 25 000 daltons correspond to the "group II polypeptides" known from unidimensional SDS electrophoresis [14]. One or two of these polypeptides in the 25 000—23 000 daltons range have been reported to represent the apoprotein(s) of chlorophyll-protein complex II [1,6,14].

In addition to these major polypeptides, several additional spots appeared on the PS I and PS II patterns. PS I fractions showed a series of six spots in the 44 000—33 000 daltons range focusing between pH 5.8 and 7 (Fig. 3A). The polypeptides of 42 000 and 33 000 daltons may represent cytochrome *b* [3] and cytochrome *f* [3,16], respectively. In addition to the fact that cytochromes are very well extracted by Triton X-100 [17], the isoelectric focusing method permits to concentrate these molecules at their pI. As an example, the strong isoelectric focusing peak at pH 6.2 (Fig. 1A) corresponds to a conspicuous spot of 33 000 daltons (33a) in the second dimension (Fig. 3A). Thus, in the second dimension system, this polypeptide is quite characteristic for PS I fraction whereas in the unidimensional SDS electrophoresis it appears weakly stained (Fig. 2A). This is also true for all the polypeptides in this molecular weight range. Moreover, the 33 000 daltons spot of PS I fractions is distinct from the strong 33 000 polypeptide of the PS II fractions, the two spots appearing at a totally different position on the two-dimensional maps. This resolution of polypeptides differing in charge only represents an important advantage of the two-dimensional system over the conventional SDS electrophoresis.

PS II fractions showed a series of polypeptides (64 000, 58 000, 48 000 and 39 000 daltons), focusing at the same pH as the strong 23 000 daltons spot (Fig. 3B). All of these polypeptides arise from the strong peak at pH 6.3, domi-

nant in PS II fractions (Fig. 1B). Upon incubation in SDS/mercaptoethanol before the separation in the second dimension, the peak at pH 6.3 was dissociated into polypeptides of different molecular weights (64 000, 58 000, 48 000, 39 000 and 23 000). The 23 000 dalton polypeptide thus probably exists in oligomeric forms.

In conclusion, the two-dimensional approach has permitted to correlate the thylakoid membrane proteins separated by isoelectric focusing in the presence of Triton X-100 with the known SDS-polypeptides. The comparison shows that: (a) chloroplast membrane proteins can be separated by isoelectric focusing without having to use SDS, which may be important for functional studies, (b) the components of PS I and PS II fractions of thylakoids can be distinguished more clearly than with any unidimensional method.

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References

1 Anderson, J.M. (1975) Biochim. Biophys. Acta 416, 191—235
2 Remy, R. (1971) FEBS Lett. 13, 313—317
3 Klein, S.M. and Vernon, L.P. (1974) Photochem. Photobiol. 19, 43—49
4 Anderson, J.M. and Levine, R.P. (1974) Biochim. Biophys. Acta 357, 118—126
5 Nolan, W.G. and Park, R.B. (1975) Biochim. Biophys. Acta 375, 406—421
6 Machold, O. (1975) Biochim. Biophys. Acta 382, 494—505
7 Novak-Hofer, I. and Siegenthaler, P.A. (1975) FEBS Lett. 60, 47—50
8 Siegenthaler, P.A. and Depéry, F. (1976) Eur. J. Biochem. 61, 573—580
9 Vernon, L.P. and Shaw, E.R. (1971) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. XXIII (A), pp. 277—289, Academic Press, New York
10 Bruinsma, J. (1961) Biochim. Biophys. Acta 53, 576—578
11 Maurer, R. (1971) Disc-electrophoresis, 2nd edn., Walter de Gruyter, Berlin
12 O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007—4021
13 Bhakdi, S., Knüfermann, H. and Wallach, D.F.H. (1975) in Progress in Isoelectric Focusing and Isochophoresis (Rhigett, P., ed.), pp. 281—291, North-Holland Publ. Co., Amsterdam
14 Anderson, J.M. and Levine, R.P. (1974) Biochim. Biophys. Acta 333, 378—387
15 Bhakdi, S., Knüfermann, H. and Hoelzl-Wallach, D.F. (1975) Biochim. Biophys. Acta 394, 550—557
16 Süss, K.H. (1976) FEBS Lett., 70, 191—196
17 Wasserman, A.R. (1974) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds.), Vol. XXXII (B), pp. 406—422, Academic Press, New York