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COMPOSITIONAL CHARACTERISTICS OF A CHLOROFORM/METHANOL SOLUBLE PROTEIN FRACTION FROM SPINACH CHLOROPLAST MEMBRANES

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SUMMARY

Extraction of an aqueous suspension of spinach chloroplast lamellae with a chloroform/methanol mixture leads to solubilization of about 1/3 of the total membrane protein. Amino acid analysis of the chloroform/methanol-soluble protein shows that this fraction is largely enriched in the hydrophobic residues proline, leucine, alanine and phenylalanine and considerably depleted in polar amino acids, namely lysine and arginine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the solubilized material reveals the presence of a variety of low molecular weight polypeptides (molecular weight $\leqslant$ 25 000), with more than 50 % of the total fraction being contributed by a 25 000 dalton band. This band, which accounts for about 25 % of the total chloroplast lamellar protein, has recently been identified as the main component of the light-harvesting chlorophyll-protein complex. The physiological role of most of the chloroform/methanol-soluble protein fraction is not known at present. From its chemical properties and apparent biological inertness, we propose that it plays mainly a structural role in situ, interacting with the lipid moiety of the chloroplast membrane. The material insoluble in the aqueous chloroform/methanol mixture is largely enriched in manganese, iron, cytochrome and water-soluble proteins, such as chloroplast coupling factor and ribulose diphosphate carboxylase.

INTRODUCTION

The large number of polypeptides associated with the internal chloroplast membranes greatly exceeds the number of known components required to carry out the photosynthetic functions of these membranes. As we pointed out elsewhere [1], only a small fraction of the membrane protein moiety directly participates in electron transport and related reactions of the photosynthetic process; most of the remaining protein probably plays mainly a structural role, providing for a proper insertion and spatial continuity of the functional components. Within this category of structural protein, there must be a group of polypeptides closely associated with the pigments and uncolored lipids of the membrane; particularly in those cases where polypeptides form tightly bound complexes with the lipid moiety, it is conceivable that the whole

association would show lipid-like characteristics and could, thus, be solubilized by organic solvents under appropriate conditions.

As reported recently [2], our laboratory has been involved for the past few years in the development of a fracturing technique that, splitting the membrane along the inner hydrophobic regions similarly to the freeze-fracturing technique, allows the recovery of the membrane halves for their chemical and functional characterization. Analysis of polypeptide composition of both inner and outer membrane halves, freed of the lipid moiety, revealed that some of the peptides were not recovered in a quantitative way. Following this observation, it was subsequently found that chloroform/methanol extraction of chloroplast membranes, suspended in aqueous solutions, led to the solubilization of a few membrane peptides into the organic solvent mixture.

We have refined this differential solubilization procedure further, to isolate what we believe is a major hydrophobic portion of spinach chloroplast membranes. A description of this procedure, as well as some characteristics of the fractionated material, is reported here.

MATERIALS AND METHODS

Fresh market spinach (*Spinacia oleracea* L.) plants were used in this work. Broken, class II chloroplasts were isolated according to Sane et al. [3] and twice washed in a high-salt medium (0.15 M KCl, 0.05 M potassium phosphate buffer, pH 7.4). The pellet of the last washing was gently homogenized in a few ml of distilled water and its chlorophyll concentration determined by Arnon's method [4]. The homogenate was brought to 1 mg chlorophyll/ml and 2.5 ml fractions each placed in 30-ml Corex tubes; 25 ml of chloroform/methanol mixture (1 : 2, v/v) was added to each tube, the suspension thoroughly homogenized with a glass mortar and centrifuged at 15 000 $\times g$ for 20 min. The pooled supernatants were evaporated to dryness in a rotatory evaporator, under vacuum, and the residue was subjected to lipid extraction with dry chloroform/methanol; the pellet from the initial aqueous chloroform/methanol extraction was twice extracted with dry chloroform/methanol, the supernatant discarded and the residual protein used for further analysis. In a typical experiment, using 8 Corex tubes, we obtained 26 mg protein from the pooled supernatants and 66 mg protein from the initial pellet.

Amino acid analysis of the protein fractions was performed as described by Koshland [5] using a Beckman automatic analyser. For peptide analysis, a portion of membrane protein was resuspended in 0.0625 M Tris · HCl (pH 6.8), 5 % glycerol, 5 % β mercaptoethanol and 2 % sodium dodecyl sulfate [6] and dissolved at a concentration of 2 mg protein/ml by heating in boiling water for 2 min. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, was essentially according to Laemmli's procedure [6], with minor modifications [1]. Gels were prepared in cylindrical tubes (12 $\times$ 0.6 cm) and consisted of a 0.6 cm long 5 % stacking gel (pH 6.8) and a 9 cm long 9 % separating gel (pH 9.0). Sample aliquots were layered on the top of the stacking gel and the run started at 1 mA/tube; when the protein entered the lower gel the current was doubled and the run continued until the front was about 0.5 cm from the end of the tube (approx. 3 h). Gels were fixed and stained with 0.25 % Coomassie brilliant blue R in 7.5 % acetic acid/50 % methanol and the destaining was performed by several changes in Weber and Osborne solutions [7]. Destained

gels were scanned at 560 nm using a Gilford gel scanner accessory attached to a Beckman DU spectrophotometer equipped with a 50 μm slit. Molecular weights were estimated from a standard plot using bovine serum albumin (68 000 daltons), ovalbumin (43 000 daltons), carbonic anhydrase (29 000 daltons) and lysozyme (14 300 daltons) as marker proteins.

Trace metal analyses were performed by atomic absorption spectrophotometry, using a Perkin-Elmer (Model 290) spectrophotometer coupled with a recording system.

Cytochrome measurements of chloroform/methanol-extracted material were done both at room temperature and liquid nitrogen temperature, using a Cary Model 14 spectrophotometer equipped with a scattered transmission accessory. Quantitative cytochrome determinations were made from reduced minus oxidized difference spectra, at room temperature, assuming a 20 mM extinction coefficient [8]. For low temperature measurements, the cell devised by Bonner [9] was used and the membrane fractions were suspended in buffer containing 10 % glycerol.

RESULTS AND DISCUSSION

Chloroform/methanol treatment of aqueous suspensions of chloroplast lamellae results in the fractionation of the membrane components into a chloroform/methanol-soluble fraction and a chloroform/methanol-insoluble fraction. Under the conditions described here, about 30 % of the chloroplast membrane protein is extracted into the organic solvent mixture, with 70 % remaining insoluble. Complete removal of pigments from the chloroform/methanol-soluble material is very difficult to obtain and, even after extensive chloroform/methanol extraction, this fraction retains considerable color. This could be due to extensive denaturation of the pigment-bearing peptide components, but may rather reflect the firmness with which this material binds to lipids, as discussed later.

Amino acid analysis

Amino acid composition of whole chloroplast membranes and the fractioned material are reported in Table I. The amino acid composition of whole chloroplast membranes is very similar to those reported by other authors [10, 11], except for a somewhat different value of alanine and tyrosine. Comparative examination of the two fractions shows that the chloroform/methanol-soluble protein is appreciably enriched in the hydrophobic residues proline, leucine, alanine and phenylanine and significantly decreased in polar amino acids, namely arginine and lysine. The chloroform/methanol-insoluble protein, in turn, shows not only a corresponding increase in polar amino acids but also an enrichment in the sulfur-containing residues methionine and cysteine (cysteine data are not quantitated here).

The percent polar amino acids [12] calculated for the two fractions reveals a value of 37.5 for the chloroform/methanol-soluble protein and 43 for the chloroform/methanol-insoluble fraction, whereas the whole chloroplast membrane exhibits an intermediate value of 41. The low polarity index of the chloroform/methanol-soluble fraction clearly points to the "intrinsic" nature of these membrane proteins, suggesting that this highly hydrophobic fraction could indeed comprise those membrane peptides interacting with the lipids to make up the structural framework of the

TABLE I

AMINO ACID COMPOSITION (MOLAR PERCENTAGE) OF SPINACH CHLOROPLAST INTERNAL MEMBRANES, CHLOROFORM/METHANOL-SOLUBLE PROTEIN AND CHLOROFORM/METHANOL-INSOLUBLE PROTEIN

Amino acid	Whole chloroplast membrane	Chloroform/methanol-soluble fraction	Chloroform/methanol-insoluble fraction
Lys	5.0	4.3	5.4
His	1.2	1.0	1.3
Arg	4.1	3.1	4.5
Asp	9.0	8.8	9.5
Thr	5.1	4.1	5.6
Ser	5.4	5.2	5.4
Glu	9.8	9.0	10.3
Pro	6.0	7.9	5.1
Gly	10.8	11.3	10.3
Ala	9.4	10.5	9.0
Val	7.0	7.0	7.0
Met	1.8	1.4	2.0
Ile	5.2	4.8	5.2
Leu	10.9	11.9	10.7
Tyr	3.3	3.0	3.4
Phe	5.8	6.6	5.3
% polar value	40.8	37.5	42.7

chloroplast lamellae. On the other hand, the 43 % polarity value found for the chloroform/methanol-insoluble protein is consistent with the mixed nature of this fraction, composed of both intrinsic peptides, of polarity below 40 %, and peripheral components of high polarity, usually above 45 % [12].

Peptide analysis

Densitometric tracings of whole chloroplast membranes, chloroform/methanol-insoluble and chloroform/methanol-soluble peptides, after sodium dodecyl sulfate-acrylamide gel electrophoresis, are shown in Figs. 1A, 1B and 1C, respectively. The molecular weights assigned to the major bands of chloroplast membranes were estimated from a comparison of their relative mobilities with those of known proteins and are rounded to the nearest 0.5 kdalton. Some of the peptides seen in the electrophoretic pattern of whole chloroplast membranes are largely decreased by EDTA-washing of these lamellae and belong to the category of peripheral proteins; this is particularly conspicuous for the two high molecular weight components, at 56 and 52 kdalton and, to a slightly lesser extent, also for the peptides at 34 and 32 kdalton. The peak at 52 kdalton represents two unresolved peptides, a subunit of the chloroplast coupling factor and the large subunit of ribulose-1,5-diphosphate carboxylase, two water-soluble proteins known to be adsorbed to the outer surface of the photosynthetic lamellae [13]. The majority of the remaining peptides are not significantly affected by aqueous treatments of the membranes and should, indeed, be considered integral membrane peptides. Of these, the 25 kdalton peak, accounting for about 25 % of the total lamellar protein fraction, is generally recognized as the major structural peptide of the spinach chloroplast membranes [14, 15], and was recently identified as the main

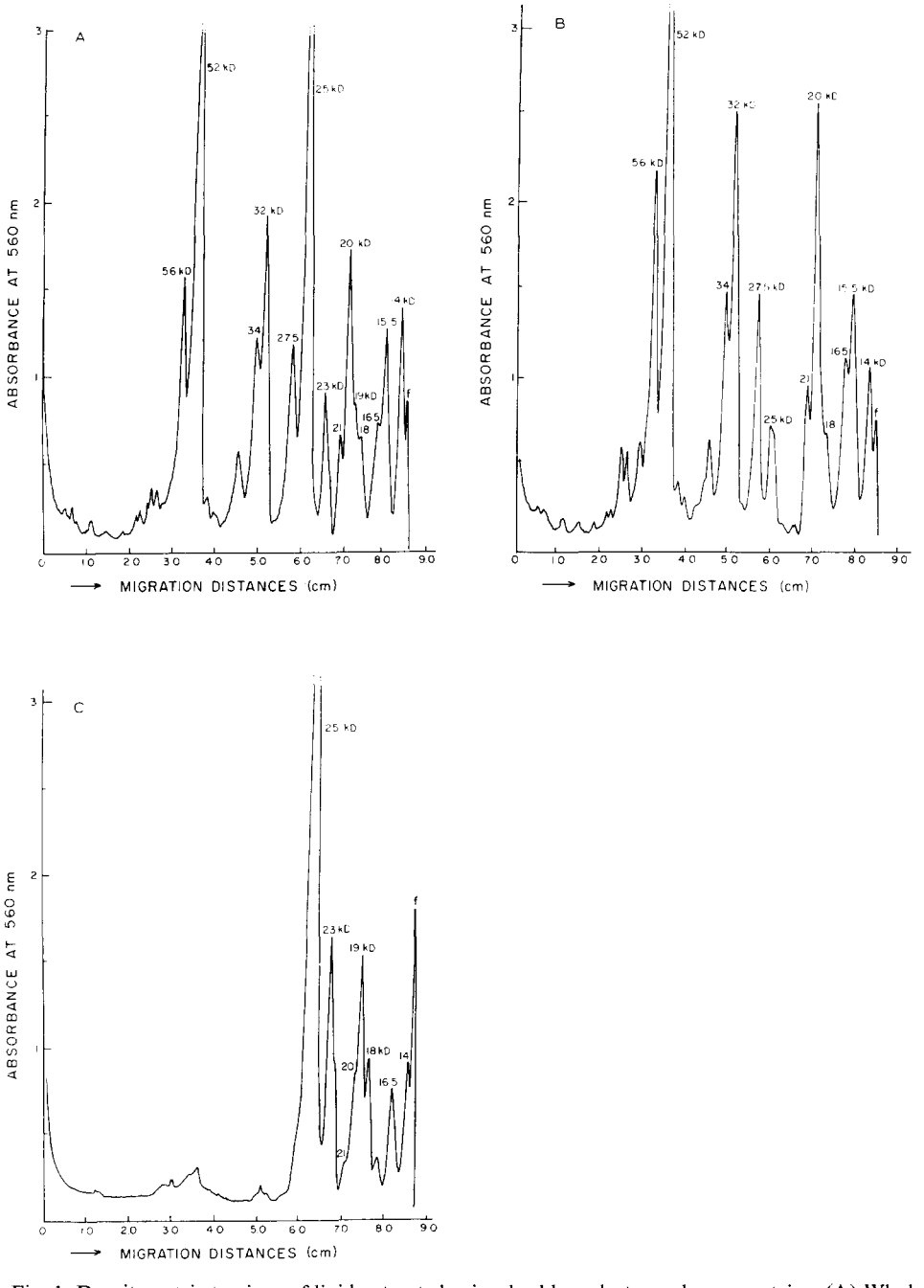

Fig. 1. Densitometric tracings of lipid-extracted spinach chloroplast membrane proteins. (A) Whole chloroplast membrane proteins. (B) Chloroform/methanol-insoluble protein fraction. (C) Chloroform/methanol-soluble protein fraction. Protein samples were solubilized in 2 % sodium dodecyl sulfate, 5 % β-mercaptoethanol and electrophoresed on 9 % acrylamide gels containing 0.1 % sodium dodecyl sulfate, as described under Material and Methods.

protein component of the light-harvesting chlorophyll protein complex [1, 16, 17].

The polypeptide profile of the chloroform/methanol-insoluble fraction shows an overall similarity with that of whole membrane, but differs markedly from this in the complete absence of the 23 kdalton peak and a substantial depletion of the large 25 kdalton band, which is reduced to a rather insignificant doublet. Minor alterations can also be seen in the low molecular weight region, namely a relative increase in the 20 and 16.5 kdalton components and a decrease of 19 and 14 kdalton peaks.

In contrast, the peptide pattern of the chloroform/methanol-solubilized material is strikingly different; here, we find no high molecular weight components (molecular weight $> 25\,000$) with the 25 kdalton band contributing more than 50 % of the total fraction. All of the 23 kdalton peptide is present here and also the 19 kdalton peak is largely enriched in this fraction. It may also be of significance that peptide(s) of very low molecular weight ($\leqslant 14\,000$), running with the front in our electrophoretic system, are preferentially removed with this fraction, whereas the bands at 20, 16.5 and 15.5 kdalton are largely decreased. Though no particular spatial relationship among the chloroform/methanol-soluble peptides can be inferred from our observations they may, indeed, reflect some sort of association in situ, as one should keep in mind that multi-peptide complexes within the membrane would be completely dissociated by the sodium dodecyl sulfate solubilization procedure used here. In a chlorophyll b-less mutant of barley, which completely lacks the 25 kdalton peak, we have recently shown that the absence of this component was accompanied by alteration in two other low molecular weight peptides [1].

Trace metal analysis

Trace metal analysis of whole chloroplast membranes, chloroform/methanol-insoluble and chloroform/methanol-soluble fractions are shown in Table II. The value of 170 ng Mn/mg protein found for whole chloroplast membranes, corresponding to approx. 1 Mn per 70 chlorophylls, agrees well with recent literature data [18–21]. Our data also support the reports that Mn is not extractable by lipid solvents

TABLE II

MANGANESE, IRON AND CYTOCHROME CONTENTS OF CHLOROFORM/METHANOL-EXTRACTED CHLOROPLAST INTERNAL MEMBRANES, CHLOROFORM/METHANOL-SOLUBLE FRACTION AND CHLOROFORM/METHANOL-INSOLUBLE FRACTION

	Mn (ng Mn/mg protein)	Fe (ng Fe/mg protein)	Cytochrome (mol cytochrome/mg protein)
Chloroform/methanol-insoluble fraction	240	1850	$8.5 \cdot 10^{-10}$
Chloroform/methanol-soluble fraction	40	250	$0.7 \cdot 10^{-10}$
Chloroform/methanol-insoluble + chloroform/methanol-soluble fraction	180	1350	$6.2 \cdot 10^{-10}$
Whole chloroplast membranes	170	1250	$7.8 \cdot 10^{-10}$

[19], since all of original Mn is accounted for by the two fractions although they have been subjected to rather extensive chloroform/methanol treatment. As it can be clearly seen, the chloroform/methanol-insoluble protein is largely enriched in Mn, comprising about 95 % of the total lamella-bound cation; on the other hand, the chloroform/methanol-soluble fraction contains only about 0.25 as much Mn, on a protein basis, as the complete membrane. In connection with these results, it is important to note the recent report by Lagoutte and Duranton [11] claiming that the 25 kdalton peak, in maize chloroplasts, is a manganese protein with Mn content 6 times larger than that of whole chloroplast lamellae. This conclusion is in clear contradiction with our results showing that the chloroform/methanol-soluble fraction, of which the 25 kdalton component constitutes more than 50 %, is largely depleted in Mn whereas the chloroform/methanol-insoluble fraction, almost completely lacking this component, is proportionally enriched in this cation.

The distribution of iron between the fractionated material shows a pattern rather similar to that for manganese; the chloroform/methanol-insoluble fraction contains 50 % more Fe/mg protein than the original material and, again, a strong depletion of this metal is observed in the chloroform/methanol-soluble protein. The iron content of whole chloroplast membranes of 1250 ng Fe/mg protein, equivalent to about 1 Fe per 10 chlorophylls, agrees with the values reported by other authors [20, 22], though a large variation in iron content associated with the chloroplast lamellae is found in the literature [23].

Cytochromes

Cytochrome content of whole chloroplast membranes, chloroform/methanol-soluble fraction and chloroform/methanol-insoluble fraction is reported in the last column of Table II. The cytochrome content found for lipid-extracted whole chloroplast membranes of $7.8 \cdot 10^{-10}$ mol cytochrome/mg protein, corresponding to a molar ratio chlorophyll/cytochrome of about 250, is considerably higher than ratios reported in the literature [24]; this is undoubtedly due to extensive denaturation of the cytochrome apoprotein by the chloroform/methanol extraction, and certainly represents a considerable drawback of solubilization procedures based on the use of organic solvents. As compared with whole chloroplasts, the chloroform/methanol-insoluble fraction shows a relative increase of cytochrome content, per mg of its protein, whereas the chloroform/methanol-soluble protein exhibits a considerable loss of this electron carrier. These results, suggesting a drastically asymmetric apportionment of cytochromes between the two fractions have, however, to be interpreted rather cautiously, as the two fractions account for only 80 % of the cytochrome content of the control material. There is no way we can, with certainty, estimate the partition of the unaccounted cytochromes; however, even assuming that all this cytochrome would have gone with the chloroform/methanol-soluble fraction, this would still show a relative depletion in this membrane component.

CONCLUSION

In this work we have used an aqueous chloroform/methanol extraction of the chloroplast lamellae to solubilize a major hydrophobic portion of these membranes, accounting for about 30 % of their total protein. We believe that, in situ, the integral

membrane peptides comprising the chloroform/methanol-soluble fraction are tightly associated with the membrane lipids and that, because of the hydrophobic character of these associations, they become soluble in organic solvents [25, 26]. Although this point would bear further investigation, the fact that this fraction contains practically all the chloroplast 25 kdalton peptide component, which is known to bind about half of the chloroplast chlorophyll, supports our contention. The paucity of the chloroform/methanol-soluble fraction in manganese and iron, two ions functionally involved in the photosynthetic process, its low cytochrome content and its tendency to tightly bind pigments and uncolored lipids of the membrane suggest that this fraction plays no role in the photosynthetic electron transport of these lamellae, rather it may serve primarily to organize spatially other membrane components.

Our data show that the various peptides of the complex photosynthetic lamellae fall into three major groups; the hydrophobic fraction soluble in aqueous chloroform/methanol, the less hydrophobic fraction containing known electron transport components and the hydrophilic proteins extracted in aqueous solvents. A possible relationship among these fractions is one in which the hydrophobic peptides, together with the lipid moiety, organize the electron carriers and other functional components within the thylakoid lamellae, the enzymes involved in the terminal stages of phosphorylation and NADP reduction being attached to the external portion of these membranes.

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