PROTEINS OF THE ENVELOPE AND THYLAKOID MEMBRANES OF SPINACH CHLOROPLASTS

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Received 24 October 1974

1. Introduction

Mackender and Leech [1] first reported on the isolation of a fraction enriched in envelope membranes from *Vicia faba* chloroplasts by differential centrifugation. More recently, the envelopes of spinach chloroplasts, isolated by density gradient centrifugation, have been characterized with regard to enzymatic properties and pigment and lipid composition [2,3]. These envelopes have also been reported to be the sites of biosynthesis of galactolipids [4] and zeaxanthin [5]. Mackender and Leech also reported on the galactolipid, phospholipid and fatty acid composition of *V. faba* chloroplast envelopes [6], but we know of no reports on the proteins of the chloroplast envelope.

Employing a new method for the isolation of functional chloroplasts from spinach [7], and a procedure of Poincelot for separating single and double membranes from chloroplast envelopes [8] we have studied the electrophoretic behavior of the proteins of these envelope fractions on SDS-polyacrylamide gels, and have compared these with proteins of the whole chloroplast, the soluble proteins and the lamellar membranes. Our results show that the proteins of the envelope membranes are quite distinct from those of the other compartments of the chloroplast, such as the soluble fraction and the thylakoid membranes. Although the two envelope fractions contained qualitatively identical polypeptides, there were distinct quantitative differences with respect to two of the polypeptides.

2. Materials and methods

2.1. *Preparation of membranes.*

Chloroplasts from greenhouse spinach (*Spinacea oleracea* L.) were isolated by centrifugation into a density gradient of Ludox AM, a silica sol manufactured by du Pont. This procedure yields pure, intact chloroplasts, which have complete photosynthetic activity [7].

The purified chloroplasts were lysed osmotically by resuspension in 50 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) at pH 6.8. This treatment appeared to have stripped most of the chloroplasts as judged by phase contract microscopy. The envelopes were then isolated from the lysed chloroplasts by centrifugation through discontinuous gradients of sucrose according to the method of Poincelot [8]. The gradients were prepared by successive layering in centrifuge tubes of the following sucrose solutions also containing 50 mM HEPES: 1 ml 30%; 1.5 ml 23% and 2.5 ml 12% (w/v) sucrose. 5–6 ml samples of the lysed chloroplasts, corresponding to about 1 mg of chlorophyll, were layered on top of each gradient and centrifugation carried out at 21 500 rev/min for 1 hr (Damon/IEC B-60, SB-283 rotor).

There was a faint yellow band at the interface between 12 and 23% sucrose and a distinct yellow band at the interface between 23 and 30% w/v sucrose, which we shall refer to as the light and heavy envelope fractions, respectively. Poincelot had

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reported these fractions to correspond principally to single- and double-membrane vesicles. The material from these bands each yielded a bright yellow pellet upon dilution with buffer and centrifugation at 50 000 rev/min for 30 min. In contrast to the condition of the envelope fraction obtained from purified chloroplasts, both the light and heavy fractions of envelopes prepared from crude chloroplasts contained small amounts of green material, which remained with the envelope fractions even after rebanding in a second sucrose gradient.

The sample zone, containing soluble proteins, was colorless; virtually all the green, thylakoid membranes sedimented to the bottom of the tube. These membranes were extracted with acetone [9] and the final pellet designated as thylakoids. About 55% of the total protein was recovered as soluble protein in the sample zone and 40% in the pellet, whereas all of the chlorophyll was recovered in the pellet.

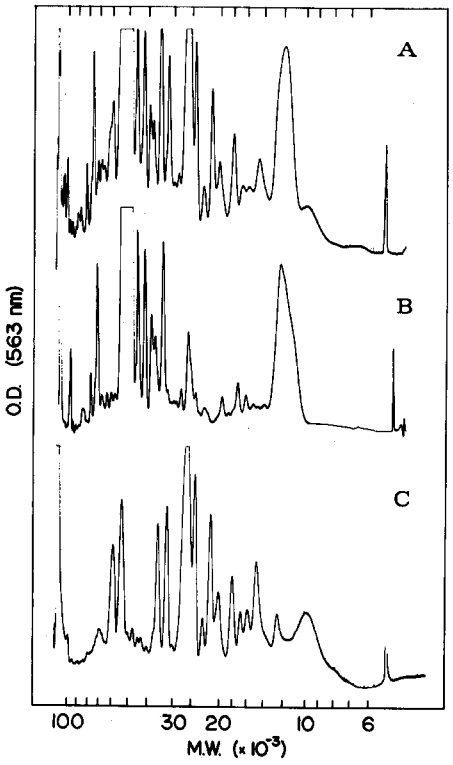

Fig. 1. SDS-polyacrylamide gel electrophoretic patterns of polypeptides of spinach chloroplast; A. Whole chloroplast; B. Soluble fraction; C. Thylakoid membranes. Mol. wts were estimated from the migration of marker proteins [10].

2.2. *Analysis of polypeptides by electrophoresis on SDS-polyacrylamide gels.*

Polyacrylamide gels containing 0.1% SDS (w/v) (sodium dodecylsulfate) were prepared essentially according to the procedure of Laemmli [10]. The separation gel contained 12.5% acrylamide and 0.33% *N,N*-methylene bis-acrylamide, while the stacking gel contained 3% acrylamide. The gels were set in glass tubes, 17 cm long with an internal diameter of 0.5 cm.

The samples for electrophoresis were taken up in sample buffer consisting of 0.0625 M Tris–HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 3% (w/v) SDS and 10% (v/v) glycerol and heated at 70 to 80°C for 10 min. Aliquots containing up to 100 μg of protein were mixed with tracking dye (2 μl of 0.1% Bromphenol Blue) and layered on top of the stacking gels. Electrophoresis was carried out with a current of 1 mA/gel until the samples had just entered the lower gel. Electrophoresis was continued at 2.5 mA/gel until the tracking dye was about 1 cm from the bottom of the tubes. Total electrophoresis time was about 5 hr.

The gels were fixed in 50% (w/v) TCA, and subjected to two cycles of staining and destaining, using Coomassie Brilliant Blue according to the modified procedure of Eikenberry [11].

3. Results

When the proteins of the envelope and thylakoid fractions were analyzed by electrophoresis on SDS-polyacrylamide gels, the resulting polypeptide patterns of the different fractions were easily distinguishable. As might be expected, the profile for polypeptides of the whole, unfractionated chloroplasts is very complex, showing a wide range of polypeptides from about 10 to 100 kilodaltons (fig. 1A). The large peaks at approximately 55 and 12 kilodaltons correspond to the large and small subunits, respectively, of spinach ribulose-1,5-diphosphate carboxylase, as confirmed by electrophoresis of purified samples of the enzyme on similar gels. This enzyme is also present in large amounts in the soluble fraction of the chloroplasts, as can be seen in fig. 1B.

The green pellet obtained after centrifugation of osmotically shocked chloroplasts through gradients of sucrose appeared to contain mainly thylakoid

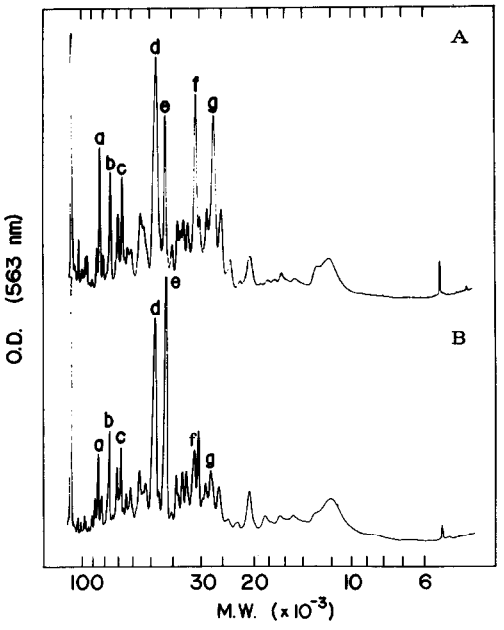

Fig. 2. SDS-polyacrylamide gel electrophoretic patterns of the polypeptides of the chloroplast envelope membranes; A. Heavy envelope fraction; B. Light envelope fraction.

membranes, as shown by the similarity of the polypeptide profile (fig. 1C) to the patterns reported by others for lamellar proteins of spinach chloroplasts [12], as well as of membrane preparations we obtained by successive extraction of isolated chloroplasts with water, dilute EDTA and acetone.

A profile of the polypeptides of the heavy envelope fraction is illustrated in fig. 2A. Although there are again numerous polypeptides, the pattern is distinct from those of other compartments of the chloroplast. The polypeptides of the envelope membranes are confined to the regions of the gel corresponding to relatively low mobilities and consequently high mol. wts. There are seven predominant polypeptides, designated a through g, and ranging from approximately 85 to 27 kilodaltons. The profile of the heavy envelope fraction is remarkably reproducible including most of the minor components.

The profile obtained for the polypeptides of the light envelope fraction is qualitatively identical to that of the heavy envelope fraction in both major and minor components, but the major polypeptides f and g (32 and 27 kilodaltons, respectively) are present in greatly reduced amounts. We have observed this decrease in all our preparations.

We also looked at the polypeptide profiles of envelope preparations obtained from crude chloroplasts. The patterns were similar to those of the envelopes for purified chloroplasts with some minor variations in peak heights of some of the proteins. However, polypeptide e was missing or drastically reduced in both the light and heavy envelope fractions obtained from crude chloroplast preparations.

4. Discussion

The proteins of the envelope fractions of spinach chloroplasts are a distinct set among the total proteins of the chloroplast, as shown by the pattern of their constituent polypeptides on SDS-gel electrophoresis. The principal polypeptides are relatively few in number and correspond to relatively high mol. wts compared to those in the thylakoids and stroma. The polypeptides of both the major and the minor components yield highly reproducible patterns in different preparations of envelopes.

We have confirmed Poincelot's observation [8] that envelopes may be resolved into two fractions in step gradients of sucrose, fractions which he has identified as single and double membranes. We find qualitatively the same complement of polypeptides in the two fractions. If Poincelot is correct in identifying the light fraction as single membranes and the heavy fraction as double membranes, it follows that the light fraction must contain both inner and outer membranes. However, since we do find quantitative differences, namely decreased amounts of polypeptides f and g, it would seem that one of the membranes must undergo specific losses during fractionation. We suggest, as a purely ad hoc explanation, that since the inner membranes have been seen to be attached to the thylakoids [13], they might tend to co-sediment with the latter membranes. According to this reasoning, polypeptides f and g are components of the inner membrane, while a through e are components of the outer membrane or of both membranes. The only observation to support this explanation is that recentrifugation of heavy fraction envelopes on fresh gradients yields small amounts of

a light fraction, which then yields a polypeptide profile indistinguishable from the heavy fraction; i.e., the double membranes, once separated from the thylakoids, break down into equal numbers of inner and outer membranes.

We should like to note that Poincelot's fractionation procedure also provides a simple and efficient means for separating the stroma and thylakoids cleanly from one another as well as from the envelopes. The polypeptide profiles of each fraction showed little evidence of cross-contamination.

Acknowledgements

We wish to thank Dr C. A. Price for his helpful discussions and criticisms and Erik Breden for his technical assistance.

This work was supported in part by grants from the USPHS No. HD-05602 and the Charles and Johanna Busch Memorial Fund.

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