Further Chemical and Morphological Characterization of Chloroplast Membranes from a Chlorophyll b-less Mutant of *Hordeum vulgare*

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ABSTRACT

A comparative study of peptide composition and freeze-fracture morphology of chloroplast membranes from a chlorophyll b-less mutant and a normal barley plant (*Hordeum vulgare* L.) is reported in this work. Using a high resolution, discontinuous sodium dodecyl sulfate—acylamide gel electrophoretic system, we show that the mutant chloroplast membranes not only completely lack the 25-kilodalton peak, which accounts for about 18% of the chloroplast membrane protein in the normal plant, but also exhibit gross reduction in other components at 27.5- and 20-kilodalton regions. Despite such extensive deletions in the peptide composition of the mutant chloroplast lamellae, no alteration could be detected in either density or size of the intramembranous particles, visualized by freeze-fracturing.

In 1950 Highkin reported a chlorina strain of barley which apparently lacked Chl b (11). Several years later, Highkin and Frenkel (12), using more sensitive techniques, confirmed the absence of Chl b and undertook the first metabolic studies of this unusual higher plant. In 1966, two studies (5, 10) appeared simultaneously, dealing with the structure and photochemistry of the chloroplasts from the Chl b-less barley mutant and its wild type. The mutant chloroplasts were reported to have a rather disorganized internal membrane system, with less lamellae per granum and fewer grana per chloroplast than the wild type (10). These changes in chloroplast structure did not impair the photochemical abilities of the mutant as shown by Boardman et al. (5). Indeed, all membrane-associated functions were active in the mutant and displayed rates which were comparable to those of chloroplasts from the wild type.

For over 20 years the only demonstrated chemical deficiency in the barley mutant was the absence of Chl b, although it remained a definite possibility that some other lesions on the photosynthetic apparatus might exist. Recently, chloroplast membrane polypeptides have been analyzed by SDS-acylamide gels and it was found that the chloroplasts of the mutant plants lack certain polypeptides (2, 9, 23).

The number of missing polypeptides and their electrophoretic mobilities vary somewhat in these reports. Thornber and Highkin (23), using a rather crude system for peptide fractionation, observed the absence of a large band in the mutant profile; Genge et al. (9) obtained essentially the same result; Anderson and Levine (2, 3) in two recent publications, using quite different solubilization procedures and electrophoresis techniques, succeeded in showing that two of the group II polypeptides, IIb and IIc, were missing from the mutant pattern whereas two new peptides with slightly different mobilities from IIb and IIc were reported. These apparent discrepancies are undoubtedly the result of differences in the analytical techniques used, and these conflicting data should be resolved with further comparable observations.

In this paper we explore the peptide composition of mutant chloroplast membranes in greater detail than reported previously. So that our results are comparable to previous studies, we analyzed the mutant and wild-type membranes by polyacrylamide disc gel electrophoresis. These preparations were then further analyzed using a high resolution SDS-acylamide slab gel electrophoretic technique.

A freeze-fracture study of the photosynthetic membranes of both the Chl b-less mutant and the wild type is also presented. This study was prompted, in part, by recent observations in our laboratory (19) that in spinach the peaks in the 25 kD region, which may be assumed homologous with those in barley, are associated with the inner fracture face (B) of the membrane which is the site of the large particles. If a similar correlation holds for barley chloroplasts, one would expect that the mutant chloroplast, lacking this group of polypeptides, might exhibit some modification of the large B-face particles.

MATERIALS AND METHODS

Seeds of the Chl b-less mutant and normal barley plants (*Hordeum vulgare* L.) used in this study were kindly supplied by Dr. Harry R. Highkin, San Fernando Valley State College. The plants were grown at 25 °C under artificial illumination (2000 ft-c) with an 8-hr photoperiod. Leaves from one-month-old plants were used in these experiments.

Chloroplast Isolation. Chloroplasts were isolated in 0.05 M potassium phosphate buffer (pH 7.4)–0.01 M KCl–0.5 M sucrose following the method of Sané et al. (21); after isolation, the chloroplast pellet was washed three times with a 0.05 M phosphate buffer (pH 7.4) containing 0.15 M KCl, and finally was incubated for 30 min in 1 mM EDTA (pH 8). This suspension was then centrifuged at 20,000 g for 20 min to yield the final pellet used in these studies. Chloroplasts were iso-

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Abbreviation: kD: kilodalton.
lated according to Jensen and Bassham (14) for freeze-fracture observations.

**Extraction and Solubilization of Proteins.** The chloroplast membrane protein fraction was recovered by centrifugation after extensive lipid extraction with chloroform-methanol (1:2; v/v), followed by three washes with anhydrous methanol.

After drying under vacuum for 3 hr, the protein pellet was re-suspended in 0.0625 M tris-HCl (pH 6.8), 5% glycerol, 5% mercaptoethanol, and 2% SDS (15) at a concentration of 1.5 mg of protein/ml and was heated in boiling water for 2 min.

**Gel Electrophoresis.** The electrophoretic method employs a discontinuous SDS buffer system (15); gels were prepared either in cylindrical glass tubes or in a thin slab apparatus (Hoefer Scientific Instruments, San Francisco). The tube (0.6 x 10 cm) gel technique uses a 1-cm long 5% stacking gel (pH 6.8) and a 8-cm long 9% separating gel (pH 8.8), prepared according to Laemmli (15). Forty microliters of sample solution were layered on the top of the gels and the run was started at 1 mAmp/tube. When the protein entered the lower gel, the current was doubled and the run continued until the tracking dye (pyronin Y) was about 1 cm from the end of the tube. The slab apparatus consists of 2 glass plates (30 cm long, 18 cm wide) which sandwich a 0.75-mm thick slab gel between them. An upper 5% gel (pH 6.8) and a 9% lower gel (pH 9) as described by Studier (22) and Ames (1) were used.

By increasing the pH of the separating gel, a considerable increase in resolution of low mol wt components was obtained. Sixty microliters of sample solution were layered on each well and voltages applied as follows: 15 min at 50 v, 15 min at 300 v, 2 hr at 500 v, and 2 hr at 650 v.

Gels were stained for protein with Coomassie brilliant blue R and destained sequentially (8). Densitometric tracings were made using a Gilford gel scanner attached to a Beckman Model Du spectrophotometer equipped with a 50 μm slit.

Molecular weights of sample peaks were estimated from a standard plot using phosphorylase a (93,000 daltons), BSA (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and Cytc (12,400 daltons) as marker proteins; relative electrophoretic mobilities were calculated according to Weber and Osborn (24).

**Freeze-fracture.** Immediately after isolation, small droplets of the chloroplast pellet were placed on copper discs, frozen in "Freon 22" at -150 C and freeze-fractured in a Balzers apparatus at -110 C. A platinum-carbon replica of the fracture face was prepared. The replica was cleaned on H2SO4 and Clorox bleach and was examined in a Siemens Elmiskop 1A electron microscope.

**RESULTS**

**Gel Electrophoresis.** When analyzed by SDS-acrylamide gel electrophoresis technique, the membrane proteins of barley chloroplasts reveal a complex electrophoretic pattern, comprising a large number of components with mol wt ranging from approximately 10,000 up to 100,000 daltons (Figs. 1 and 2). The apparent mol wt shown in these figures were assigned from comparison of relative mobilities of sample peaks with those of known mol wt proteins, according to Weber and Osborn (24) and are expressed in kD. Because of the rather extensive washing procedure to which the chloroplast lamellae have been subjected, the polypeptides seen in the gel profiles are considered tightly bound components of the photosynthetic membranes.

Examination of the densitometric tracing of Figure 1A shows that four components predominate the gel profile, viz., the peaks at 55, 54, 31.5, and 25 kD; of these, the 25 kD band is probably a composite, as can be seen in Figures 1B and 2. The peak marked f contains a small group of low mol wt proteins, not resolved by the separating gel, which co-migrate with the glycine-chloride front. The last peak on the right hand side of the gels is attributable to absorbance of the nichrome wire that indicates the marker dye position.

Comparison of the polypeptide patterns for chloroplast membranes from normal barley and the Chl b-less mutant (Fig. 1A and B) clearly shows that striking dissimilarities exist...
between the two strains, particularly at the middle region of the electrophoretogram. The most remarkable difference is the virtual absence of the band at 25 kD in the mutant profile. The presence of two, or perhaps three, small components in this region of the mutant seems to indicate that the broad 25 kD band of the wild type may indeed comprise a group of distinct peptides with very similar mobilities, impossible to resolve with the disc tube gel technique.

Another significant difference is the large decrease in the 27.5 kD peak, as well as its shoulder, which are reduced to less than one third of their amounts in the wild type. A less obvious difference, but consistently observed in our experiments, was found in the reduction of the 20 kD component in the mutant. This was a minor peak and because of the poor resolution of the disc gel technique in the low mol wt range it was usually not seen or obscured by large neighboring bands (2, 3). In contrast with previous reports using the disc gel technique (2, 3), we have not been able to find any new peptides specific to the mutant chloroplast membranes or any relative increase in its components with respect to the wild type. It is important to stress the over-all similarity of peptide patterns for chloroplast membranes from both sources, excluding the differences mentioned above.

To find whether additional differences could be observed with higher resolution techniques we reexamined the peptide profiles for the mutant and wild-type chloroplast by SDS-slab gel electrophoresis. Besides the higher resolution achieved, this technique introduces some other important advantages, especially a more satisfactory comparison of bands due to elimination of variations from tube to tube which occur in disc gel experiments. An SDS-slab gel photograph is presented in Figure 2. Through an appropriate change in pH of the separating gel, we were able to spread the bands below the 25 kD peak, so that they were now more discretely separated and minor differences were more easily detected. The number of bands was almost doubled as can be seen when Figure 2 is compared with the disc patterns in Figure 1, A and B. The original slab gel showed more than forty components. Not all of these, however, are visible in the photograph. The slab gel data confirm the differences already detected with the disc gels. A possible additional difference was noticed in the polypeptide immediately above the 20 kD peak, which was not visible in the mutant profile.

**Freeze-fracture.** After some initial dispute it is now generally accepted that the freeze-fracture splits the membrane along an internal fracture plane, yielding two complementary faces (6, 25). Application of this technique to chloroplast lamellae led to visualization of these membranes as particulate structures. One interesting observation was that the particles are not distributed at random within the membrane but rather are arranged in such way that one can differentiate two complementary fracture faces. Following the nomenclature of Branton and Park (7), B is the face bearing a relatively small number of large particles (average 175 Å in diameter and about 90 Å in height) and C is the face packed with large numbers of particles which are heterogenous in size but smaller than those of face B. Images of the freeze-fracture chloroplast membranes from the wild type and Chl b-less mutant barley plants are shown in Figure 3. Apparently, the deletion of polypeptides in the mutant has little or no effect on the fracture plane; the two electron micrographs are very similar, not only to each other, but to other published work on barley (13) and higher plants in general (7, 16, 17, 20).

**DISCUSSION**

Thornber and Highkin (23) were the first to report the absence of a major component in an electrophoretogram of chloroplast membrane proteins from the barley Chl b-less mutant. They showed that this component comprises the protein moiety of the photosystem II Chl-protein, which they renamed light-harvesting Chl-protein complex. Because of the poor resolution of the gel system they used, no further differences could be detected between the mutant and wild type strain. Genge et al. (9) also addressed themselves to the problem of Chl-protein complexes, essentially confirming the conclusions of Thornber and Highkin in reporting the loss of the pigment-protein complex II. Levine and Anderson (2) using more suitable electrophoretic techniques for polypeptide separation, were able to show that two of the group II polypeptides, the components IIb and IIC (Levine's group nomenclature) were lacking in the mutant profile. Two rather prominent peaks were, in turn, present in the mutant in a seemingly correspondent position to bands IId and IIC of wild type, but...
according to those authors they have different electrophoretic mobilities from those peaks and can not be considered homologues of components IIb and IIc. In a recent publication, Anderson and Levine (3) reexamined the polypeptide profile of both mutant and wild-type chloroplast membranes and reaffirmed their view that the two polypeptides present in the mutant profiles are not coincident with those in the wild type. They also reported an increase in polypeptide IIa relative to the wild-type profile, which none of the above authors found in their gels.

Our results disagree to a certain extent with these reports. We show the virtual absence of the 25 kD component as well as a large decrease in the 27.5 and 20 kD components in the mutant profile. We have not found any new polypeptide or enrichment in any of the mutant components relative to the wild-type pattern. These discrepancies are probably attributable to differences in the analytical procedures used. Under the conditions in which membrane-proteins are most often solubilized the process is incomplete, with the consequence that small aggregates may be mistaken for single polypeptides. This may explain some of the variations in results reported in the two papers by Anderson and Levine (2, 3) discussed above. When Figure 1 of reference 2 is compared with Figure 3 of reference 3, numerous differences are apparent, even though the same solubilization and separation procedures were used. The group I polypeptides, for example, while separated in Figure 3B (3) exist as a single peak in Figure 1B (2). Also striking disparity in the ratios of group II peak heights can be seen between Figures 1 (2) and 3 (3) as well as a large variation in the amount of low mol wt components in the two experiments. The solubilization procedure used by us not only gives a reproducible pattern from run to run, but also resolves a large number of peaks on disc gels, allowing a finer and more reproducible analysis of polypeptide composition, which better characterizes the differences between mutant and wild-type membranes.

From compositional data (4, 18) on chloroplast membranes, we know that the electron transport components of chloroplast lamellae account for less than 15% of the total membrane protein. Thus, it is not surprising that extensive deletions of polypeptides can occur in the Chl b-less mutant without seriously affecting electron transport. However, we tacitly assume that the “structural polypeptides,” although not directly involved in electron transport are essential to the proper spatial configuration of the membrane. In this sense, the finding that their omission has no major effect on the functional integrity of the chloroplast membrane is unexpected. The observations by Thornebr and Highkin (23) and Genge et al. (9) give some indications of the role played in the wild type by the missing peaks. Their data show that the peaks in the 25 kD region comprise the protein moieties of the light-harvesting Chl-protein complex. The physiological significance of most of the other peaks seen in our electrophoretogram, however, is not known. The peptides at 55 and 54 kD are extensively washed out by 1 mM EDTA and represent, most probably, subunits of the coupling factor and RuDP carboxylase enzymes. Though some other peaks can be tentatively identified by comparing their mol wt with those of known electron carriers present in the photosynthetic membrane, a more detailed knowledge of individual polypeptides is necessary before such assignment can be done with certainty.

For many years the absence of the Chl b was implicitly attributed to the omission of an essential step in the conversion of this pigment from its precursor, Chl a. The present observations, as well as data from others (23), suggest that, alternatively, the lack of the pigment could be attributable to the absence of an appropriate environment in the chloroplast membrane for that reaction to occur; this causes the pheno-
typic expression of the mutation. A third possibility, already evoked by Highkin and Frenkel (12) and the one that most easily would explain the multiple differences between the mutant and wild-type strains, is that several sites have been affected by mutation. The present data do not allow us to select among these different possibilities, and more detailed genetic and biochemical analysis must be performed before the primary effects of the mutation become understood.

The observation that the mutant chloroplast membranes do not show any apparent alteration of their intramembranous particles, either in size or in number, is unexpected because there is evidence that these particles are largely protein in nature (17) and because the missing polypeptides account for a large fraction (about 20%) of the membrane protein. This result can be tentatively explained by assuming the large particles contain a multitude of different polypeptides and that the removal of only a few do not lead to any morphological alterations detectable by our techniques. Alternatively, they may be taken to suggest that some of the membrane protein is not in the particulate form, but rather differently arranged in the structural framework of the photosynthetic membrane. Further studies are necessary before a comprehensive understanding of the relationship between the peptides seen in the gel patterns of chloroplast membranes and the architecture of these membranes, as visualized by the freeze-fracture technique, is possible.
LITERATURE CITED