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Higher plant chloroplasts: Evidence that all the chlorophyll exists as chlorophyll–protein complexes

(*Zea mays* L./zwitterionic detergent/electrophoretic fractionation)

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ABSTRACT **By using the polyacrylamide gel electrophoresis system described in this report, it was possible to fractionate all the photosynthetic pigments of maize (*Zea mays* L.) thylakoids into chlorophyll–protein complexes with negligible formation of free or detergent-complexed chlorophyll. Identical sodium dodecyl sulfate extracts of thylakoids have previously resulted in up to 50% of the chlorophyll migrating as free chlorophyll after electrophoresis. The major difference from previous gel electrophoresis systems is the replacement of sodium dodecyl sulfate in the electrophoresis buffer by Deriphat 160 (disodium *N*-lauryl-β-iminodipropionate), a zwitterionic detergent. The results suggest that: (*i*) no significant amount of free chlorophyll exists in the chloroplast thylakoid membranes *in vivo*, and (*ii*) most of the free pigment seen previously on gels was generated during the electrophoresis and was not a result of the solubilization technique. Additionally, the new chlorophyll–protein complexes resolved appear to have different characteristics (pigment content and size) than those observed in former systems.**

Since introduction of the technique in 1966 (1, 2), sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis has been widely used in the fractionation and study of chlorophyll–protein complexes. A typical fractionation pattern exhibits two major chlorophyll–protein complexes, representing as much as 50% of the total pigment, and a rapidly migrating band of chlorophyll that does not appear to contain protein (3). This rapidly migrating band has been termed the "free pigment zone" (4) and is thought to consist of detergent-complexed chlorophyll and carotenoids. The two major chlorophyll–protein complexes have been termed the P700–chlorophyll *a*–protein complex (5) and the light-harvesting chlorophyll *a*/*b*–protein complex (6) on the basis of their pigment content and function. There have been a number of reports in the last few years that indicate resolution of additional chlorophyll–protein complexes (7–14). Although these chlorophyll–protein complexes have generated much interest, little work has been done with the free pigment material. A recent report (15), in which the goal was to fractionate chlorophyll–protein complexes with decreased generation of free pigment, described an electrophoretic fractionation procedure by which extracts of higher plants yielded only 10% free chlorophyll. This decrease in the percentage of free chlorophyll was accompanied by an increase in the amount of chlorophyll *a* in the P700–chlorophyll *a*–protein and the resolution of previously unobserved chlorophyll–protein complexes. These data corroborated suggestions (11, 12) that the majority of the free pigment had been generated by breakdown of the P700–chlorophyll *a*–protein. It has also been suggested that at least some of the free chlorophyll is generated from heretofore unresolved chlorophyll–protein complexes that are denatured by the currently used methods of preparation (16).

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The present report describes a system for solubilization and fractionation of higher plant chlorophyll–protein complexes from thylakoid membranes. The procedure appears to be less harsh than conventionally used procedures. The amount of free pigment observed during fractionation is negligible. In addition, a radically different pattern of chlorophyll–protein complexes is observed after fractionation.

MATERIALS AND METHODS

All plants were grown in a greenhouse with available light. Leaf material was collected from 10-day-old seedlings of maize (*Zea mays* L.). For the preparation of washed thylakoid membranes, approximately 5 g of leaves was collected and rinsed in cold distilled water. The leaves were cut into small segments and homogenized in a mortar with a pestle and washed sand. The cold grinding medium contained 400 mM mannitol, 20 mM *N*-tris(hydroxymethyl)methylglycine (pH 7.6), and 10 mM NaCl. All further steps were conducted at 0–4°C. The resulting brei was filtered through one layer of Miracloth (Chicopee Mills, Inc., Milltown, NJ), and the filtrate was centrifuged momentarily at 270 × *g* to remove whole cells and large debris. The supernatant fraction was decanted and centrifuged at 12,000 × *g* for 10 min to pellet the chloroplast fraction which contained most of the pigment. The chloroplasts were resuspended with a Ten Broeck homogenizer in 50 mM Tris, pH 8.0/1 mM Na EDTA containing 1 mM phenylmethylsulfonyl fluoride to inhibit protease activity. The solution was centrifuged at 18,000 × *g* for 10 min. This wash was repeated and an aliquot was removed for chlorophyll determination.

The pelleted membranes were suspended in 6.2 mM Tris/48 mM glycine/1% $NaDodSO_4$/10% (vol/vol) glycerol at a $NaDodSO_4$/chlorophyll ratio (wt/wt) of 10:1. The detergent solution was cooled immediately prior to use to circumvent the problem of $NaDodSO_4$ precipitation in the buffer upon cold storage. The sample was suspended with the aid of a glass homogenizer and immediately centrifuged at 40,000 × *g* for 10 min. The supernatant fraction was immediately loaded onto the gels. Samples (2–15 μl) were routinely used for electrophoretic analysis.

Gels were cast containing 5% acrylamide, 0.25% *N*,*N*′-methylenebisacrylamide, 0.125% ammonium persulfate, 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 6.2 mM Tris, 48 mM glycine, and 0.1% Deriphat 160. Slab gels were 2 mm thick and 115 mm long. Tube gels were 8 mm in diameter and 100 mm long. The upper and lower reservoirs were filled with 12.4 mM Tris/96 mM glycine/0.2% Deriphat 160, pH ≈8.3. The electrophoresis was carried out at 100 V for 30–35 min.

Densitometer tracings of the resulting gels were obtained as described (15). For molecular weight estimation, proteins of known molecular weight were denatured at 100°C for 3 min

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

in 6.2 mM Tris/48 mM glycine/1% $NaDodSO_4$/1%. 2-mercaptoethanol/10% (vol/vol) glycerol. Gels were fixed overnight with 10% acetic acid prior to staining with Coomassie brilliant blue R. The marker proteins used and their subunit molecular weight values were as follows: mollusc hemocyanin, 290,000; rabbit muscle phosphorylase *a*, 92,500; catalase, 60,000; and ovalbumin, 43,000.

The concentration of chlorophyll in samples extracted in 80% (vol/vol) acetone was determined spectrophotometrically by using Arnon's equations (17). To obtain spectra of material separated by gel electrophoresis, the appropriate section of the gel was excised and scanned directly. All absorption spectra were recorded on an Aminco DW-2 spectrophotometer.

Acrylamide (enzyme grade) and *N,N'*-methylenebisacrylamide were purchased from Eastman Kodak; ammonium persulfate, from Baker; phosphorylase *a*, from Worthington; and Tris, *N*-tris(hydroxymethyl)methylglycine, mannitol, glycine, phenylmethylsulfonyl fluoride, ovalbumin, catalase, $NaDodSO_4$, and *N,N,N',N'*-tetramethylethylenediamine, from Sigma. Deriphat 160 (disodium *N*-lauryl-β-iminodipropionate) was a generous gift of the Chemical Division, General Mills (Kankakee, IL). Hemocyanin was a gift of M. A. K. Markwell (Molecular Biology Institute, University of California, Los Angeles, CA).

RESULTS

Electrophoresis of the maize thylakoid extracts resulted in the banding pattern shown in Fig. 1. Similar results were obtained with tobacco (*Nicotiana tabacum* L.) and barley (*Hordeum vulgare* L.) extracts. When tube gels were used (to facilitate densitometer scanning), the amount of total migration during the 35 min of electrophoresis was directly dependent on the volume of the sample. This problem was not encountered with multiple-sample slab gels. The phenomenon is assumed to be due to the current passing through the tube gels being dependent on the ionic content of the sample.

Four chlorophyll–protein complexes, labeled N, O, P, and Q, and a zone of free chlorophyll (labeled F) were resolved on the gels. The amount of free chlorophyll was usually 2–3% of the chlorophyll *a* applied to the gel. In about 20% of the experiments, there was no detectable free pigment. In no case did we observe any chlorophyll-containing material that failed to enter the gel. The effect of heating at 100°C for 5 min is shown in Fig. 2A; most of the chlorophyll migrated in the free pigment region of the gel. A $NaDodSO_4$ extract incubated at 20°C for 24 hr contained all four chlorophyll–protein complexes, but the amount of free chlorophyll was then 20% (data not shown).

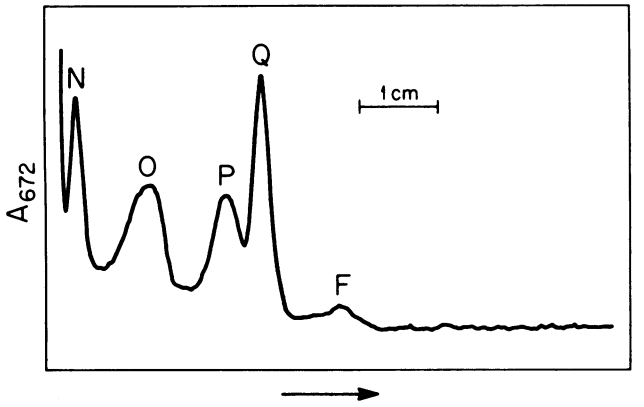

FIG. 1. Densitometric tracing, at 672 nm, of a gel immediately after electrophoresis. The sample was 3 μl of a $NaDodSO_4$ extract of maize thyalkoids. The bands are chlorophyll–protein complexes; the gel was not stained to reveal proteins. Arrow, direction of migration.

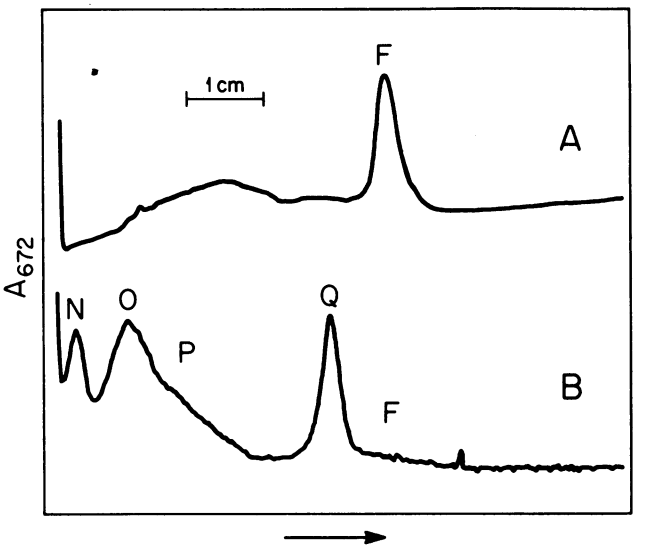

FIG. 2. Densitometric tracing of polyacrylamide gels. (*A*) Sample heated at 100°C for 3 min. (*B*) Sample prepared as described in *Materials and Methods*, in which the P band migrated less than usual.

The chlorophyll–protein complexes that are resolved appear to be different from any previously reported complexes. The migration of the N, O, and Q bands was constant, but that of the P band was not. This band sometimes appeared to split into two or three bands, all intermediate in migration between the O and Q bands. At other times the P band migrated more slowly and appeared as a shoulder on the faster migrating side of the O band (Fig. 2*B*). Additionally, a minor chlorophyll-containing band was occasionally seen that migrated more slowly than the N band. Spectra for the four chlorophyll–protein complexes are shown in Fig. 3. The red absorption maximum for all four complexes was 671–673 nm. The P band is seen to contain significant amounts of chlorophyll *b* (substantial absorbance at 470 and 650 nm) in addition to chlorophyll *a*, whereas the N, O, and Q bands contain predominantly chlorophyll *a*.

When the gels were treated with Coomassie brilliant blue, stained bands were located in the positions where the N, O, P, and Q bands had migrated, corroborating that these are indeed chlorophyll–protein complexes. By using slab gels, the migra-

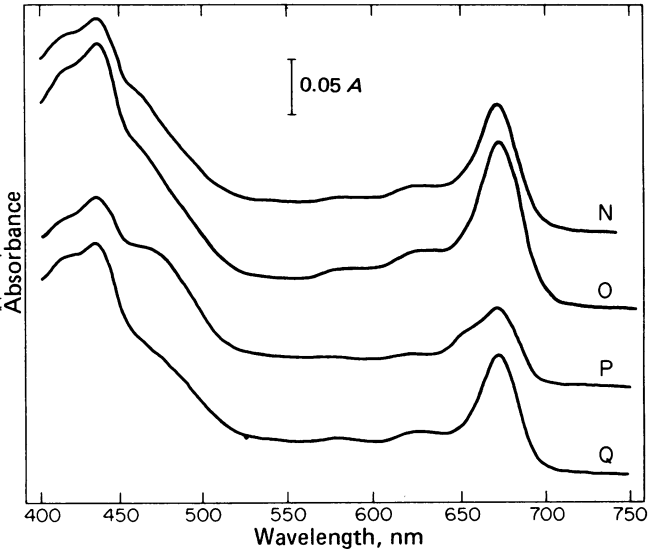

FIG. 3. Absorption spectra of the N, O, P, and Q bands at room temperature.

tion of the various chlorophyll–protein complexes was compared to that of polypeptides of known molecular weights. Values equivalent to molecular weight were assigned to each of the chlorophyll–protein complexes as follows: Q, approximately 50,000; P, variable between 60,000 and 100,000; O, approximately 150,000; and N, approximately 250,000.

DISCUSSION

A major obstacle to obtaining a detailed description of the total organization of the photosynthetic pigments in plants has been the large amount of free pigment that is observed after fractionation of the thylakoid membranes. Some years ago it was believed that all the chlorophyll was contained in the lipid bilayer of photosynthetic membranes. The advent of polyacrylamide gel electrophoretic techniques showed conclusively that at least the majority of chlorophyll was conjugated with protein, but still a $NaDodSO_4$ extract of higher plants would normally exhibit 30–50% free chlorophyll upon electrophoresis (3). Although it seemed most reasonable to suppose that this free chlorophyll resulted from denaturation of chlorophyll–protein complexes that existed in the photosynthetic membrane (16), it was still possible that a significant pool (about 10%) of non-protein-complexed chlorophyll occurs *in vivo*. We therefore sought a fractionation system that would yield all of the photosynthetic pigment in chlorophyll–protein complexes without generation of free chlorophyll in an attempt to substantiate the supposition that all the chlorophyll is conjugated with protein. It would then not be necessary to postulate additional, highly labile complexes that do not survive the electrophoresis, and a determination of the total organization of pigments *in vivo* would become a possibility. Such a system would also do much to alleviate the concern that the complexes studied to date have been partially denatured and may not be an accurate reflection of the *in vivo* state of the complexes. The system described in this report demonstrates that fractionation without significant denaturation is possible. The amount of free chlorophyll generated by the gel fractionation system was very low (2–3%). In many cases, the amount of free pigment was negligible (e.g., Fig. 2*B*). Similarly, the amount of carotenoid found unassociated with protein is greatly reduced. Although not studied quantitatively, it appears that the amount of carotenoid found in the chlorophyll–protein complexes is increased over the amounts found previously.

The drastic reduction in the percentage of free pigment was accompanied by a concomitant change in the chlorophyll–protein complex profile. Previously, the largest generally observed chlorophyll–protein complex was the P700–chlorophyll *a*–protein (5). The molecular weight equivalence of this complex is generally reported as between 100,000 and 130,000 (3, 16). There has been a report of a presumed dimer or oligomer of this complex (14), but data on its molecular weight equivalence are lacking. The P700–chlorophyll *a*–protein, which comprises the photochemically active core of photosystem I, contains chlorophyll *a* but not chlorophyll *b*. None of the bands described in this report seem to have characteristics identical to those of the previously characterized complex. Two of the other new complexes, the N and O bands, appear to have molecular weight equivalencies in excess of any previously studied complexes. The designations N, O, P, and Q are tentative. As soon as these complexes are better characterized and their functions are determined, more appropriate designations will be assigned.

Although estimation of polypeptide size by electrophoresis on polyacrylamide gels is accepted as valid with fully denatured samples (18), its accuracy in assigning absolute values to partially denatured chlorophyll–protein complexes is uncertain (cf. ref. 16). The values given here are provided only as a preliminary estimate of the molecular size. The molecular weight standards were denatured in the same detergent solution (with the addition of 2-mercaptoethanol) used to solubilize the chlorophyll–protein complexes. These standards migrated distances proportional to the logarithm of their respective molecular weights even though the gel contained Deriphat 160, rather than $NaDodSO_4$. Thus, the migration of the chlorophyll–protein complexes, although modified by the extent of detergent bound and molecular shape, should be dependent on size (i.e., larger complexes will migrate more slowly than smaller complexes). It seems likely that the N and O complexes are larger than any previously characterized chlorophyll–protein complex and may represent supramolecular associations of smaller complexes. If this is the case, these supramolecular complexes may be more representative structural and functional units of the *in vivo* photosynthetic apparatus than any previously studied components.

It is significant that the $NaDodSO_4$ extract contained negligible amounts of free pigment. This extract is essentially the same as extracts used previously. The initial work with fractionation of chlorophyll–protein complexes by polyacrylamide gel electrophoresis resulted in approximately 30–50% of the chlorophyll as free pigment. Recent modifications of the electrophoretic system reduced the amount of free pigment to approximately 10% (15). The further modifications presented in this report, using the same $NaDodSO_4$ extract, resulted in negligible amounts of free pigment. Thus, it would appear that the large amounts of free pigment observed earlier resulted from denaturation of the chlorophyll–protein complexes during the electrophoretic, and not during the solubilization, process.

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