CHLOROPHYLL–PROTEIN COMPLEXES FRACTIONATED FROM INTACT CHLOROPLASTS

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1. Introduction

Higher plant chlorophyll(chl)–protein complexes have been subjects of intensive investigation for almost 15 years [1–3]. The technique of most general usefulness for fractionation of these detergent-solubilized complexes has been polyacrylamide gel electrophoresis (e.g., [4,5]). Since 1965, the techniques and formulations used for electrophoretic fractionation have been improved markedly by empirical manipulations [6]. Current procedures fractionate at least 2 chl *a*-containing complexes from higher plant photosynthetic membranes, one associated with each photosystem, as well as 3 complexes containing chl *a* and *b*. Many investigators in this research area favor the notion that the multiple banding of the chl *a* + *b*-complexes on electrophoresis is due to the separation of different oligomeric forms of the fastest migrating complex; i.e., higher plants possess only 1 fundamental type of light-harvesting chl *a* + *b*-complex. Recent evidence showing that 1 of the 3 complexes does not behave as expected for an oligomer [7] and that 2 different chl *a* + *b*–proteins occur in the membranes [8,9] indicates that microheterogeneity may exist among biochemically similar complexes. If this is the case, slightly different functions or specificities of association may eventually be ascribed to the complexes.

Most studies have used chloroplast material which, prior to detergent extraction, has lost the chloroplast envelope, resulting in loss of stromal contents, and has received varying amounts of washing with buffer to remove extrinsic components from the surface of the thylakoids. One effect of this treatment is to allow a reorganization of the thylakoid components within the plane of the membrane from the functionally active in vivo state to a less ordered state [10]. With this in mind, we conducted a study of chl–protein complexes in intact chloroplasts; i.e., plastids which have not lost their envelope.

2. Materials and methods

Plant species used were pea (*Pisum sativum* var. Feltham First) and tobacco (*Nicotiana tabacum* var. Turkish Samsun). Peas were germinated and grown in vermiculite for 8–10 days with a 12 h light–dark cycle. Tobacco seed was germinated and grown for 4–6 weeks in a soil–vermiculite mixture in a greenhouse in Los Angeles.

The method used for preparation of intact pea chloroplasts was a modification of the procedure in [11] designed to increase the yield of intact chloroplasts. Harvested pea leaves (35 g) were macerated with a Polytron disintegrator (Brinkmann, Inc.) in 100 ml of a partially frozen slurry of 0.33 M sorbitol, 0.2 mM $MgCl_2$ and 20 mM 2(*N*-morpholino)ethanesulfonic acid brought to pH 6.5 with Tris base. The resulting brei was filtered through 10 layers of muslin with layers 2 and 3 separated by a thin layer of cotton wool. The filtrate was centrifuged in an MSE bench top centrifuge at 2200 × *g* for 30 s with the total time allowed for reaching the maximum speed, fractionation and hand-breaking being ~90 s. The supernatant fraction was decanted or aspirated. Within the pellet could be observed a light (upper) layer and a dense (lower) layer. The lighter portion of the pellet

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was resuspended by gentle swirling in ~25 ml 'cation-free' medium containing 0.33 M sorbitol adjusted to pH 7.5 with Tris base, and then decanted into a clean centrifuge tube. The firm pellet was resuspended in 'cation-free' medium with the aid of a small paint brush. These suspensions were centrifuged at 2200 × *g* for 20 s with the total time allowed for reaching maximum speed and hand-breaking being ~60 s. The tubes were tilted while aspirating off the supernatant fraction. In both cases the pellet separated again into a distinctly soft (upper) and a firm pellet. The soft pellets were either pooled and washed again in the 'cation-free' medium for maximum yield or discarded. The firm pellets were used as intact chloroplast preparations. Intactness was best preserved if cations (1 mM Mg^{2+} and K^{+}) were present in the resuspension buffer. In the case of older plants, greater intactness was obtained from the chloroplasts in the soft pellets. The chloroplasts used here were judged to be 92% intact by the ferricyanide test [12,13].

Broken, washed chloroplasts were prepared from intact chloroplasts by addition of 4 vol. distilled water or 5 mM EDTA, 25 mM tris(hydroxymethyl)methylglycine–NaOH (pH 8.0), a hypotonic medium. After 10 min, the chl-containing membranes were recovered by centrifugation at 5000 × *g* for 10 min. Chlorophyll concentrations were determined as in [14]. Intact and disrupted chloroplasts were extracted prior to electrophoresis with sodium dodecylsulfate (SDS) at 1% (w/v) final conc. and a detergent/chlorophyll ratio of 10:1 (w/w). Also added were Tris, glycine and glycerol to 6.2 mM, 48 mM and 10% (v/v) final conc., respectively. The solutions were homogenized with a glass or glass–teflon hand homogenizer and centrifuged at 40 000 × *g* for 5 min. The extraction of chl-containing components was essentially quantitative [15]. Samples of 2–25 μl were applied to polyacrylamide gels for electrophoretic fractionation in the system of [15]. Absorption spectra of the various chl–protein complexes were determined directly on excised gel slices with an Aminco-Chance DW-2 spectrophotometer.

Tobacco leaves (35 g) were deveined and macerated with a Polytron disintegrator in 100 ml ice-cold solution of 0.4 M sorbitol, 25 mM tris(hydroxymethyl)-methylglycin–NaOH (pH 8.0). The homogenate was filtered through 2 layers of Miracloth (Chicopee Mills, Milltown, NJ) and centrifuged at 2500 × *g* for 60 s. The pellet was resuspended in the same buffer. These chloroplasts were judged to be 70% intact by microscopic examination. In addition to the above extraction procedure, some tobacco chloroplast fractions were solubilized in a similar manner except that the extraction solution contained 0.9% (w/v) *N*-lauroylsarcosine and 0.1% (w/v) SDS rather than 1.0% SDS.

Ammonium persulfate, *N,N,N',N'*-tetramethylmethylenediamine, *N*-lauroylsarcosine, 2(*N*-morpholino)ethanesulfonic acid, Tris, and tris(hydroxymethyl)-methylgycine were purchased from Sigma (St Louis, MO). Acrylamide, *N,N'*-methylenebisacrylamide, SDS, sorbitol and glycine were purchased from BDH Chemicals, Poole.

3. Results

Electrophoretic fractionation of chl–protein complexes extracted directly from intact pea chloroplasts creates the pattern shown in fig.1A. The depicted bands represent the green of chl; 5 bands are visible. The existence of the band labelled 'L' has not been previously reported [7,15]. If the chloroplasts were

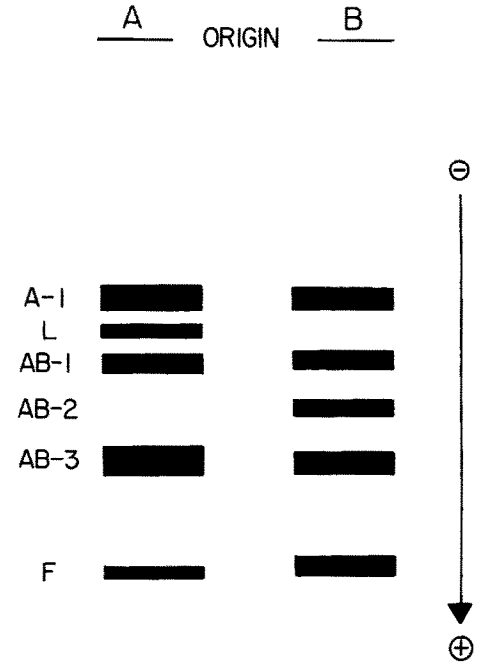

Fig.1. Diagrammatic depiction of chlorophyll–protein complexes after 50 min electrophoretic fractionation. Detergent extracts were prepared from intact chloroplasts (A) or chloroplasts that had been hypotonically disrupted prior to extraction (B).

hypotonically disrupted prior to extraction, the resulting pattern changed (fig.1B). Five green bands are still visible, but now the band labelled AB-2 is present and the L band is absent. The latter pattern is that normally observed for detergent extracts of broken chloroplasts or thylakoid fragments using this fractionation system [15]. The absorption spectrum of 2 chl *a*-containing bands (A-1,A2) observed in this system appeared in [7,15]; the A-2 band migrates coincidentally with the AB-3 band in fig.1 [7]. The spectra of the AB-1, AB-2 and AB-3 zones from broken chloroplasts are shown in fig.2. The absorption maxima at ~672 and 650 nm indicate that they all contain chl *a* and substantial quantities of chl *b*, respectively. The L zone has a similar absorption spectrum (fig.3) which also indicates that it contains both types of chl.

To demonstrate that the presence of the L band was not confined to pea chloroplasts, but was a more general phenomenon, the same experiments were performed with tobacco chloroplasts. The isolation method, which worked so well with pea chloroplasts, gave less than optimal results with the tobacco chloroplasts (i.e., a fraction containing mostly broken chloroplasts). Because of this problem, the less elegant isolation procedure described in section 2 was employed. Extraction of 70% intact tobacco chloroplasts with the standard extraction buffer containing SDS resulted in the appearance of a barely perceptible amount of

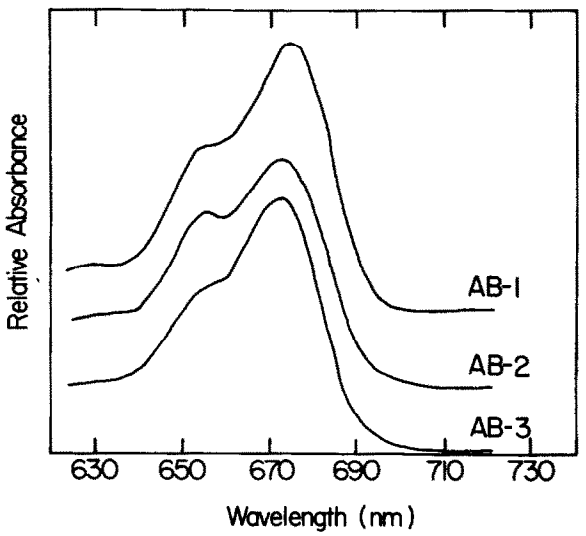

Fig.2. Room temperature absorption spectra of excised gel slices containing the indicated chlorophyll–protein complexes from hypotonically disrupted chloroplasts.

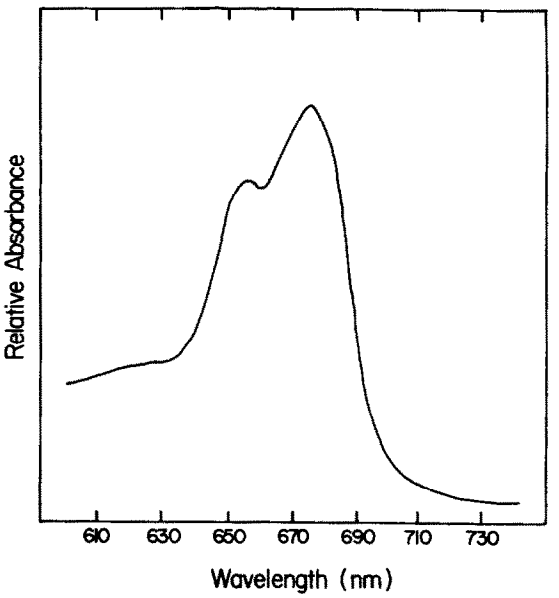

Fig.3. Room temperature absorption spectrum of an excised gel slice containing the L chlorophyll–protein complex from intact chloroplasts.

the L chl–protein complex. Results similar to those observed in pea chloroplasts, appearance of the L band and decrease of the AB-2 band, were observed when the intact, but not when the broken, tobacco chloroplast fraction was extracted with a mixture of SDS and *N*-lauroylsarcosine.

4. Discussion

Direct extraction of intact chloroplasts and subsequent electrophoretic fractionation have revealed the presence of a chl–protein complex, the L band, not observed when plastids were lysed prior to extraction. The concomitant appearance of the L band and disappearance of the AB-2 band, as well as the similarity of their absorption spectra, suggest that the L band is some oligomeric form containing the AB-2 complex. Extraction of thylakoid fragments with the same mixture of SDS and *N*-lauroylsarcosine as used here for the tobacco chloroplasts causes an increase in the amount of chl associated with the AB-1 complex and a decrease in the amount of chl migrating with the AB-3 complex (J. P. M., J. P. T., unpublished). These data and the evidence in [7] suggesting that the AB-2 complex is the product of a different gene

than either the AB-1 or AB-3 complexes are consistent with the notion advanced in [6] that the AB-3 and AB-1 are equivalent to the monomeric and oligomeric (perhaps dimeric) forms of 1 type of light-harvesting chl *a* + *b*-complex whereas the AB-2 and L bands represent a second type. It has yet to be determined if the AB-2 and L complexes contain completely different protein components than AB-1 and AB-3, or whether they may contain common protein constituents, either pigmented or colorless. The existence of an oligomeric form of the major light-harvesting chl *a* + *b*-complex was first reported by Hiller et al. [16] and is now routinely observed [17–20].

Recent improvements in electrophoretic fractionation techniques allowed fractionation of up to 10 chl–protein complexes in [8]. These authors also concluded that there is more than one basic type of light-harvesting chlorophyll *a* + *b*-complex. However, the minor light-harvesting complex identified in [8] (designated $Chl_{a/b}$–P1) apparently has a higher chl *a*/*b* ratio than that in the L and AB-2 complexes. A second group has recently fractionated a complex from spinach with a similarly high chl *a*/*b* ratio [9]. Thus, there may be 3 forms of light-harvesting complexes containing chl *a* and *b*.

It is natural to attempt comparison between the pigment–proteins isolated by this fractionation system and other electrophoretic fractionation systems in use [17–20]. However, we would caution readers not to assume that multiple chl *b*-containing pigment–protein complexes resolved on different electrophoretic systems are necessarily equivalent. We have recently found (J. Bennett, J. P. M., M. P. Skrdla, J. P. T. in preparation) that the most rapidly migrating chl *b*-containing complex on other fractionation systems (generally designated CPII) is not present when detergent-treated membranes are fractionated on this system; the most rapidly migrating chl *b*-containing complex in the latter system (AB-3) seems to be significantly larger than CPII. We now believe that CPII is an electrophoretically-altered form of the major pigmented constituent of the light-harvesting antenna. The AB-3 band [7,15] probably represents a less altered form. Thus we are as yet reluctant to claim equivalence to, or discuss any possible relationship between, superficially similar complexes fractionated with different electrophoretic systems.

The difference in the fractionation pattern between intact and broken chloroplasts may reveal something concerning the organization of the thylakoid membrane in vivo. The membranes are normally organized into microscopically recognizable regions of appressed multiple layers termed granal lamellae and unappressed membranes termed stromal lamellae. The granal lamellae are thought to be enriched in chl *b* and photosystem II components and depleted in photosystem I components with respect to stromal lamellae or whole thylakoids [21]. The AB-2-containing oligomer, L, occurs only in extracts from intact chloroplasts with thylakoids organized into granal and stromal lamellae. Incubation of the membranes in low ionic strength medium or EDTA, which causes an unstacking of the grana [22,23] and reorganization of components in the plane of the membrane to a less ordered state [10], resulted in the disappearance of the L complex and reappearance of the AB-2 complex. This is consistent with a conversion of the AB-2 complex to an AB-2-containing oligomer during the organization of the membrane components necessary to allow formation of grana. Thus the L complex may be present only after organization of the membrane components has resulted in locally high concentrations of the AB-2 complex. An alternate possibility is that the AB-2 containing oligomer is recovered from intact chloroplasts because of a protective role played by the stromal contents. Further work will be required to establish the dominant reason for the fractionation of the AB-2-containing oligomer complex from intact chloroplasts.

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