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The Polypeptide Patterns of Membrane Fractions from Normal and Primary Thylakoids of Bean Leaves*

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Abstract

The polypeptide pattern of primary thylakoids from bean plastids grown under a flash regime, and of their heavy and light fractions was compared with that of normal green thylakoids. The primary thylakoids are deficient in some polypeptides (especially the 23 kdalton polypeptide 9) which are characteristic of the heavy fraction of normal green thylakoids. A heavy and a light fractions were separated in most preparations from the primary thylakoids of flashed bean leaves, although these leaves did not emit oxygen when illuminated with continuous intense light for the first time.

Important differences have been noted between granal chloroplasts of green leaves and chloroplasts of leaves grown in intermittent light (= "flashed leaves"). The latter have primary thylakoids and no grana (SIRONVAL *et al.* 1968); their chlorophyll *b* content is low (AKOYUNOGLOU *et al.* 1966); they are unable to emit oxygen when illuminated for the first time with continuous, intense light (STRASSER and SIRONVAL 1972) and other photosystem II properties are lacking (DUJARDIN *et al.* 1970; MICHEL and SIRONVAL 1972). A short period of continuous illumination induces the ability to evolve oxygen in the flashed leaves; "induced-flashed leaves" are obtained in this way (MICHEL and SIRONVAL 1972; STRASSER and SIRONVAL 1972). There is evidence that the deficiency in the flashed, non-induced leaves is localized on the water splitting side of photosystem II (REMY 1973a; STRASSER and SIRONVAL 1973). It seems, however, that the functional differences between non-induced-flashed and normal leaves (review in STRASSER 1973a and in SIRONVAL 1975) are not necessarily related to qualitative features of the lamellar proteins of the chloroplast. Electrophoretic patterns of SDS-solubilized entire lamellae from etioplasts, non-induced-flashed chloroplasts and granal chloroplasts show rather quantitative than qualitative differences (REMY 1973b).

It has been suggested that a successful fractionation of the thylakoid membrane into heavy and light particles exhibiting properties of one or another of the two photosystems by the detergent digitonin requires that chloroplasts possess stacked membranes (ANDERSON and LEVINE 1974). In the grana-less primary thylakoids of flashed bean leaves, the absence of pigment-protein complex II, a chlorophyll-protein complex apparently related to photosystem II (SIRONVAL *et al.* 1967; THORNBER *et al.* 1967) has been reported (HILLER *et al.* 1973; ARGYROUDI-AKOYUNOGLOU 1974).

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In order to investigate if heavy and light particulate fractions can be separated from primary thylakoids, as they are from normal thylakoids of granal chloroplasts, we compared the behaviour of normal and primary thylakoids subjected to *Triton X-100* treatment and subsequent gradient centrifugation. *Triton X-100*, a non-ionic detergent, has been used by VERNON, BRIANTAIS and others (review in VERNON and SHAW 1971) to prepare subchloroplast fractions enriched in photosystem I or II. The fractions we have obtained by this procedure from normal and primary thylakoids have been characterized by their absorption spectra and their polypeptide constituents as resolved by SDS-gel-electrophoresis.

MATERIAL AND METHODS

Plants: *Phaseolus vulgaris* L. cv. Commodore was cultivated as described by other authors (DUJARDIN *et al.* 1970; PHUNG NHU HUNG *et al.* 1970; STRASSER and SIRONVAL 1972). The plants were grown 7 d in darkness, then they were cultivated under an intermittent light regime (1 ms flashes alternating with 15 min dark periods = flashed plants) or 7 d under continuous light (green plants).

Chloroplast isolation: 15 to 20 g of primary leaves from green or flashed plants were homogenized by a tissue chopper in 50 ml of a medium containing 140 mM sucrose, 2 mM KCl, 5 mM KH_2PO_4, 1 mM EDTA . Na_2, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$. 4 H_2O, 1.4% PVP, and 10% glycerol, pH 7.4. To this medium we added an excess of $CaCO_3$. The homogenate was filtered through two layers of large pore nylon cloth and one layer of fine pore nylon cloth. Then it was centrifuged for 2 min at 400 × *g*. The sediment with the cell debris and the nuclei was discarded and the supernatant was centrifuged for 2 min at 4000 × *g*. The chloroplast pellet was resuspended in the same medium, glycerol was added to a concentration of 30% and the chloroplasts were stored in liquid nitrogen at −196 °C. All operations were carried out at +4 °C.

Preparation of thylakoids: Chloroplast suspensions with chlorophyll (*a* + *b*) content from 0.5 to 1.0 mg ml^{-1} were sedimented by centrifugation (2 min at 4000 × *g*). The pellets were resuspended in 10 volumes of ice cold distilled water and homogenized with a *Potter* homogeniser. The disrupted chloroplasts were sedimented by centrifugation (10 min at 30000 × *g*) and the pellet was twice washed with 50 mM Tris buffer pH 7.5 including 10 mM $MgCl_2$ (recentrifugation between the washings each time 10 min at 30000 × *g*). The final pellet was suspended in 100 mM Tris. HCl pH 7.5, including 10 mM $MgCl_2$. All operations were done at +4 °C.

Preparation of particles from the thylakoid membrane: Thylakoid suspensions were treated with *Triton X-100* (*Triton*/chlorophyll = 2.5 w/w) for 30 min at 4 °C in the dark. The suspension was then layered on a linear sucrose gradient (20% to 50% sucrose in 100 mM Tris . HCl pH 7.5) and centrifuged for 60 min at 60000 × *g* at 4 °C in a *Spinco SW 25* rotor. Absorption spectra were recorded after the run with the fractions directly out of the gradient at 20 °C with a *Cary 17* spectrophotometer. The fractions were stored in liquid nitrogen until electrophoresis was performed.

Solubilisation of thylakoids and thylakoid fractions prior to electrophoresis: Sodium-dodecyl-sulphate (SDS) was used to resolve the membranes into their polypeptide constituents. Before electrophoresis generally a SDS/protein ratio of 10 was applied to the samples (in 100 mM Tris, 10 mM $MgCl_2$ pH 7.5) for 30 min at 4 °C in the dark.

Gel-electrophoresis: Gel system I according to MAURER (1971) was used, with two modifications: (*1*) the running gel was 9% acrylamide; (*2*) spacer and running gel as well as the electrophoresis buffer contained 0.1% SDS. Samples of about 100 µg of protein were run in glass tubes of 12.5 cm

length and 0.6 cm diameter, with a current of 6 mA per tube for about 3.5 h. Electrophoresis was performed in the cold (+4 °C) and in the dark. The gels were stained according to CHRAMBACH *et al.* (1967), destained in 7% acetic acid and scanned at 600 nm.

Chlorophyll contents were measured according to ŠESTÁK (1971).

Protein determination followed the method of LOWRY *et al.* (1951).

RESULTS

Action of *Triton X-100* on normal and primary thylakoids; centrifugation patterns

Normal as well as primary thylakoids were treated with the same amount of *Triton X-100* on a chlorophyll basis. The ratio of *Triton X-100* to protein was different in the preparations of the two types of lamellae; the chlorophyll/protein ratio was about four times lower in primary thylakoids than normal thylakoids.

Two green bands were separated after sucrose gradient centrifugation of the *Triton X-100* treated lamellae in normal as well as in primary thylakoids. The lower band was in 43% sucrose (= heavy fraction); the upper one was just entering the gradient at 20% sucrose (= light fraction). The supernatant of the centrifugation was green. At the bottom of the tube there was a sediment of unfractionated lamellae.

Thylakoids from green leaves were always separated into two green bands. Primary thylakoids formed the lower band in 43% sucrose in about two thirds of the 15 experiments, whereas the light fraction in 20% sucrose was always present.

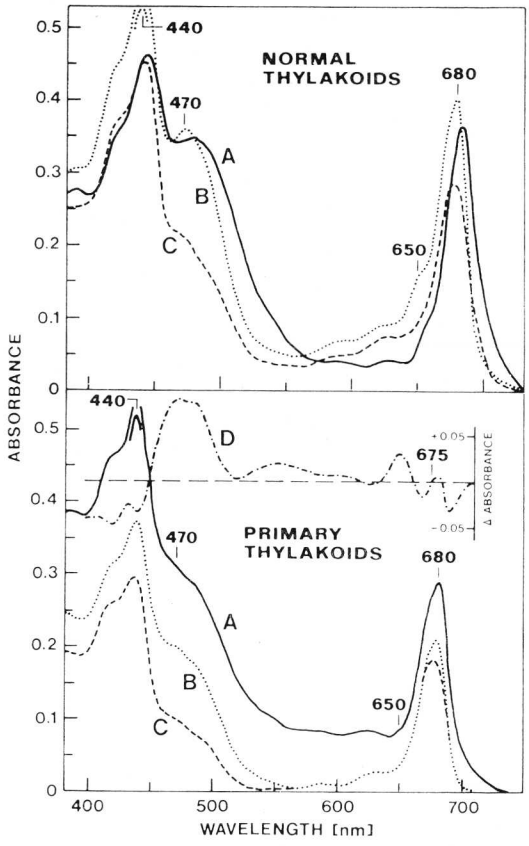

Fig. 1. Absorption spectra of membrane fractions separated by gradient centrifugation of *Triton X-100* treated, normal (*top*) and primary (*bottom*) thylakoids. *A*: Untreated thylakoids. *B*: Heavy fraction. *C*: Light fraction. *D*: Difference spectrum (heavy minus light fraction) of primary thylakoids.

Absorption spectra

Untreated normal thylakoids had absorption maximum at 20 °C in the red spectral region at 680—682 nm (Fig. 1, *top*, curve *A*). The absorptions due to chlorophyll *b* were around 650 nm and at 470 nm. The chlorophyll *b* bands were found in the heavy fraction after *Triton X-100* treatment and gradient centrifugation (curve *B*). The maximum in the red of the heavy fraction was at 678—680 nm; in the case of the light fraction (curve *C*) it was slightly shifted to shorter wavelengths (675—676 nm).

Untreated primary thylakoids had absorption maximum at 20 °C in the red at 680—682 nm (Fig. 1, *bottom*). If present, a small amount of chlorophyll *b* only was there, as seen by the absence of distinct bands at 650 and 470 nm. The absorption maximum in the red of the heavy fraction after *Triton X-100* and gradient centrifugation was at 678—680 nm; there was a shoulder at 671 to 673 nm. The light fraction (curve *C*) had its maximum in the red at about 675 nm. The difference spectrum (absorption of the heavy fraction minus absorption of the light fraction — curve *D*) after adjustment to equal absorption at 675 nm shows an excess absorption around 650 nm and between 450—500 nm which belongs to the heavy fraction.

Polypeptide patterns

Normal thylakoids (Fig. 2, *left* and *middle*): Nine components were separated (numbered from *1* — nearest to the anode — to *9* — nearest to the cathode) in normal thylakoids not treated

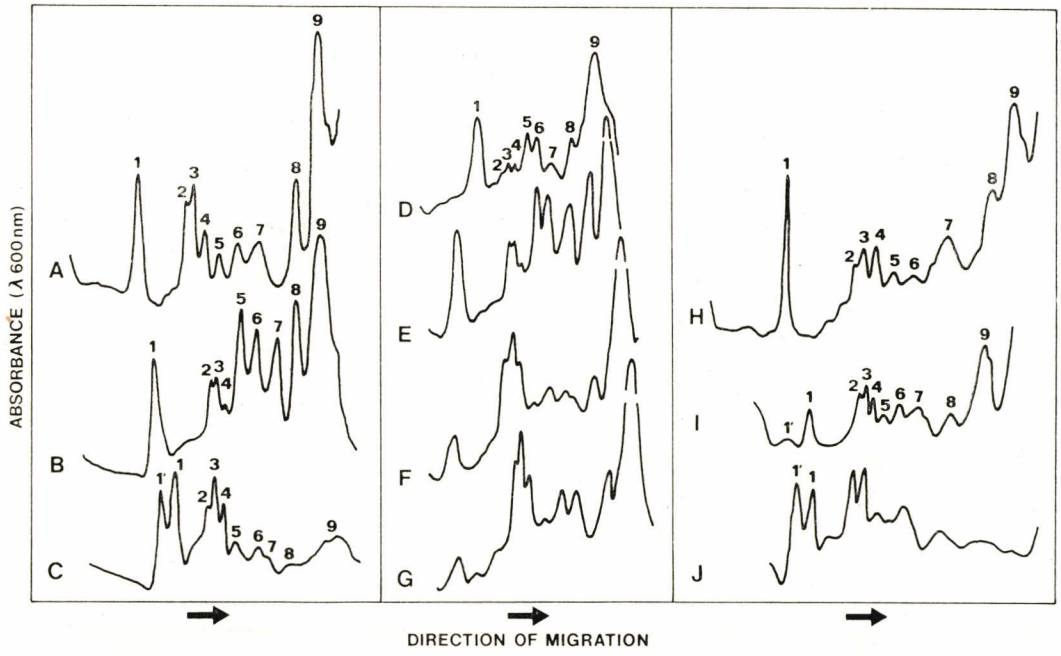

Fig. 2. Densitometer scans of polyacrylamide gel electrophoregrams. *A*: Untreated, normal thylakoids. *B*: Heavy fraction from normal thylakoids. *C*: Light fraction from normal thylakoids. *D*, *E*, *F*, *G*: Comparison of heavy fractions from normal thylakoids obtained in four different experiments. *H*: Untreated primary thylakoids. *I*: Heavy fraction from primary thylakoids. *J*: Light fraction from primary thylakoids. The fractions were obtained by the action of *Triton X-100* and subsequent gradient centrifugation. The whole thylakoids as well as the membrane fractions were dissolved in SDS prior to electrophoresis.

with *Triton* after SDS-gel-electrophoresis and staining (curve *A*). The heavy fraction obtained by *Triton X-100* treatment and gradient centrifugation (curve *B*) had relatively more of the components *5* to *9* than the corresponding light fraction (curve *C*), which had relatively more of components *1'* and *1* to *4*. There was a marked dominance of component *9* in the heavy fraction, in contrast to the light fraction. To check the presence of individual components, heavy fractions from distinct experiments with normal thylakoids were compared (Fig. 2, curves *D* to *G*). All the heavy fraction patterns fell into two groups: either with relatively much of component *1* and a low content of components *2*, *3* and *4*; or with a low content in component *1* and more of *2*, *3* and *4*. In all cases the heavy fractions were enriched in component *9*.

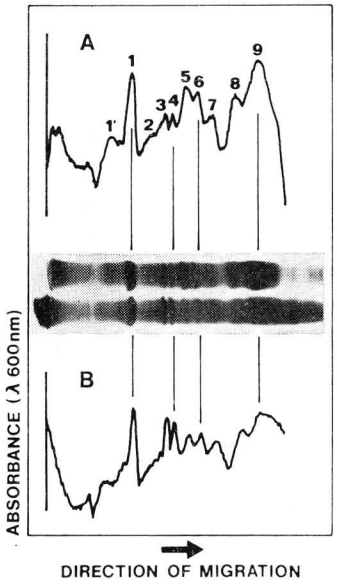

Fig. 3. Comparison of densitometer scans of photographs of parallel electrophoreses. *A*: Heavy fraction from normal thylakoids. *B*: Heavy fraction from primary thylakoids. Same preparation procedure as in Fig. 2.

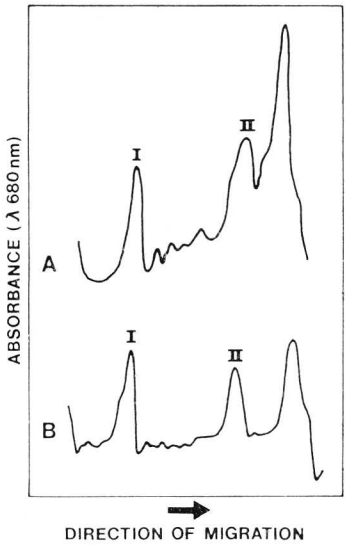

Fig. 4. Densitometer scans of green bands (680 nm) of polyacrylamide gel electrophoregrams. *A*: Normal thylakoids. *B*: Primary thylakoids. The thylakoid preparations were dissolved in SDS prior to electrophoresis.

Primary thylakoids (Fig. 2, *right*): All components *1* to *9* were present in these thylakoids as in the normal green ones (curve *H*). The distribution of the components between heavy (curve *I*) and light fractions (curve *J*) was the same as in normal thylakoids: component *9* was more abundant in the heavy fraction; components *1'* and *1* to *4* were more abundant in the light fraction.

However, there were quantitative differences between the patterns of entire green and primary thylakoids and between the patterns of the fractions from green and primary thylakoids. Generally, components *5* to *9*, — and especially *9* —, were less abundant in primary thylakoids than in green ones. Fig. 3 reproduces typical densitograms of a heavy fraction from green thylakoids (*A*) and of a heavy fraction from primary thylakoids (*B*).

Patterns of thylakoids from "induced-flashed" leaves: Flashed leaves were illuminated 6 min with actinic light before extraction. Thylakoid preparations of these induced-flashed leaves were Hill-active. Treatment with *Triton X-100* followed by gradient centrifugation resulted in a separation into heavy (in 43% sucrose) and light (in 20% sucrose) fractions as for green and primary

thylakoids. The electrophoresis patterns of the thylakoid fractions prepared from induced-flashed leaves were similar to those obtained for corresponding fractions from non-induced primary thylakoids.

Pigment-protein complexes

Scannings at 680 nm after SDS-gel-electrophoresis of non-*Triton* treated, entire green and primary thylakoids (Fig. 4) shows pigment-protein complexes I and II in both types of thylakoids. The peak nearest to the cathode was due to solubilized chlorophyll.

Photosystem II activity

Tests of Hill activity and DPC photooxidation with DCPIP as electron acceptor were done on the thylakoid preparations used in this work (see STRASSER 1973b). Isolated thylakoids from normal-green and induced-flashed leaves were Hill-active (oxygen emission coupled to DCPIP reduction in the light). Thylakoids from non-induced-flashed leaves (primary thylakoids) did not emit oxygen in the light (Hill-inactive). All three types of thylakoids (from non-induced-flashed leaves, from induced-flashed leaves and from normal green leaves) showed photooxidation of DPC coupled to DCPIP photoreduction.

DISCUSSION

The digestion of chloroplast lamellae by *Triton X-100* has been widely used for the preparation of subchloroplast fragments. We applied relatively low *Triton X-100* concentrations, similar to BRIANTAIS (1969). When applied to normal thylakoids this treatment gave two fractions: a heavy fraction, enriched in chlorophyll *b*, with an absorption maximum at 678–680 nm, which was slightly further in the red than the maximum of the light fraction. A similar spectral behaviour of heavy and light particles, prepared by the action of *Triton X-100* on normal thylakoids has been reported by BRIANTAIS (1967). The electrophoresis separation of the membrane polypeptides in the presence of SDS from normal thylakoids showed nine components. A comparison of the pattern of the whole lamellae with the patterns of the two fractions showed that polypeptides *5* to *9* are more characteristic for the heavy fraction, whereas the components *2* to *4* are more dominant in the light fraction.

Recently, a number of papers have appeared (REMY 1971; ANDERSON and LEVINE 1974; KLEIN and VERNON 1974) with a description of the distribution of chloroplast lamellar proteins between heavy and light fractions. Although different procedures were used for the electrophoresis, which may not allow a direct comparison of the patterns, generally about ten polypeptides have been separated by SDS-gel-electrophoresis. The components of the light fraction are mainly found in the higher molecular weight range (50–70 kdaltons); the major components of the heavy fraction have a lower molecular weight from 50 to 20 kdaltons (KLEIN and VERNON 1974). Our results fit into this general picture. In the heavy fraction from green thylakoids component *9* (23 kdaltons) always dominated. However, we have found variations in the relative amounts of components *1* to *4*: in cases with relatively much of *1* present,

252 *2*, *3* and *4* were less abundant, and *vice versa* (Fig. 2). It has been shown by REMY (1971) that component *1* is an aggregate: it can be separated into *2* and *3* by mercaptoethanol. We did not use mercaptoethanol in our buffers, but the characteristic variations of the patterns of the heavy fractions could be understood if component *1* and the components *2* to *4* are indeed interrelated.

The fractions produced by the action of *Triton X-100* on primary thylakoids migrate to the same density in the sucrose gradient as the fractions from green thylakoids. The heavier fraction however did not appear in all of the experiments when starting from primary thylakoids. In this respect, the fractionation of the primary thylakoids of flashed chloroplasts seems to be less reproducible than that of the normal thylakoids of granal chloroplasts.

REMY (1973b) showed that most of the chloroplast lamellar proteins are already pre-existent in the etioplast. Our results confirm that there is no qualitative difference between the polypeptide patterns of normal and primary thylakoids, which both contain pigment-protein complex I as well as II (Fig. 4), but that the thylakoids differ in their quantitative patterns. This agrees with the data of ANDERSON and LEVINE (1974) on proteins extracted from chloroplasts with unstacked membranes of barley and pea mutants, as well as from the grana-less bundle sheath cell chloroplasts of maize. These chloroplasts are deficient in at least some of the polypeptides essentially found in the heavy fraction.

Primary thylakoids differ morphologically (no grana) as well as functionally (no oxygen evolution) from normal green thylakoids. Some characteristic polypeptides of the heavy fraction (*5* to *9*) are less abundant in primary thylakoids than in normal thylakoids. Both types of lamellae, however, may be split into light and heavy fractions after *Triton X-100* treatment, and the two fractions have the same densities in both primary and normal thylakoids. It is possible to isolate heavy particles from non-induced-flashed, or induced-flashed, as well as from normal green thylakoids. On the other hand, all of these thylakoids seem to have the active centre of photosystem II since they all show electron transport from DCP to DCPIP in the light. The complete Hill reaction, however, (electron transport from H_2O to DCPIP in the light) can only be observed in normal green thylakoids, or in induced-flashed thylakoids. This permits us to conclude that the presence of heavy particles may be correlated to the presence of active centres of photosystem II in the membranes, but that it cannot be considered as a criterium for an intact, complete photosystem able to produce oxygen.

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