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THE FRACTIONATION OF PLANT PHOTOACTIVE PIGMENT-PROTEIN COMPLEXES I AND II

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Summary

The pigment-protein complexes enriched with Photosystem I (PPC-I) and Photosystem II (PPC-II) were obtained using sievorptive chromatography on DEAE-Sephadex column. Both types of complexes contain Chlorophyll *a*, β-carotene and minor quantities of Chl *b*. Red absorbance maxima are located at 676 nm and 673 nm for PPC-I and PPC-II, respectively. The degrees of reaction centre enrichment were measured by the method of differential spectrophotometry: PPC-I has one *P*-700 per 35 bulk Chl *a* molecules, PPC-II contains one *P*-680 per 18 bulk Chl *a* molecules. The yield of PPC-II is 7—10 times lower than that of PPC-I. After one chromatographic procedure the amount of *P*-680 in PPC-I preparation does not exceed 7% of that of *P*-700, the amount of *P*-700 in PPC-II preparation 2% of that of *P*-680. The product of PPC-II degradation was studied.

Introduction

Recent progress in the understanding of the primary photochemical reactions of bacterial photosynthesis has been mostly associated with isolation of purified photoactive reaction centres [1]. This was not the case with green plants due to the difficulty in removing all the antenna pigments. Several attempts have been made to separate two green plant photosystems and to

Abbreviations: PPC-I and PPC-II, pigment-protein complexes from Photosystem I and II, respectively; Chl, chlorophyll; *P*-700, reaction centre of Photosystem I; *P*-680, reaction centre of Photosystem II; LHC, light harvesting Chl *b*-containing complex; *V*, column bed volume; ΔA, absorbance change; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

prepare photoactive pigment-protein complexes from Photosystem I (PPC-I) [2—4] as well as from Photosystem II (PPC-II) [5]. These authors employed the mild action of nonionic detergents with the subsequent ultracentrifugation and the ion-exchange chromatography. It has been shown by means of differential spectroscopy that both complexes contain 30—50 Chlorophyll molecules per reaction centre [2—4,6]. The application of more severe treatments (extraction with diethyl ether [7], incubation with the mixture of ionic and nonionic detergents [8]) resulted in a decrease of this ratio to 10—20 for PPC-I. However, in contrast to PPC-I, the preparation of highly purified photoactive PPC-II is still a great problem since the method of Wessels et al. [5] requires a lot of time (one week), the use of expensive equipment and eventually yields rather a small quantity of the preparation.

In this report the method of sievorptive chromatography on DEAE-Sephadex for fast separation of green plant photosystems and simultaneous isolation of PPC-I and PPC-II is described. These complexes were characterized by the spectroscopic method. The light-induced spectra and kinetics of absorbance changes were studied in the red optical region and the enrichment of both complexes with reaction centres was estimated.

Materials and Methods

Pea chloroplasts were isolated as described elsewhere [9]. The removing of the light harvesting Chl *b*-containing complex (LHC) was carried out by the procedure, suggested by Shutilova and Kutjurin [10]. The chloroplasts were osmotically shocked in a solution of 1 mM $MgSO_4$ and 5 mM Tris-HCl buffer (pH 8) and were sedimented at $5000 \times g$ for 20 min. After incubation in a medium containing 3.3% Triton X-100, 0.4 M sucrose, 5 mM Tris-HCl buffer (pH 8) (Triton X-100/Chl 50 : 1) during 1 h at 0°C solubilized thylakoid membranes were passed over DEAE-cellulose column, equilibrated with 0.05% Triton X-100 and 5 mM Tris-HCl buffer (pH 8). Solubilized pigments were removed by washing with equilibrating buffer. The mixture of complexes was eluted by a solution containing 0.3 M NaCl and equilibrating buffer. Then after incubation in the dark at 2°C for 5 h the suspension was centrifuged at $20\,000 \times g$ for 15 min. The sediment contained primarily LHC. The mixture of PPC-I and PPC-II in the $20\,000 \times g$ supernatant was once again passed over DEAE-cellulose column and washed with 5 mM Tris-HCl buffer (pH 8) +0.5% Triton X-100 for 1.5—2 h according to Ref. [11]. Then it was eluted from the column and fractionated using the method of sievorptive column chromatography on anion-exchange Sephadex. The latter method was proposed by Kirkegaard et al. [12] and is based on a combination of adsorption chromatography and gel filtration. The fractionation conditions are usually chosen in such a way that the proteins to be purified interact weakly with the ion-exchange gel and are eluted from the column within the sieving range (0.4—0.9 *V*) by means of inherent gradient. The latter is produced by a salt added to the sample applied as a result of molecular diffusion and specific properties of the gel.

The columns (10—20 cm in length, 3 cm in diameter for initial separation, 1—2.5 cm in diameter for subsequent purification) with DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) were used, thoroughly equilibrated with 10 vols.

of 0.1 M Tris-HCl buffer (pH 8), 30 volumes of 0.02 M Tris-HCl buffer (pH 8) and finally 2 vols. of 0.02 M NaCl + 0.05% Triton X-100. The initial material was preconcentrated in the ultrafiltration cell up to 15—150 μg/Chl ml. Then 1—5 ml of it in 0.3—0.5 M NaCl + 0.05% Triton X-100 was passed over the column. Elution was accomplished by 0.1 M NaCl + 0.05% Triton X-100 at a flow rate of 0.07—0.15 ml/min per cm^2. The volume of the fractions collected was 1—5 ml depending on column sizes. All the operations were carried out at 2—4°C.

Absorbance spectra and light-induced ΔA were measured at room temperature with a Model 10 double-beam spectrophotometer [13]. The sensitivity of the difference scheme was up to $5 \cdot 10^{-5}$ absorbance units. The exciting light from a 400-W tungsten lamp was filtered with an optical filter having the transmittance maximum at 480 nm with a halfband about 50 nm. The maximal intensity of the exciting light was approx. $9 \cdot 10^4$ erg/cm^2 per s. The cut-off ($\lambda < 620$ nm) filter was placed between the sample and the phototube to protect the latter from scattered exciting light.

The chlorophyll concentration was determined by the method of Arnon [14]. The Chl *a*/*P*-700 ratio was calculated by dividing the corresponding sample absorbance in the red maximum by ΔA_{696} from the difference spectrum of *P*-700 photooxidation, both measured in the same 10 mm cuvette. The calculations were made presuming that antenna Chl *a* and *P*-700 extinction coefficients in spectrum maxima were identical [15]. In this case determination of the Chl content by extraction with 80% acetone is unnecessary, since the red band halfwidth of the complexes obtained is relatively small (about 14 nm) and has a difference which is 10% of that of acetone extracts.

Results

Absorbance and photochemical properties of the fractions after initial and repeated separations on Sephadex A-25 are summarized in Fig. 1. The absorbance spectra of the individual fractions were measured from 400 to 750 nm. They differ from the LHC spectrum by a considerable decrease in absorbance at 460—500 and 650 nm due to the lesser carotenoid and Chl *b* content (compare spectra b, c with a in Fig. 2). The shoulder near 490—500 nm is probably of β-carotene [16]. The above spectra revealed variations in the position and amplitude of the red peaks. According to Fig. 1A, the mixture of PPC-I and PPC-II was fractionated within 0.4—1.0 *V*, which is essentially the sieving range of the gel. The distribution of red peak absorbance, similarly to Chl *a* content, has a pronounced optimum around 0.65 *V*. The fractions in this range have the longest absorbance maximum at 676 nm.

The capacity for reversible photobleaching, which is characteristic of *P*-700 and *P*-680, was used as a test for photochemical activity of the fractions collected. Formal summation of the difference spectra of *P*-700 [15] and *P*-680 [6] yields a spectrum with two bleaching maxima at 680 and 700 nm. The value of $\Delta A_{700}/\Delta A_{680}$ allows estimation to be made about relative contribution of each reaction centre to the total ΔA. It is true that some uncertainty is involved due to the satellite band 680 nm belonging to *P*-700. It should be noted that the amplitude of this band varies greatly in different

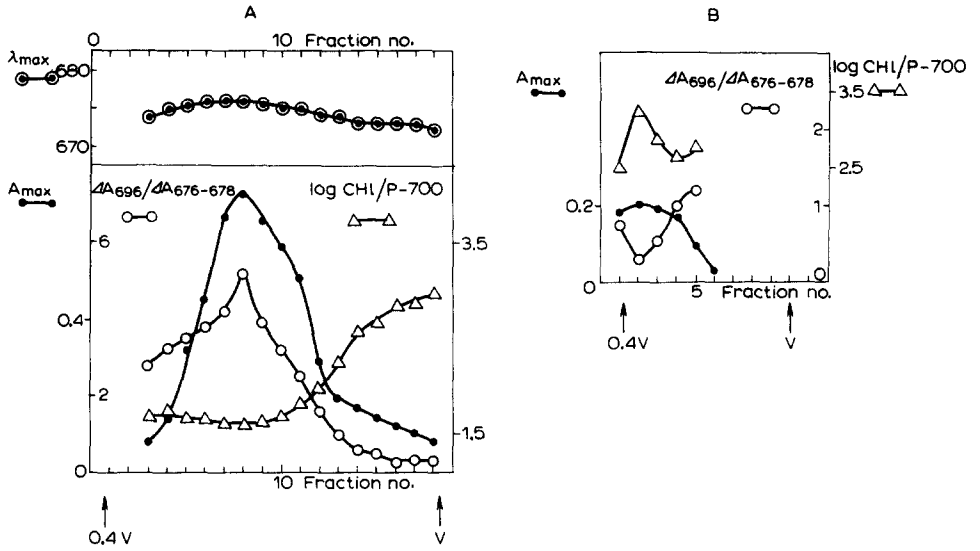

Fig. 1. Absorbance and photochemical properties of the fractions obtained by separation of PPC-I and PPC-II mixture on DEAE-Sephadex A-25; ⊙———⊙, the wavelength of red absorbance maximum; ●———●, absorbance in the red maximum; ○———○, the ratio of amplitudes ΔA_{696} and $\Delta A_{676\text{-}678}$ from light-induced difference spectrum; △———△, the concentration ratio Chl *a*/*P*-700. Intensity of exciting light half maximal. A. The first separation: material containing 0.35 mg of Chlorophyll in 3 ml of 0.5 M NaCl was applied to the 3 × 21 cm column; volume of the fractions 5 ml; flow rate 0.07 ml/min per cm^2. B. Repeated separation of the zone 0.85—1.0 *V*: material containing 0.016 mg of chlorophyll in 1 ml of 0.3 M NaCl was applied to the 1.7 × 9 cm column; volume of the fractions 1.5 ml; flow rate as in A.

preparations [2—4,15], depending on the isolation procedure and measuring conditions. In this respect the kinetic analysis of ΔA_{680} renders assistance in identification of its portions belonging to *P*-700$^+$ and *P*-680$^+$ because the lifetimes of the latter differ considerably [17].

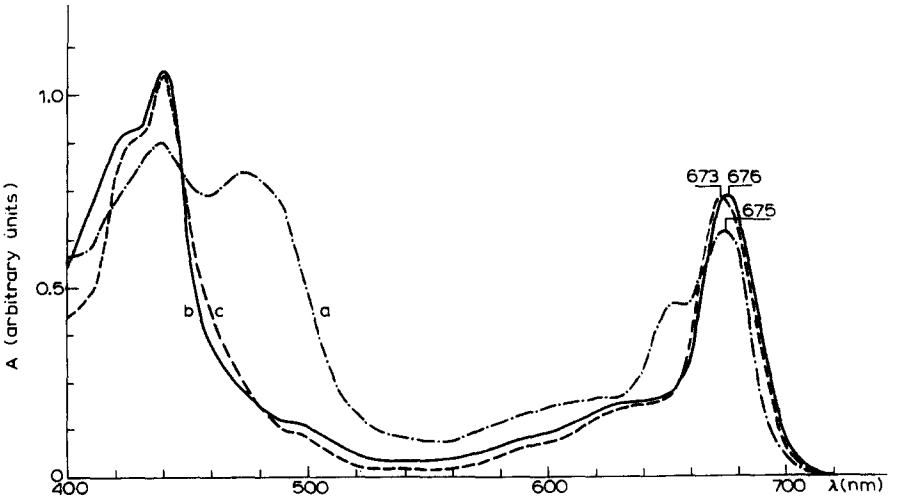

Fig 2. Absorbance spectra of LHC (a), as well as of fractions No. 8 (b) and No. 16 (c) (see Fig. 1A).

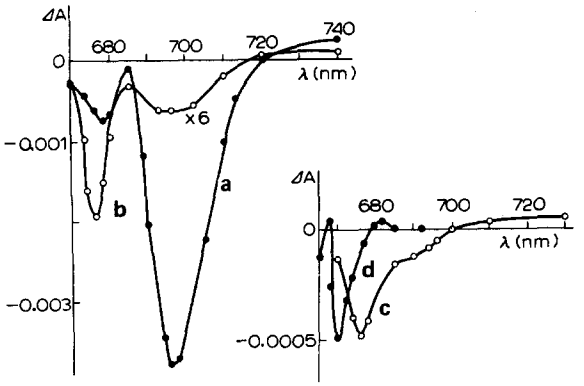

Fig. 3. Light-induced difference spectra of fractions No. 8 (a), No 16 (b,c) (see Fig. 1A), No. 2 (d) (see Fig. 1B). Additions: a and b, 5 μM TMPD, 50 mM Tris-HCl buffer (pH 8); c, 1 mM ferricyanide, 50 mM succinate buffer (pH 4); d, 50 mM Tris-HCl buffer (pH 8). Intensity of exciting light halfmaximal. Sample absorbance in the red maximum 0.15.

The ΔA spectra were measured under light saturating conditions for *P*-700 and in the presence of 5—10 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), cyclic electron transport cofactor (see, for example, spectra a and b in Fig. 3). The addition of TMPD induced some changes in the ΔA kinetics: (1) under saturating light the concentration of photooxidative *P*-700 increased 1.5- to 2-fold, up to its maximal level, (2) the halftime of *P*-700 dark reduction accelerated from several tens to 1—6 seconds. The former circumstance enabled us to estimate the portion of *P*-700 in every fraction and thereby to reveal the degree of *P*-700 enrichment.

According to Fig. 1A, the zone of *P*-700 higher concentration has a maximum at 0.65 *V* (Chl *a*/*P*-700 39), similarly to the Chl *a* content. Besides, at the same *V*, $\Delta A_{696}/\Delta A_{678}$ reaches its maximal value of approx. 5.3. The absorbance changes in both photobleached bands have the same rate of dark relaxation and are saturated with light in a similar way (Fig. 4A).

The concentration of *P*-700 diminishes 25-fold between 0.85 and 1.0 *V*. In this zone: (1) the red absorbance maximum is shifted to shorter wavelengths from 676 to 673 nm; (2) the ratio $\Delta A_{696}/\Delta A_{676\text{-}678}$ is decreased from 5.3 to 0.3; (3) light curves for ΔA_{696} and ΔA_{676} are different (see Fig. 4B): in contrast to the curve for ΔA_{696}, the one for ΔA_{676} is very steep and not saturated under experimental light conditions (The latter circumstance results in the ratio $\Delta A_{696}/\Delta A_{676}$ being essentially higher than that for saturating light.); (4) in the dark ΔA_{676} decays about 10 times faster than ΔA_{696}; (5) contrary to ΔA_{696}, the amplitude of ΔA_{676} hardly decreases when the pH value lowers from 8 to 4 and the redox potential rises on addition of potassium ferricyanide (Fig. 3c). It may be possible that the spectral and kinetic heterogeneity of ΔA in this zone is associated with the appearance of predominant spectral changes of *P*-680, having the maximum at 676 nm. This point of view is supported by the original results of Pulls et al. [18] and Haveman and Mathis [19].

The analysis of repeated separations on Sephadex A-25 revealed some interesting features. Purification of *P*-700-containing fractions resulted in enrichment with reaction centres so that the ratio Chl *a*/*P*-700 became 35 and

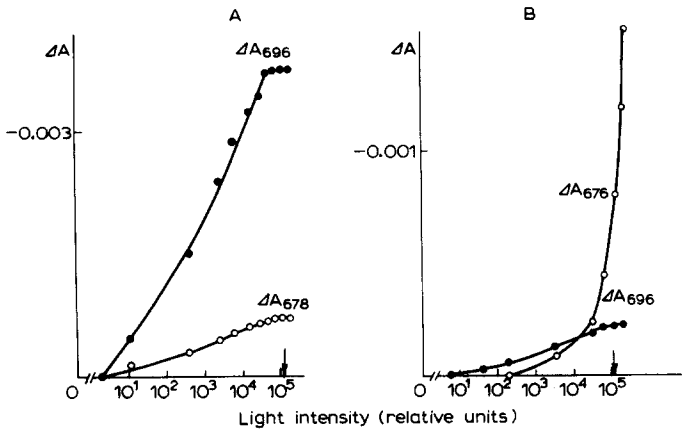

Fig. 4. Light intensity dependence of absorbance changes corresponds to fractions No. 8 (A) and No. 16 (B) (see Fig. 1A) in the presence 5 μM TMPD, 50 mM Tris-HCl buffer (pH 8). Sample absorbance in the red maximum 0.15. Light intensity used in Figs. 1 and 3 is shown by arrows.

$\Delta A_{696}/\Delta A_{678}$ 6. If the material from the 0.85—1.0 *V* zone was subjected to a similar procedure, it completely lost coloured fractions in this range (see Fig. 1B). Instead, some coloured protein appeared in the 0.4—0.6 *V* zone. This protein exhibited the activity inherent in *P*-680 during 2—3 days then the characteristic ΔA diminished. Sometimes the position of the ΔA maximum shifted to 670 nm (Fig. 3d) along with a similar shift of the red absorbance peak. In the case of the initial sample, which was not washed with 0.5% Triton X-100, the protein zone of 0.4—0.6 *V* was in the first separation on Sephadex A-25. The remains of LHC were also eluted in this zone if the initial material was purified insufficiently. Sometimes the blue absorbance maximum of the complexes eluted in the 0.4—0.6 *V* zone was shifted from 436—440 to 421—425 nm, probably due to a substantial proportion of pheophytin with the absorbance maximum at 410 nm [20].

ΔA_{676} dependence on the light intensity was taken from Fig. 4B and drawn up in double reciprocal plots (not shown). Correction for the contribution of *P*-700 satellite band, accounting for 1/6 of ΔA_{696} was performed. It made possible the estimation of ΔA_{676} amplitude in saturating light. A value of 18 ± 4 was obtained for the Chl *a*/*P*-680 ratio assuming that the differential extinction coefficients of *P*-680 and *P*-700 are equal. This value is twice as low as the minimal one for Chl *a*/*P*-700 in the 0.6—0.7 *V* zone. The amount of *P*-700 in the complexes from the 0.85—1.0 *V* zone does not exceed 2% of that of *P*-680 (Chl *a*/*P*-700 800 for the fraction No. 16, Fig. 1A).

Discussion

It follows from the above data that three types of pigment-protein complexes may be successively eluted in the sieving range of DEAE-Sephadex.

(1) 0.4—0.6 *V* range. The degradation product of PPC-II forms either after the initial solubilization of chloroplasts by 3.3% Triton X-100 and removing of LHC, or by repeated purification of PPC-II. It is likely that the shifts of

absorbance and ΔA maxima to shorter wavelengths indicate partial pheophytinization of antenna pigments and loosening of PPC-II structure (it results in easy extraction of these pigments from the complexes by 0.5% Triton X-100). Loss of *P*-680 changes observed above may be associated with: (a) destruction of reaction centres; (b) decrease in the efficiency of energy migration to reaction centres as a result of loosening of complex structure. In this case, further increase in light intensity must restore the ΔA signal.

(2) 0.6—0.7 *V* range. Here PPC-I prevails, which is resistant to purification, with the concentration of one photoactive *P*-700 per 35 Chl *a* molecules being maximal and the ratio $\Delta A_{696}/\Delta A_{678}$ being equal to 6. It should be emphasized that the ratio of the basic and satellite band of *P*-700 in the difference spectrum under specific conditions (pH 8, 5—10 μM TMPD, saturating light) can be a criterium for PPC-I purity. We assumed that the preparations with this ratio being equal to 6 had no *P*-680. Thus for fraction No. 8 (Fig. 1A) with $\Delta A_{696}/\Delta A_{678}$ 5.3 the amount of *P*-680 does not exceed 7% of that of *P*-700.

(3) 0.85—1.0 *V* range. Here PPC-II is the major constituent. This complex is supersensitive to repeated treatments. It is eluted at about the end of the sieving range where ionic interactions are stronger. According to our calculations, PPC-II contains one photoactive *P*-680 per 18 Chl *a* molecules. Increase in the molecular weight and (or) decrease in the negative surface charge of protein may be responsible for the rise of PPC-II migration rate and eluation into the 0.4—0.6 *V* zone. This point of view is confirmed by the fact that the remains of LHC, that tend to protein-protein interaction in the ionic medium [21], are eluted in the same zone. The protein content of the fraction collected was not characterized in this work and it is possible that some colourless products of PPC-II degradations are eluted in the zone of 0.85—1.0 *V*.

So far isolation of photoactive PPC-II by electrophoresis could not be achieved because of the great lability of this complex. Recent investigations of Wessels and Borchert [22] have shown that PPC-II loses photoactivity as well as Chl *a* after incubation with 1% sodium dodecyl sulphate. This process is accompanied by rearrangement of the complex: initial PPC-II with its molecular weight approximating to 39 000—42 000 yields three polypeptides at 50 000, 46 000 and 42 000. This is in agreement with the results of the present work. In this respect it is possible that the express-method of PPC-I isolation with a high *P*-700 content [8] is based on direct destruction of Photosystem II complexes. In the further studies of physical and chemical properties of PPC-II one should take into account the possibility of its functional and structural variations. It cannot be excluded that the separation of membrane-bound LHC and PPC-II reduces the stability of the latter in water solution.

The absorbance spectra of isolated complexes are similar to those reported in the works [4,5]. The absorbance of Chl *b* does not exceed 10% of that of Chl *a* in Soret bands.

It should be emphasized that our method used for obtaining simultaneously purified photoactive PPC-I and PPC-II has several advantages over the one developed earlier [5]: it does not require prolonged gradient centrifugations and all the procedures may be accomplished within two days. The degree of PPC-I purity after the first separation is, at least, two times higher than after two-fold chromatography on DEAE-cellulose according to the method [10].

The Chl *a* content of PPC-II approximates to 0.3% of that in the chloroplast material used (as in the method [5]) and to 10—15% of that in the PPC-I obtained (see the curve of A_{max} in Fig. 1A). However, the relative concentration of *P*-680 in PPC-II is twice as high as that of *P*-700 in PPC-I. Supposing: (a) each pigment-protein complex has one reaction centre; (b) the concentrations of *P*-680 and *P*-700 in the noncyclic electron transport chain are equimolar; (c) *P*-680 exists only in grana; (d) the amounts of *P*-700 in grana and stroma lamellae are equal, the *P*-700/*P*680 ratio in chloroplasts is equal to 2 and the total Chl *a* content in Photosystem II is 4 times lower than that in Photosystem I. This conclusion is in good agreement with literature data: there are approx. 30% of chloroplast Chl *a* in Photosystem I and 5—9% in Photosystem II (without LHC) [21,23,24]. It follows that more then 90% of chlorophyll is lost in the process of complex isolation.

The task of further investigations on fractionation of plant green photosystems is to decrease of losses and, particularly, to raise the PPC-II stability.

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