THE SEPARATION OF PHOTOCHEMICALLY ACTIVE PS-I AND PS-II CONTAINING CHLOROPHYLL–PROTEIN COMPLEXES BY ISOELECTRIC FOCUSING OF BEAN THYLAKOIDS ON POLYACRYLAMIDE GEL PLATES

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1. Introduction

While preparative methods for the isolation of chl–protein complexes from thylakoids have provided tools for considerable insight into properties of LHCPC and reaction centers of PS-I and PS-II [1–8], microscale analytical methods have been less successful. The latter methods usually include solubilization of thylakoids by anionic detergents like SDS and separation of the solubilized chl–protein complexes by SDS–PAGE [9,10]. SDS–PAGE allows to separate 2–6 chl–protein complexes and a variable fraction of 'free pigments' [9–11]. However, exposure of thylakoids to SDS or LiDS destroys their photochemical activities [4,12–14] thus rendering the physiological identification of isolated complexes difficult; it also affects binding between proteins and pigments [4,15,16] which raises the question whether the 'free pigment' fraction has biological significance or is an isolation artifact.

After preparative isolation procedures for chl–protein complexes have shown non-ionic detergents like Triton X-100 or digitonin [4–8] to be more gentle than SDS, an analytical method using Triton X-100 and avoiding SDS was introduced [17]. In their original procedure, acrylamide solutions containing Ampholines, Triton X-100 and thylakoids were polymerized and subjected to IEF. From mature chloroplasts, 4–5 green bands can be separated by this method; spectral shifts observed in the separated bands, however, indicate that the native environment of the chl is altered during separation (D.S.-H., H.N., unpublished).

Here an analytical method for the isolation of photochemically active chl–protein complexes is described, including short-time solubilization of thylakoids with Triton X-100 and separation of the solubilized proteins by IEF on digitonin-containing Ampholine–PAG plates. The separated chl–protein complexes bind almost all thylakoid pigments and can be correlated to functionally distinct parts of the photosynthetic apparatus. An abstract of part of this work is [18].

Abbreviations: chl, chlorophyll; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; DPIP, 2,6-dichlorophenolindophenol; IEF, isoelectric focusing; LHCPC, light-harvesting chlorophyll *a*/*b* protein complex; LiDS, lithium dodecylsulphate; PAG, polyacrylamide gel; PS, photosystem; SDS, sodium dodecylsulphate; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis

2. Materials and methods

Beans (*Phaseolus vulgaris* L. 'commodore', LSBR Warsage) were grown in a greenhouse at 25°C under 12 h light–12 h dark cycles for 25 days. Before harvesting, the plants were kept in the dark for 12 h to reverse light-induced zeaxanthin accumulation [19]. All preparations were performed at 4°C. For preparation of washed thylakoid membranes, leaves (~20 g) were homogenized in 150 ml medium containing 300 mM sorbitol, 50 mM Hepes–Na buffer (pH 7.5), 1 mM EDTA, 10 mM NaCl, 1 mM $MgCl_2$ and 0.1% defatted bovine serum albumin, filtered through nylon cloth (36 μm mesh width) and centrifuged for 3 min at 3000 × *g*. The pelleted membranes were

suspended in 20 ml glass distilled water, mixed with 5 ml 25 mM EDTA solution (pH 7.8) [3], centrifuged for 3 min at 6000 × *g*, washed in glass distilled water and, after centrifugation, adjusted to 4 mg chl/ml. Portions of 250 μl were stored at −20°C without significant loss of activities. Immediately before IEF, the thylakoid membranes were solubilized at 0°C for 5 min with 3% Triton X-100 at a chl/Triton X-100 ratio of 1:15. Since Triton X-100 caused an absorbance shift of the membranes to shorter wavelengths, longer solubilization times were avoided.

For IEF, PAG plates were made by pouring fresh solutions of 3.0% (w/v) acrylamide, 0.33% (w/v) *N,N'*-methylenebisacrylamide, 0.07% (v/v) *N,N,N',N'*-tetramethyldiaminoethane, 0.08% (w/v) ammoniumperoxodisulfate, 1.6% (w/v) digitonin, 1.3% (v/v) LKB Ampholine (pH 5–7) and 1.3% (v/v) LKB Ampholine (pH 3.5–5) on supporting glass plates and polymerizing them under N_2 for 15 min. (The digitonin, Serva 19550, incorporated into the gels to prevent reaggregation of the chl–protein complexes, was added from 5% stock solutions that after boiling had been clarified by centrifugation.) For glass plates of 9 × 12 cm, 15.4 ml solution were used; sample troughs were formed in the polymerizing gel by incorporating glass capillaries which were later removed.

IEF was performed in the LKB Multiphor 2117 chamber, with a constant wattage power supply. Electrode solution was water. Gel plates, oriented with their sample troughs towards the cathode, were cooled to 2°C and pre-run at 10 W/gel for 15 min. After application of the solubilized samples (250 μl or 600 μg chl/gel), the run was continued at the same wattage for 15 min, ensuring that most of the chl–protein complexes left the trough, then at 100 V for 10–12 h. After the run, the pH gradient was read with a LKB Multiphore electrode. For densitometric tracing, the gel was moved in 0.5 mm steps in front of the photomultiplier of a two-wavelengths double-beam spectrophotometer while monitoring absorbance in the dual mode with 740 nm as reference (monitoring beams 0.25 × 2 mm; pass band 1 nm). For all spectrophotometric measurements, a Perkin-Elmer Model 356 instrument was used.

For extraction of chl–protein complexes, the green bands were cut from the gel and homogenized in a potter with 3–5 ml 50 mM Tris–Cl buffer (pH 7.8); the resulting green solutions were separated from the gel material by centrifugation. Total chl content of the chl–protein complexes was determined in aliquots extracted with 80% acetone according to [20]; chl *a*/*b* ratios were determined after TLC analysis of the pigments. *P*-700 oxidation was assayed as light-induced A_{700} decrease [4,21] using an extinction coefficient for *P*-700 of 64 mM^{-1} cm^{-1} [22]. DPIP reduction was assayed as light-induced A_{590} decrease [23] using an extinction coefficient for DPIP of 16 mM^{-1} cm^{-1}. For pigment analysis, the protein solutions were extracted according to [24]. The organic phase was concentrated under vacuum in the presence of a few drops of 50 mM Tris–Cl buffer (pH 7.8) (to avoid acidification due to traces of HCl in the chloroform) and analysed using the reverse-phase TLC system [25].

Experiments were reproduced 3–5 times; the data presented in tables 1,2 are from 1 typical experiment.

3. Results

When Triton X-100-solubilized thylakoids were subjected to IEF on PAG plates, their chl accumulated in 8 bands of chl–protein complexes with acidic isoelectric pH values (fig.1). The complexes extracted

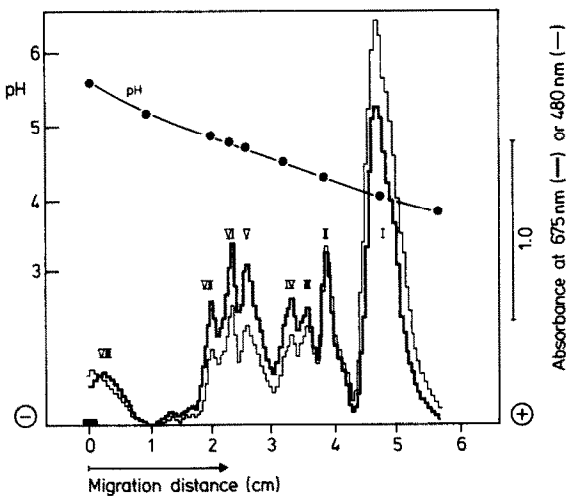

Fig.1. Densitometric traces at 675 nm and 480 nm of the chl–protein complexes of bean thylakoids after IEF on a digitonin-containing Ampholine–PAG plate. The black bar represents the sample trough. For experimental details see section 2.

Table 1
Spectral properties, chl distribution and photosynthetic activities of chl–protein complexes (fractions I–VIII) isolated from bean thylakoids by IEF on PAG plates

Fraction	Red maxima (nm)	Rel. chl content (%)	chl *a*/*b* ratio (mol/mol)	*P*-700 oxidation (mol *P*-700 ox./ 100 mol chl *a*)	DPIP-reduction rate (μmol DPIP red. .mg chl^{-1} h^{-1})
I	674, 651	41.7	1.6	0	0
II	675, (651)	6.8	2.3	0	12.9
III	675, (651)	4.8	3.0	0	15.7
IV	676.5	7.6	4.4	0.17	11.2
V	677	11.7	5.9	0.39	2.4
VI	677	7.6	6.3	0.48	0
VII	677	8.9	6.3	0.50	0
VIII	672	10.9	6.3	0.53	0
'Recombined' fractions, after IEF	–	–	2.95[a]	0.233[a]	2.76[a]
Solubilized thylakoids, before IEF	–	–	3.15	0.295	4.04
		% recovery after IEF		79.0%	68.3%

[a] Data calculated from relative chl content and photosynthetic activities of fractions I–VIII

from these bands (fractions I–VIII) will be characterized and correlated to functionally distinct parts of the photosynthetic apparatus.

3.1. *Spectral properties, chl distribution and photosynthetic activities of the chl–protein complexes*

Fraction I represented the major fraction (~40% of total chl) and was enriched in chl *b* (table 1). Its spectrum with 2 peaks in the red spectral region (fig.2) was similar to the spectrum of LHCPC isolated by other methods [3,26]. Like LHCPC [26], the major red maximum of fraction I was at 674 nm as compared to the maximum of intact bean thylakoids at 677 nm. Fraction I showed no *P*-700 oxidation or PS-II mediated DPIP reduction (table 1, figs.3,4), and its isoelectric pH 9pH 4.15 ± 0.10) was similar to the value of pH 4.25 reported [8] for LHCPC preparation. Therefore, fraction I is identified as LHCPC.

Fractions II–IV contained ~20–25% of total chl. With increasing fraction number, the relative chl-*b* content decreased (table 1) and the chl-*b* related maximum at 651 nm disappeared in the spectra (table 1, fig.2). Also, a shift of the red maximum to longer wavelengths was seen in these fractions. We observed scarcely any or no *P*-700 oxidation in fractions II–IV while all 3 fractions showed

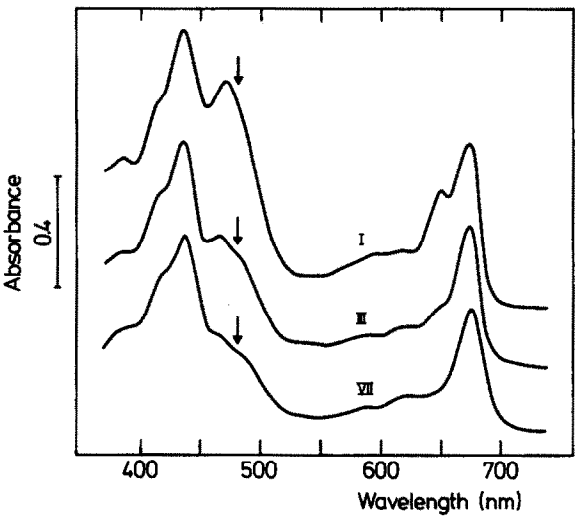

Fig.2. Room temperature absorbance spectra of chl–protein complexes extracted from gel bands I, III and VII of fig.1. Arrows indicate A_{480} (compare fig.1).

PS-II mediated electron transport from DPC to DPIP (table 1, figs.3,4). The fact that DPIP reduction was inhibited by DCMU and did not occur without DPC (fig.4b) indicates that these fractions contain the DCMU-sensitive endogenous electron acceptor of PS II and have lost the water-splitting enzyme system. Based on their photochemical activities and their isoelectric pH values (pH 4.30–4.55) which are similar to pH 4.55 reported [8] for PS-II preparation, fractions II–IV are identified as PS-II containing chl–protein complexes.

Fractions V–VII (with 25–30% of total chl) contained a low amount of chl *b* (table 1), though the presence of chl *b* could not be deduced from the shape of their spectra (fig.2). Maximal absorbance of the 3 fractions occured at 677 nm, i.e., at the maximum of PS-I preparations obtained by other methods [4,27]. The isoelectric pH values of fractions V–VII (pH 4.65–4.95) were close to pH 4.7 reported [8] for a PS-I preparation. Most conclusive, however, was the observation that all 3 fractions showed *P*-700 oxidation but were practically inactive in PS-II-mediated electron transport (table 1, figs.3,4). Therefore fractions V–VII are identified as PS-I containing chl–protein complexes.

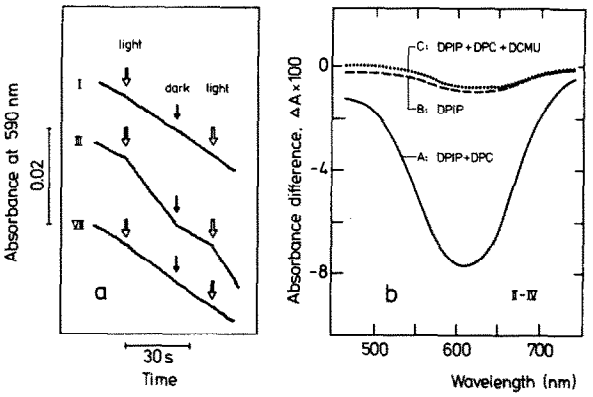

Fig.4. DPIP reduction of chl–protein complexes extracted from gel bands I, III and VII (a) and of combined gel bands II–IV (b) of fig.1. (a) Kinetics of the light-induced ΔA_{590}. (b) Light–dark difference spectra of the ΔA. Actinic light was at 430 ± 20 nm and saturating intensity. In fig.4a, the photomultiplier was protected by a monochromatic filter (Schott) transmitting at 590 nm; the reference was water. In fig.4b, difference spectra were taken in the dark after 4 min (curve A) or 7 min (curves B,C) of illumination, with a dark sample as reference. Reaction mixtures (3 ml) contained 50 mM Tris–Cl buffer (pH 7.8), 30 μM DPIP, chl–protein complexes with 5 μg chl/ml (a) or 7.5 μg chl/ml (b) and, where indicated, 1 mM DPC (plus 1% ethanol) and 4 μM DCMU (plus 0.4% ethanol).

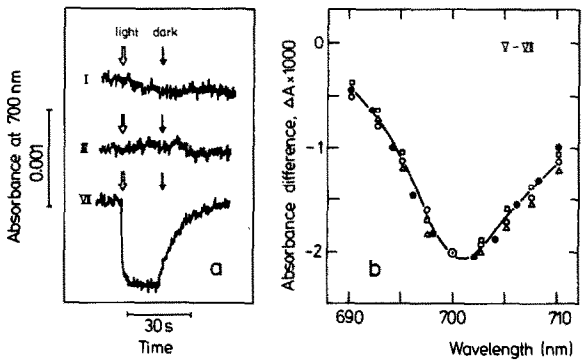

Fig.3. *P*-700 oxidation of chl–protein complexes extracted from gel bands I, III and VII (a) and of combined gel bands V–VII (b) of fig.1. (a) Kinetics of the light-induced ΔA_{700}. (b) Light–dark difference spectrum of the ΔA change. Actinic light was at 430 nm and saturating intensity, the photomultiplier being protected by a red-transmitting filter (Schott RG-665). Reference wavelength: 725 nm. Reaction mixtures (3 ml) contained 50 mM Tris–Cl buffer (pH 7.8), 10 mM Na-ascorbate, 0.1 mM methylviologen and chl–protein complexes with 2.5 μg chl/ml (a) or 5 μg chl/ml (b). In fig.3b, different symbols indicate different complex preparations; their ΔA are normalized based on av. ΔA_{700}.

Fraction VIII photochemical activities agreed with those of fractions VI, VII (table 1). However, fraction VIII occured as a rather diffuse band (isoelectric pH 5.3–5.6), and the position of its red maximum is shifted to 672 nm. This position indicates that fraction VIII did not consist of free chl, since free thylakoid pigments (after organic extraction, solubilization in aqueous detergent and diffusion into the gel during IEF) showed maximal absorbance at 668 nm. Instead, the similarity between the extents of *P*-700 oxidation of fraction VIII and of fractions V–VII suggests that the chl of fraction VIII were organized in PS-I containing chl–protein complexes like the chl of the other fractions. The wavelength shift of fraction VIII may be explained by prolonged exposure to Triton X-100 that was present in the nearby sample trough (cf. section 2). Triton X-100 induced wavelength shifts have been observed for both, purified PS-I preparations [8] and bean

thylakoids (D.S.-H., H.N., unpublished). Finally, it should be mentioned that the amount of fraction VIII (8–12% of total chl) could be increased by decreasing the pore size of the PAG. Therefore it seems probable that the chl–protein complex(es) of fraction VIII which remained near the sample trough was inhibited in mobility, perhaps due to incomplete solubilization or clogging of the gel by other proteins.

3.2. *Carotenoid content of the chl–protein complexes*

The densitometric traces of the PAG plate at 675 nm (monitoring the chl) and at 480 nm (monitoring the carotenoids and chl *b*) behaved similarly (fig.1) indicating that the carotenoids of solubilized thylakoids did not penetrate the gel by free diffusion but were associated with the chl–protein complexes. This association seems to be specific, since the chl–protein complexes differed significantly in their carotenoid content (table 2).

In agreement with other LHCPC preparations [9,10], fraction I was enriched in lutein and neoxanthin but contained only traces of carotene, thus differing from fractions II–VIII. The carotenoid content of fractions II–IV varied: with increasing fraction number, carotene content increased while lutein and neoxanthin content decreased. Also a slight accumulation of violaxanthin was observed (the level of violaxanthin being unusually low in the leaf material studied). Among fractions V–VII the carotenoid content was quite similar; in accordance with other PS-I preparations [28] these fractions were characterized by high carotene and low xanthophyll levels. The relatively high lutein and violaxanthin levels of fraction VIII might be caused by 'free pigments' which are either not bound to chl–protein complexes in situ or were separated from their protein during isolation. About 5% of the total lutein and 7.5% of the total violaxanthin (estimated from the pigment increase in fraction VIII as compared to fractions V–VII) were possibly in a free state, while the major part of the carotenoids remained organized in chl–protein complexes after IEF.

4. Discussion

IEF of solubilized thylakoids of PAG plates allows to isolate several photochemically active PS-I or PS-II containing chl–protein complexes by a fast one-step

Table 2
Carotenoid content of the chl–protein complexes isolated from solubilized bean thylakoids (fractions I–VIII) in comparison with untreated thylakoids

Fraction	Carotenoid content (mol pigment/100 mol chl *a*)			
	Carotene	Lutein	Viola-xanthin	Neo-xanthin
I	0.6	30.7	2.0	10.0
II	7.1	18.8	3.0	5.5
III	9.3	14.6	3.5	4.7
IV	10.5	9.6	2.0	2.2
V	12.0	6.5	1.4	1.2
VI	13.8	7.3	1.6	1.3
VII	14.0	7.6	1.3	1.5
VIII	15.5	14.2	3.1	1.2
'Recombined' fractions I–VIII	8.4[a]	17.4[a]	2.1[a]	4.8[a]
Untreated thylakoids	8.7	15.8	2.1	4.6

[a] Data calculated from pigment/chl *a* ratios (this table) and chl content (table 1) of each fraction

procedure. The gentle approach with our procedure is demonstrated by the amount of recovery of photosynthetic activities (table 1) and from the carotenoid/chl ratios (table 2) of the combined complexes after IEF as compared to membranes not subjected to IEF; it also is shown by the fact that the thylakoid pigments are almost completely bound to the isolated pigment–protein complexes. Using this method, it has now become possible to determine the distribution of chl between functionally distinct parts of the photosynthetic apparatus without additional assumptions on the origin of 'free' pigments (table 1). In particular, our data indicate that ~20–25% of the bean thylakoids chl are organized in PS-II containing complexes.

Not only the chl but also most or all carotenoids seem to be complexed with specific proteins rather than being dissolved in the lipid core of the thylakoid membrane. This organization of the photosynthetic pigments implies that they are concentrated at specific sites in the membrane, an arrangement supported also by immunological studies [29]. The close packing of pigments that results from such arrangement seems to provide a basis for the efficient energy transfer between carotenoids and chl observed in isolated chl–protein complexes [11].

The occurence of 3 complexes with PS-I activity and of 3 complexes with PS-II activity each with a distinct isoelectric point (table 1, fig.1) has not been described before. The biological significance of these complexes remains to be established. After SDS–PAGE, 2 bands were observed [11], the spectral properties of which were typical for PS-I complexes and 3 bands with spectral properties of LHCPC. It was suggested [11] that these bands might represent oligomers of the PS-I complex and of LHCPC. A similar explanation for the occurence of 3 PS-I containing complexes in our experiments cannot be excluded since the 3 complexes are rather similar in *P*-700, chl *b* and carotenoid content, and since their isoelectric points might be altered by oligomer formation. The fact, however, that in case of the LHCPC only one band was obtained with our procedure, does not favor this view. In contrast to our PS-I complexes, our PS-II complexes differed significantly from each other in their pigment content (table 2). Therefore, they probably do not represent oligomers of the same basic complex. The question whether they may be subunits of a highly organized PS-II complex present in the thylakoid membrane in vivo, remains to be investigated.

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