SEPARATION OF THYLAKOID PIGMENT–PROTEIN COMPLEXES BY SDS–SUCROSE DENSITY GRADIENT CENTRIFUGATION

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1. Introduction

The pigment-protein complexes of SDS-solubilized chloroplast thylakoids can be separated by polyacrylamide gel electrophoresis (PAGE). Earlier methods resolved 2 complexes: CPI, the chl *a*-rich P700–protein complex, and CPII or LHCP, the chl *a* + *b*-rich light-harvesting complex [1,2]. Milder methods resolve 6 main complexes and some minor bands, with >90% of the thylakoid chlorophyl bound to them: the monomers CPI and $LHCP_3$, their oligomers CPIa, and $LHCP_1$ and $LHCP_2$, respectively, and a minor chl *a* containing complex (CPa, complex IV, A, or chl *a*-P_2–chl *a*-P_3) [3–8], thought to originate in the PSII reaction center. The oligomers increase during greening [9] and are transformed to their monomers by extensive SDS action or cation neutralization of the electrostatic forces, which seem to prevail [10].

The question arose, however, whether the oligomers are not real higher M_r forms, but rather unfolded forms of their monomers, exhibiting lower mobility on the gel, and whether their transformation to the monomer by cation addition reflects a conformational change to the more compact form and not a change in size [10]. One way to distinguish between a change in size from a change in conformation of a macromolecule is to use a combination of PAGE and sucrose density gradient centrifugation. Less compact forms have lower electrophoretic mobility and thus appear to have higher M_r but when analyzed by sucrose density gradient centrifugation they exhibit an S-value corresponding to lower M_r [11].

We thus tried to separate the pigment–protein complexes by sucrose density gradient centrifugation. This procedure resolves 4 pigmented bands from SDS–solubilized thylakoids, containing in order of increasing density: $LHCP_3$, $LHCP_1$ + $LHCP_2$, CPI and CPIa (as judged by PAGE, pigment composition and P700/chl).

In all cases the electrophoretic mobility of the complexes was found to be inversely proportional to their sedimentation rate. Furthermore, only 2 bands were resolved by sucrose density gradient centrifugation in the presence of cations (those of $LHCP_3$ and CPI) in agreement with the results of the SDS–PAGE [10]. This speaks in favor of the existence of real higher M_r forms of the complexes, which are transformed to their monomers in the presence of cations.

The SDS–sucrose density gradient centrifugation provides a convenient alternative to PAGE, whenever preparative schemes are required.

2. Methods

Chloroplasts and thylakoids were prepared from *Phaseolus vulgaris* leaves as in [7]. The thylakoids, washed twice in 0.05 M Tricine–NaOH (pH 7.3) were solubilized in 0.3 M Tris–HCl (pH 8.6)–10% glycerol–1% SDS to have ~700 μg chl/ml, SDS/chl = 6–7, and 0.4–0.5% SDS. This sample (0.3 ml) was layered onto a 5 ml linear sucrose density gradient (5–22% sucrose in 0.05 M Tris–borate (pH 9.5)–0.1% SDS) and was centrifuged at 390 000 × *g* for 5.5 h or at 170 000 × *g* for 17 h at 17°C (SW 65L or 50.1 rotors). The resolution is better with the short centrifugation but the overnight centrifugation is more convenient. The gradients were fractionated (ISCO model 640 density gradient fractionator) and the fractions analyzed for chlorophyll [12], carotenoids [13], protein [14] and P700 [15,16], absorption difference of the ferricyanide oxidized minus

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ascorbate-reduced samples, $\Delta\epsilon = 64$ mequiv.$^{-1}$. cm^{-1}). Absorption spectra were recorded on a Perkin Elmer 356 double beam spectrophotometer and low temperature fluorescence spectra in a fluorometer setup described in [17]. SDS–PAGE was done as in [7,18]. The electrophoretic profiles were recorded in a Joyce-Loeble Chromoscan, using a cut-off filter transmitting light above 620 nm.

3. Results

Fig.1 shows the resolution of the SDS-solubilized thylakoid pigmented components after 5.5 h centrifugation at 390 000 × g on a linear sucrose density gradient. Four pigmented bands are clearly distinguished (I–IV). After fractionation bands II and IV are further resolved into 2 components each. PAGE profiles of each band are shown in fig.2. Comparison of these results with those obtained from total thylakoids ([3,7] and fig.2) suggests that band I contains $LHCP_3$ and free chlorophyll, band II a mixture of the oligomers $LHCP_1$ and $LHCP_2$, which upon PAGE resolve in addition, their monomer $LHCP_3$, band III contains only CPI and band IV a mixture of CPI and CPIa. The latter may contain only CPIa, but due to its tendency to form CPI upon extensive SDS action, it may resolve both CPI and CPIa.

The results of further analyses lead to the same conclusion (fig.3; tables 1–3). The two lower M_r bands (I,II) have low chl a/chl b ratio, no P700, trace

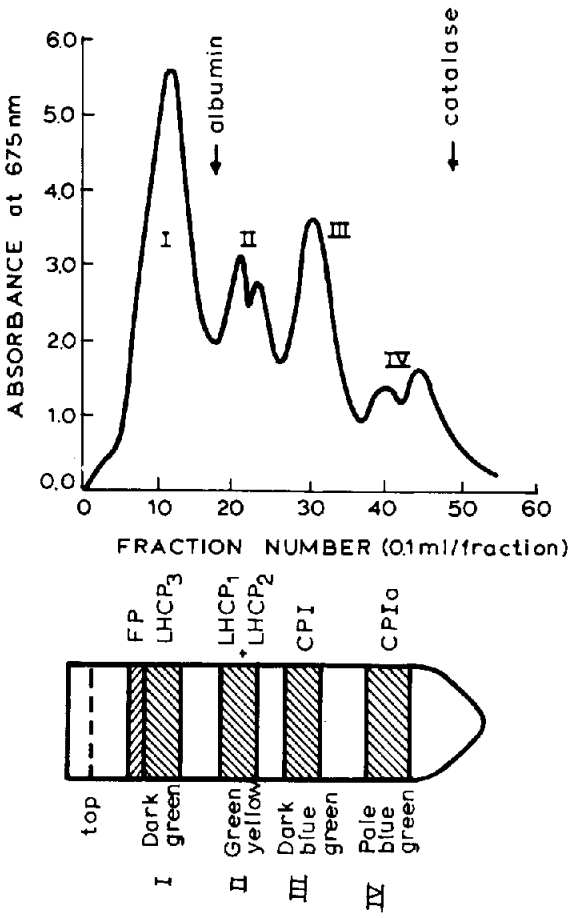

Fig.1. Separation of the pigment–protein complexes of SDS-solubilized *Phaseolus vulgaris* thylakoids by SDS–sucrose density gradient centrifugation (390 000 × g for 5.5 h at 17°C). The solubilized material applied on the gradient had chl a/chl $b = 2.6$, SDS/chl = 5.1, SDS = 0.5%, chl/ml = 980 μg, and total chlorophyll equal to 300 μg. Arrows show the location of the M_r markers (bovine serum albumin, 67 000 and catalase, 250 000).

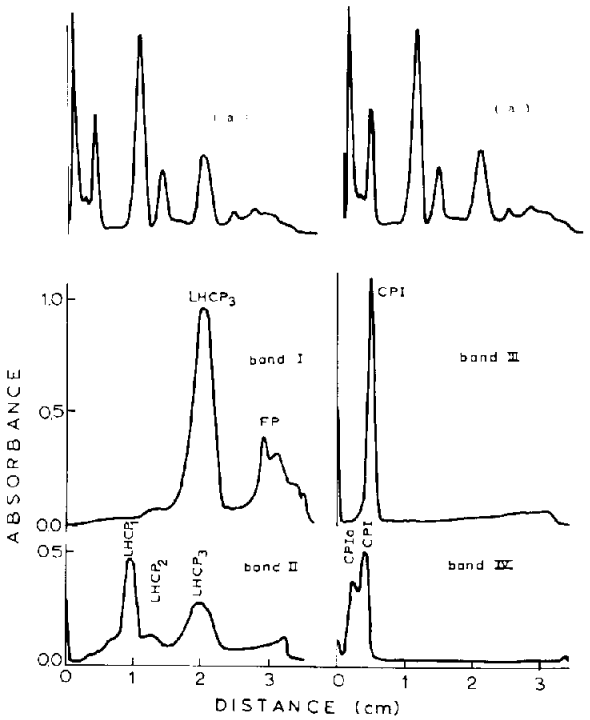

Fig.2. Densitograms of PAGE profiles of the bands separated by SDS–sucrose density gradient centrifugation: 0.1 ml each band was applied on the gel containing 11 μg chl (band I), 7 μg chl (band II), 6.7 μg chl (band III) and 4.4 μg chl (band IV). The gels are not stained for protein. The PAGE profile of SDS-solubilized thylakoids prior to centrifugation is shown on top.

Table 1
Pigment composition of *Phaseolus vulgaris* thylakoids and of their pigment–protein complexes separated by SDS–sucrose density gradient centrifugation

Sample	Chl *a* / Chl *b*	P700 / Chl	Xanth. / *b*-car. (w/w)	Carot. / Chl	Chl *a* / *b*-car. (mol/mol)	Chl *a* + *b* / Carot.	Carotenoid distribution (%) *b*-car	Lu	Vx	Nx
Band I (LHCP[3])	1.4	1:1300	23.4	0.114	74	5.5	4.1	70.5	trace	25.4
Band II (LHCP[2] + LHCP[1])	1.2	1:3000	high	0.132	high	4.7	0	76.5	trace	23.5
Band III (CPI)	>9	1:30	0.9	0.05	21	11.4	52.5	47.5	trace	trace
Band IV (CPIa)	5	1:100	1.8	0.16	10.4	3.78	35.6	29	trace	35.4
Thylakoids in SDS	2.33 (2.28)	1:300	3.36 (2.6)	0.15 (0.13)	13.1 (11.4)	6.25 (4.8)	23.0 (27.7)	56.5 (45.9)	7.45 (13.1)	13.0 (13.1)

Values in parentheses are calculated from the data in [20] for spinach lamellae

Table 2
Chlorophyll: protein ratio of the pigment–protein complexes separated by sucrose density gradient centrifugation from SDS-solubilized *Phaseolus vulgaris* thylakoids

Sample	Chl *a* + *b*/protein (μg/mg)	Protein/chl *a* + *b* (g/mol)		Chl/protein (mol/M_r)		
Band I	121	7440	5200[a]	4.7	(35 000)	6[a]
Band II	159	5662		6.1	(35 000)	
Band III	234	3846	7000–8000[a]	28	(110 000)	14–21[a]
Band IV	71	12 690		14	(180 000)	
Thylakoids in SDS	197	4570				

[a] Values from [1,2] for the light harvesting chl *a* + *b* protein and for the P700–chl *a* protein. Values in parentheses show the M_r on which the chl:protein molar ratio is calculated

Table 3
Spectral characteristics of the pigment–protein complexes of SDS-solubilized *Phaseolus vulgaris* thylakoids, separated by SDS–sucrose density gradient centrifugation

Sample	A_{max} (273 K) nm				F_{max} (77 K) nm		F_{730}/F_{685} (or 720/680)
Band I	435	465	654	672	689	730	0.129
Band II	435	472	654	673	685	730	0.13
Band III	435	–	–	676	685	722	0.45
Band IV	435	–	–	674	686	718	0.89
Chloroplasts (in tricine)					695	738	4.1

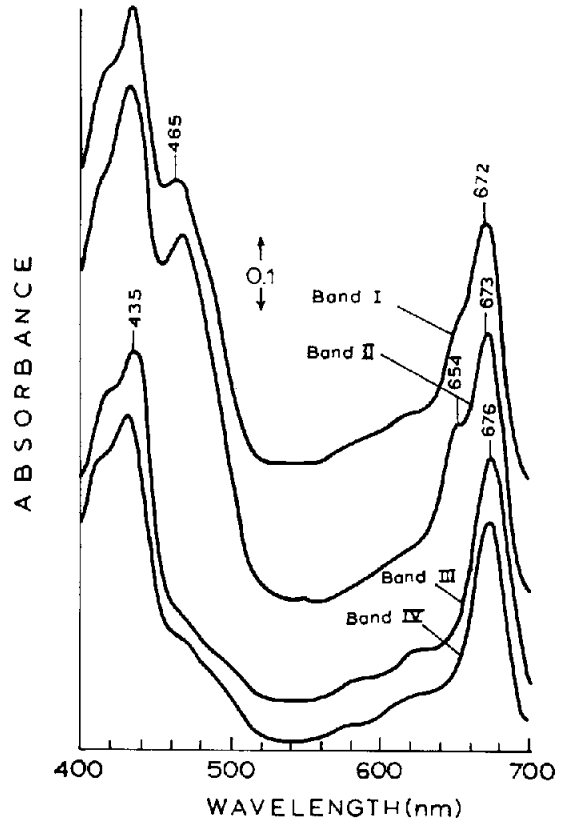

Fig.3. Visible absorption spectra of the pigment–protein complexes separated by SDS–sucrose density gradient centrifugation of SDS-solubilized thylakoids.

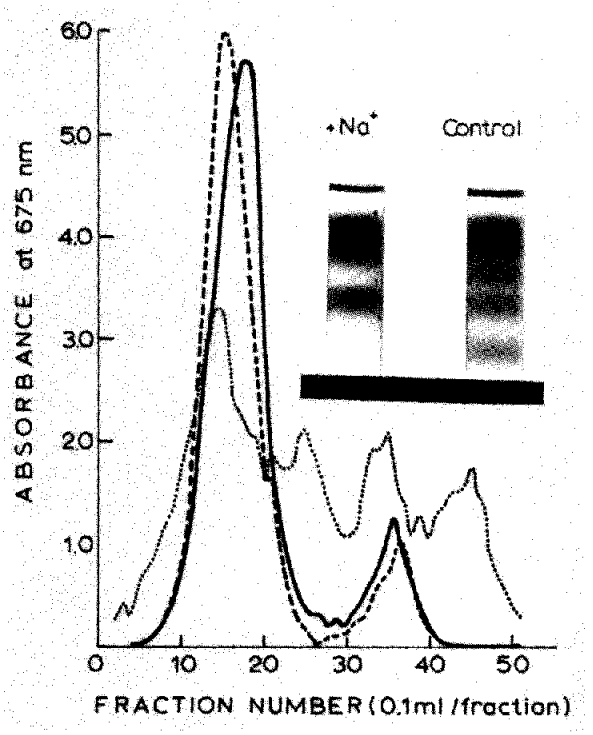

Fig.4. Sucrose density gradient centrifugation profiles obtained from thylakoids in the absence (. . .) or presence of Na^+ at 40 mM (—·—) or 80 mM (– – –) in the gradients. Centrifugation at 170 000 × *g* for 17 h in a SW 50.1 rotor.

of *b*-carotene, absorption maxima in the red at 672–674 and 654 nm, and low temperature fluorescence maxima at 685 and 730 nm (low F_{730}/F_{680}). On the contrary, bands III and IV have high chl *a*/chl *b* ratio (much higher band III), are enriched in P700 (especially band III) and in *b*-carotene, their absorption maxima in the red are at 674–676 nm, while the ratio F_{720}/F_{680} is higher than that of bands I and II. The values are in good agreement with those found for LHCP or CPI isolated by PAGE or column chromatography [1,2]. The data suggest therefore that bands I and II are the LCH components, while bands III and IV the CPI and CPIa, respectively.

Using as M_r markers albumin (67 000 M_r) and catalase (250 000 M_r) and based on the relationship $S_1/S_2 = (M_r t_1/M_r t_2)^{2/3}$ [20], where S is the distance travelled by a protein from the meniscus, we have estimated the M_r band I to be 29 000 (based on albumin) or 32 000 (based on catalase), and of band III to be 117 000 or 130 000, respectively. These values agree with those found by SDS–PAGE [10]. The values in parentheses (table 2) are tentative M_r-values of the monomers, assumed to be present in the bands. However, the CPIa complex contains in addition to the 110 000 M_r CPI complex, smaller polypeptides of M_r 26 000, 24 000, 16 000 and 12 000 (not shown). The chl/protein (mol/M_r) ratio, shown for this band, is therefore estimated on the app. M_r of 180 000 [10].

The pigment–protein complexes after isolation from the gradients can be conveniently concentrated by Mg^{2+} precipitation (at 50 mM) [21].

Fig.4 shows the resolution of the pigment–protein complexes of thylakoids after sucrose density gradient centrifugation in the absence or presence of 40 or 80 mM Na^+. Although the thylakoids in the absence of Na^+ resolve all 4 bands, in its presence they resolve only bands I ($LHCP_3$ + free chl) and III (CPI), in agreement with the SDS–PAGE study [10].

4. Discussion

These results showed that the electrophoretic mobility of the complexes is indeed inversely proportional to their sedimentation rate. We can therefore conclude that the oligomers of the pigment–protein complexes are real forms of higher M_r and not unfolded forms of their monomers.

Furthermore, the simple method of SDS-sucrose density gradient centrifugation offers a convenient alternative to PAGE whenever preparative schemes are required. The PAGE methods are excellent for analytical purposes but inadequate for preparative purposes, since laborious and often destructive elution of the complexes from the gels is required. In addition, the sucrose density gradient centrifugation offers a one-step isolation procedure for the P700–pigment–protein complex from SDS-solubilized thylakoids. The only disadvantage of the method at this point seems to be its inability to detect the CPa band, which is distinctly resolved by PAGE [3–8]. This is probably due to the ease with which the CPIa complex loses its chlorophyll upon SDS action.

The method has been applied successfully in the analysis of the pigment–protein complexes of stroma and grana thylakoids obtained by differential centrifugation of French press disrupted chloroplasts, as well as of the non-appressed lamellae of the blue-green alga *Nostoc muscorum*. The *Nostoc* thylakoids contained only band III, stroma lamellae were deficient in bands I and II and grana thylakoids were enriched in bands I and II, as expected. The sucrose density gradient centrifugation can therefore be used successfully in all types of SDS-solubilized thylakoids, with results comparable to those obtained by PAGE.

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