CHLOROPHYLL–PROTEIN COMPLEXES OF SPINACH AND BARLEY THYLAKOIDS

Spectral characterization of six complexes resolved by an improved electrophoretic procedure

Jan M. ANDERSON, J. C. WALDRON and S. W. THORNE
CSIRO, Division of Plant Industry, PO Box 1600, Canberra 2601, Australia

Received 9 June 1978

1. Introduction

The chlorophyll–protein complexes of higher plant and green algal chloroplast thylakoid membranes can be resolved by SDS–polyacrylamide gel electrophoresis (PAGE) into 3 chlorophyll-containing zones:

(i) Chlorophyll–protein complex 1 (CP1) or P700-chlorophyll *a*–protein.
(ii) The light-harvesting chlorophyll *a*/*b*–protein complex (LHCP).
(iii) Free chlorophyll complexed to SDS at the electrophoretic front [1–4].

Several minor bands have also been reported [5–10]; one of these has been characterized as a dimer of LHCP [6,8,10]. All of these methods [5–10] give 25–50% free chlorophyll, which probably arises from the dissociation of chlorophyll from chlorophyll–protein complexes yet to be identified.

Spinach and barley thylakoids were solubilized with SDS at 4°C and immediately electrophoresed on discontinuous tube SDS–PAGE gels at 4°C. Some 90% of the total chlorophyll and carotenoid is now associated with protein, whereas earlier procedures had only about 70% of the chlorophyll associated with protein. The 6 resolved chlorophyll–protein complexes were characterized by in situ absorption and fluorescence spectroscopy at 77°C. The pigment complexes resolved are CP1a, an oligomer of CP1, the P700-chlorophyll *a*–protein complex, CP1, $LHCP^1$ and $LHCP^2$, oligomers of LHCP ($LHCP^3$), and CPa, a chlorophyll *a*–protein complex which may represent the photosystem (PS) 2 reaction centre complex. A chlorophyll *b*-deficient barley mutant which lacks CP1a, $LHCP^1$, $LHCP^2$ and $LHCP^3$ has 25% of its total chlorophyll associated with CPa. The higher oligomeric forms, CP1a and $LHCP^1$, more closely resemble the in vivo state than the monomers. Carotenoids are integral components of all 6 complexes; it is likely that all of the photosynthetic pigments of higher plants are complexed to protein.

2. Materials and methods

Spinach plants were grown in water culture [11] and normal barley and a chl *b*-deficient barley mutant [12,13] were grown in vermiculite. Chloroplasts were isolated [10,11] and thylakoid membranes washed as in [10]. Washed membranes were resuspended in 50 mM tricine buffer (pH 8.0) (2–4 mg chl/ml) and either used immediately or stored in liquid N_2. Total chl and chl *a*/*b* ratios were determined in 80% acetone [13].

Discontinuous tube SDS–PAGE was performed using a modification of the procedure in [14]. The acrylamide to *N,N'*-methylenebisacrylamide ratio was 30:0.8 and the acrylamide was 8.0% in the resolving and 4.0% in the stacking gel with 0.1% SDS (BDH), 0.0005% (v/v) *N,N,N',N'*-tetramethylenediamine and 0.1% ammonium persulphate and the gel and reservoir buffers [14] where the gel stacks at pH 8.64 and runs at pH 9.5. Analytical gels (0.7 × 7 cm) had 2.0 ml resolving gel and 0.3 ml stacking gel and preparative gels (1.3 × 8 cm) had 8.0 ml resolving gel and 1.0 ml stacking gel.

Chloroplast membranes were solubilized at 4°C,

without prior lipid extraction, in 0.3 M Tris–HCl (pH 8.8) containing 10% glycerol and 1% SDS to give a final SDS/chl weight ratio 10/1 and chl conc. 1 mg chl/ml. In some cases, Triton X-100 was also included in the solubilization buffer at a Triton X-100 : SDS : chl ratio of 20:10:1. Solubilized membrane extracts were applied immediately without centrifugation to gels at 4°C. Extract, 10–20 μl, was run at 3 mA/analytical gel, and 150–200 μl extract was run at 12 mA/preparative gel, for 30–60 min at 4°C. Gels were stained with Coomassie blue as in [10].

The distribution of chlorophyll was determined by scanning unstained gels in situ at 675 nm and 650 nm on a Varian 635 spectrophotometer with a gel-scanning attachment [7]. Chl *a*/chl *b* ratios were determined by extraction of gel segments according to [8]. High chl *a*/chl *b* ratios were determined by fluorescence emission spectroscopy of ethanol-extracted gel slices [15]. Absorption spectra of gel segments were recorded directly on a Cary model 14R spectrophotometer using the scattered transmission attachment. Fluorescence emission and excitation spectra were recorded as in [16] with gel segments which had been vacuum-infiltrated with glycerol : 50 mM tricine buffer (pH 8.0) (66:33) for 1 h.

3. Results

When spinach thylakoids were solubilized at 4°C at a SDS/chl ratio 10/1, and subjected to discontinuous SDS–PAGE at 4°C, 7 chlorophyll zones were resolved, 6 of which stained for protein (fig.1a). The resolved unstained zones in order of increasing mobility were designated CP1a and CP1 (both apple-green), LHCP[1] and LHCP[2] (yellow-green), CPa (apple-green), LHCP[3] (yellow-green) and a faint green-yellow zone corresponding to free chlorophyll and carotenoids complexed to SDS. CP1 and LHCP[3] correspond to the main complexes seen in earlier separations [1,3], and the other 4 complexes have been designated CP1a, LHCP[1], LHCP[2] and CPa on account of their

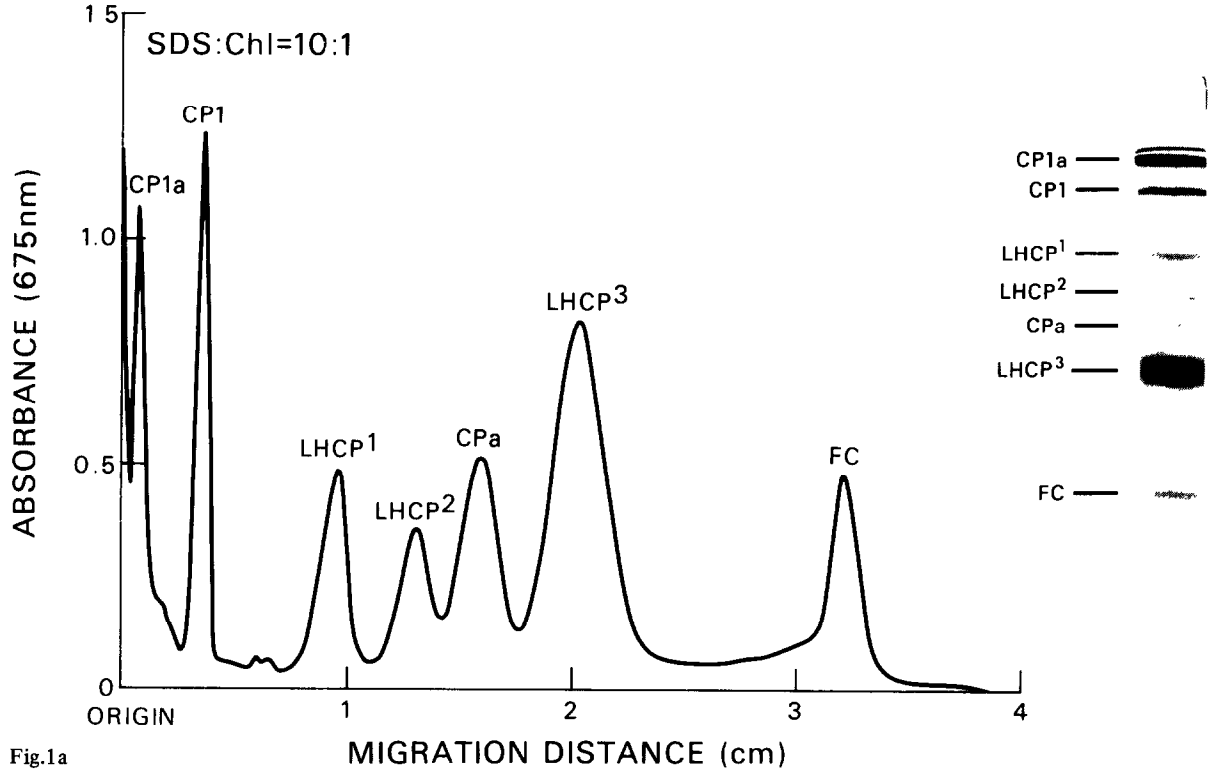


Fig.1a

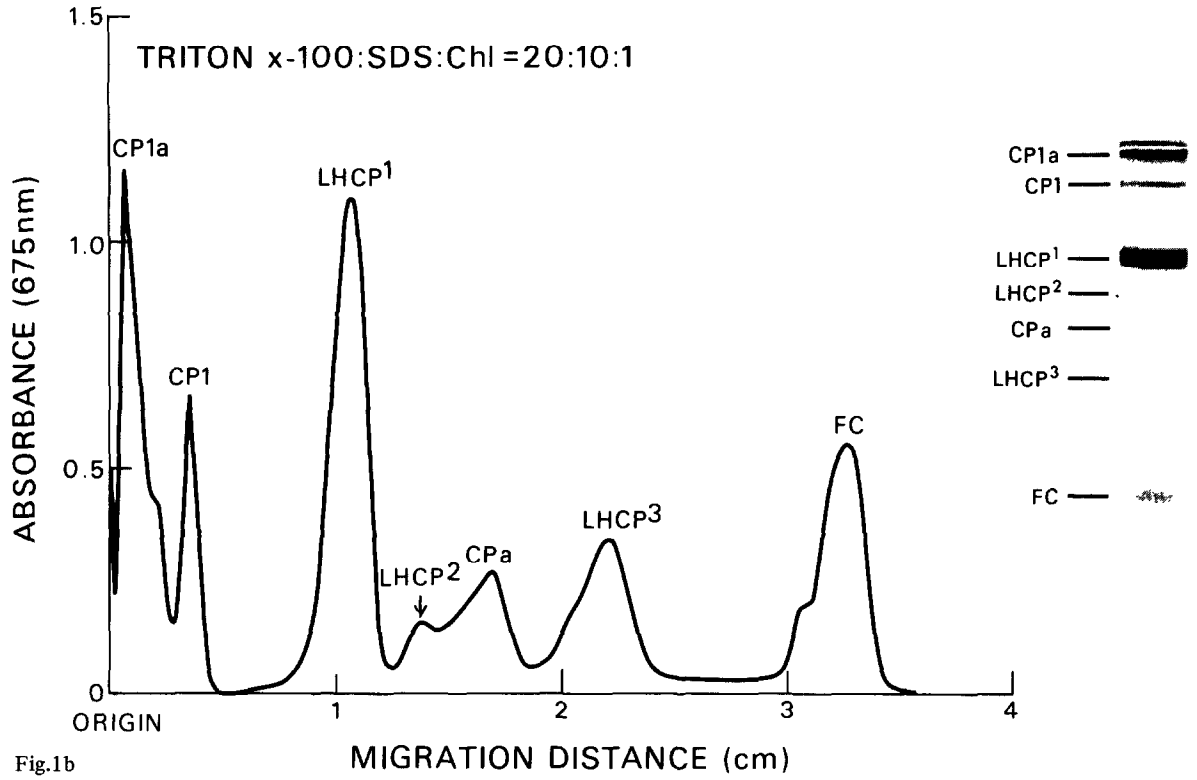


Fig.1b

Fig.1. Distribution of chlorophyll in chlorophyll–protein complexes resolved by discontinuous SDS–PAGE after solubilization of spinach thylakoids with (a) SDS in the ratio (w/w) SDS: chl = 10 : 1; (b) Triton X-100 plus SDS in the ratio Triton X-100 : SDS : chl = 20 : 10 : 1.

spectral properties described below. Some 22–28% of the total chlorophyll was associated with CP1a and CP1, 52–58% with LHCP[1], LHCP[2] and LHCP[3], 10% with CPa and only 10% in the free chl zone (table 1). With earlier methods [1,3], 10–15% of the total chlorophyll was associated with CP1, 55–60% with LHCP and 30% was with the free chlorophyll zone. Although the addition of Triton X-100 to the solubilization buffer, in the ratio, Triton X-100 : SDS : chl of 20:10:1, resulted in an increase in free chloro-

Table 1
Distribution of chlorophyll in chlorophyll–protein complexes of spinach and barley thylakoids

Thylakoids	Solubilization[a]	% Chlorophyll in complexes						
		CP1a	CP1	LHCP[1]	LHCP[2]	CPa	LHCP[3]	FC
Spinach	SDS	14	11	12	10	10	31	11
Spinach	TNX + SDS	12	7	34	3	7	15	22
Barley	SDS	6	17	9	7	8	37	16
Barley	TNX + SDS	7	16	23	4	5	20	25
Barley mutant	SDS		28			25		47
Barley mutant	TNX + SDS		27			19		54

[a] SDS is SDS/chl = 10:1; TNX + SDS is Triton X-100 : SDS:chl = 20:10:1

phyll, there was a significant increase in the amount of chlorophyll associated with LHCP[1], and a concomitant decrease in LHCP[3] (fig.1b). The stabilizing effect of Triton X-100 on LHCP[1] was seen in [10].

For comparison thylakoids from normal barley and a chlorophyll *b*-deficient barley mutant [12,13] which lacks LHCP[3] [17] and LHCP[1] [10] were also electrophoresed on discontinuous tube SDS–PAGE. Normal barley had the same complexes as were resolved with spinach, whereas the barley mutant lacked CP1a, LHCP[1], LHCP[2], and LHCP[3] and had increased free chlorophyll compared to barley or spinach. However CPa was present in the barley mutant and a significant amount of the total chlorophyll was associated with it (table 1).

We found that variations in the solubilization procedure for spinach thylakoids, such as 25°C instead of 4°C, higher SDS/chl ratios and increased time of solubilization caused a reduction in the amounts of CP1a, LHCP[1], LHCP[2] and CPa, while CP1, LHCP[3] and free chlorophyll increased. Similar effects were observed by variations in electrophoresis which included increased running times, higher currents and 25°C instead of 4°C. The most labile complex was LHCP[2], followed by CPa, then CP1a and finally LHCP[1], which was clearly resolved before [6,8,10].

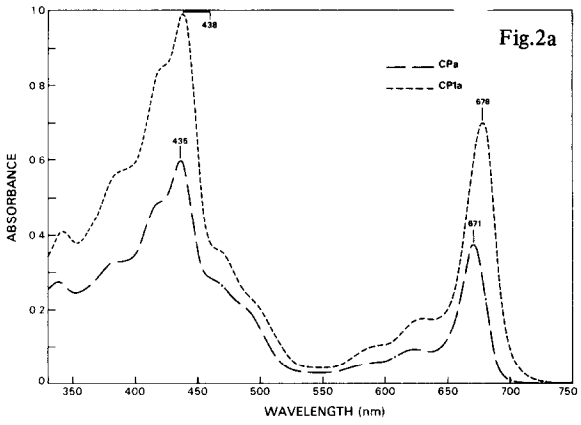
3.1. *Absorption spectra of gel segments in situ and chl* a/b *ratios*

The absorption spectrum of a gel segment containing CP1a was similar to that of CP1, both having red maxima at 678 nm and peaks in the blue at 438 nm (fig.2a). Clearly these spectra resemble that of the P700-chlorophyll *a*–protein complex [1,3]. LHCP[1] and LHCP[2] have very similar absorption spectra to that of LHCP[3] described earlier [3,17]. The high amount of chl *b* relative to chl *a* is seen in the enhanced absorption at 652 nm and 470 nm (fig.2b). In contrast, CPa with an absorption maximum in the red at 671 nm has much less chl *b* and resembles the spectrum described for complex IV [9].

Multiple determinations of the chl *a*/chl *b* ratios of freshly eluted gel segments from SDS–PAGE of spinach thylakoids (table 2), show that CP1a and CP1 have very little and no chl *b*, respectively. LHCP[1] has nearly equal amounts of chl *a* and chl *b*, LHCP[2] a slighly higher chl *a*/chl *b* ratio, while that of LHCP[3] is 1.28. The amount of chl *b* in CPa is lower and more variable. The free chlorophyll zone contained chl *a* and carotenoids only. The ratios obtained with complexes resolved after solubilization of the membranes with Triton X-100 and SDS were similar to those of table 2.

3.2. *Fluorescence emission and excitation spectra of complex in situ at 77°K*

Fluorescence spectroscopy at 77°K provides information on the nature of the various chlorophylls and the energy transfer between absorbing and fluorescing molecules. Intact chloroplasts have three main



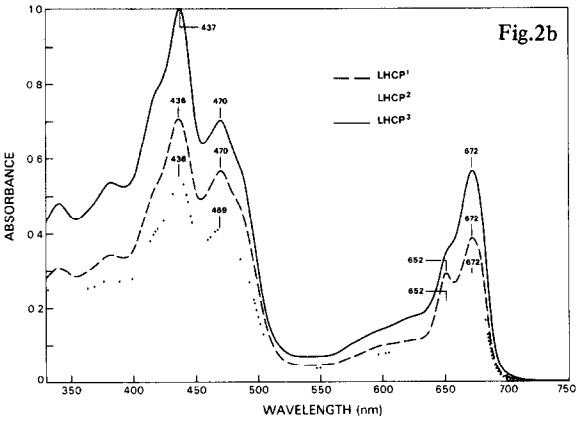


Fig.2. Absorption spectra of gel segments in situ of chlorophyll–protein complexes resolved by SDS–PAGE after solubilization of spinach thylakoids with SDS in the ratio (w/w) SDS : chl = 10 : 1. (a) CP1a and CPa; (b) LHCP[1], LHCP[2] and LHCP[3].

Table 2
Chlorophyll *a*/chlorophyll *b* ratios of chlorophyll–protein complexes from spinach thylakoids

Complexes	CP1a	CP1	LHCP[1]	LHCP[2]	CPa	LHCP[3]	FC
Chl *a*/chl *b*[a]	> 20	chl *a*	1.09 ± 0.06	1.14 ± 0.10	3.66 ± 1.42	1.28 ± 0.04	chl *a*

[a] Twice standard error

emission bands at 77°K; those at 685 nm and 695 nm being mainly associated with PS 2 and that at 735 nm being mainly associated with PS 1 [4]. Fluorescence spectra were obtained in situ on vacuum-infiltrated gel slices as we found that electrophoretic elution of the complexes from the gel segments caused partial dissociation of some complexes, and the free chlorophyll distorted the fluorescence spectra. Both CP1a and CP1 have very low fluorescence yields with CP1a having a main emission peak at 730 nm and a minor peak at 680 nm, while CP1 has a slightly lower peak at 723 nm with no distinct 680 nm peak (fig.3a). The excitation spectra of CP1a and CP1 have similar peaks but the relative heights differ; the ratio of E_{678}/E_{708} is 1.7 for CP1a and 3.4 for CP1.

The fluorescence emission spectra of LHCP[1], LHCP[2] and LHCP[3] are very similar, all having maxima at 681 nm and very small bands at 735 nm. The excitation spectra also resemble one another and clearly show the contribution of both chl *b* and carotenoids to the chl *a* fluorescence emission (fig.3b). The fluorescence emission spectrum of CPa is not unlike that of the three LHCPs, while the excitation spectrum shows lesser contributions from chl *b* and carotenoids. All of these complexes (fig.3b) have much higher fluorescence yields than those of CP1a and CP1 and there was no separate emission of chl *b* at 650 nm showing that chl *b* has not undergone structural disorganization. No trace of the 695 nm emission, thought to come from the antenna chlorophyll *a* of the PS 2 reaction centre [18] was seen with any of the resolved complexes.

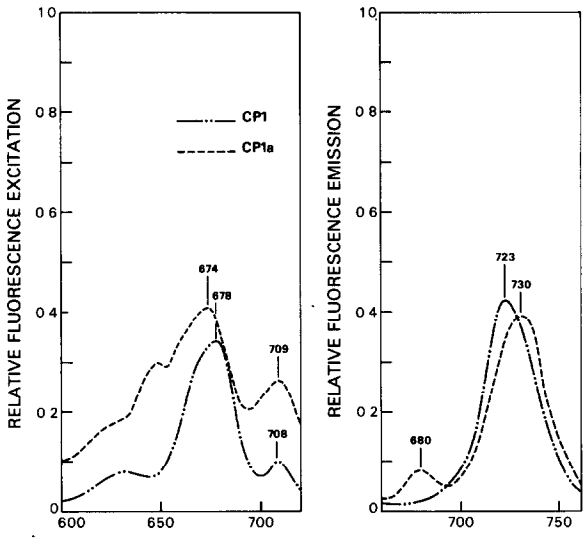


Fig.3a

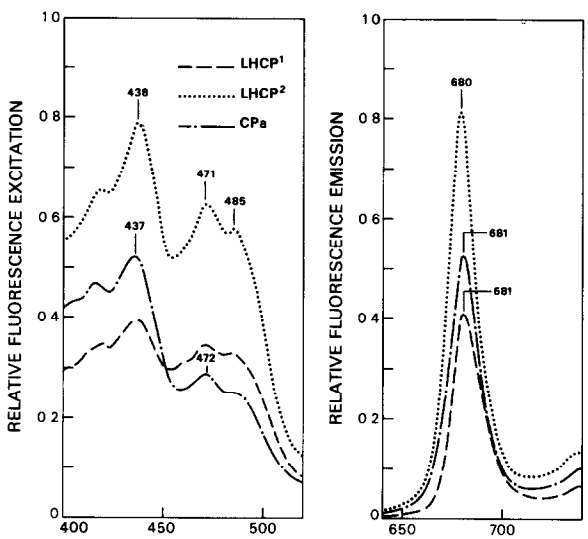


Fig.3b

Fig.3. Fluorescence emission and excitation spectra of spinach chlorophyll–protein complexes on SDS–PAGE gel slices at 77°K. (a) CP1a and CP1; (b) LHCP[1], LHCP[2], and CPa. The excitation wavelength for fluorescence emission was 436 nm and the emission wavelength for fluorescence excitation was 730 nm for (a) and 680 nm for (b).

4. Discussion

The chlorophyll–protein complexes resolved here account for nearly 90% of the total chlorophyll of spinach and barley thylakoids. We have characterized the minor complexes which were either unstable or present in trace amounts in several methods, which gave 30–50% [5–8] or at best 20% [9,10] free chlorophyll. As suggested [5] several of these are oligomers of the two main complexes, CP1 and LHCP. The absorption characteristics of CP1a and CP1 are very similar. Both complexes show marked fluorescence emission in the far-red at 77°K with the maximum for CP1a at 730 nm, being closer to the in vivo peak at 735 nm for PS 1, than that for CP1 at 723 nm (fig.3a). Previously SDS–CP1 complexes had marked fluorescence emission at 680 nm at 77°K [4]. The Triton X-100 P700-chl *a*–protein complex prepared by hydroxylapatite chromatography also has a very different emission spectrum with main peaks at 683 nm and 695 nm and lower shoulders in the far-red region [19]. Thus CP1a and CP1 for the first time have marked far-red fluorescence emission similar to that of the PS 1 digitonin particles which had a main peak at 735 nm and only a minor peak at 683 nm [20].

The spectral properties of $LHCP^1$ and $LHCP^2$ are similar to those of $LHCP^3$ [1,3] and they appear to be oligomeric forms of LHCP. The addition of Triton X-100 to the SDS solubilization buffer results in an increase in $LHCP^1$ and a concomitant decrease in $LHCP^3$. The stabilization of $LHCP^1$ may result by Triton X-100 preferentially replacing the boundary lipid and thereby affording protection against SDS [10]. Previously we termed $LHCP^1$ a dimer due to its apparent molecular weight [10]; however, with the isolation here of three LHCPs, it is more likely to be a trimer. The apparent molecular weights of all of the chlorophyll–protein complexes are anomalous and dependent on polyacrylamide gel concentration as found for CP1 [21,22] and LHCP [22]. Currently, we are characterizing the polypeptides of the complexes under both dissociating and non-dissociating conditions, using two dimensional SDS–PAGE. Although LHCP is said to have equimolar amounts of chl *a* and chl *b* [1–4], there are reports of higher ratios [9,10]. $LHCP^1$ has a low chl *a*/chl *b* ratio of 1.09 while that of $LHCP^3$ at 1.28 is higher. Curiously, on tube SDS–PAGE [10] we found a ratio of 1.24 for barley $LHCP^1$. Variability in the chl *a*/chl *b* ratios may depend on the SDS–PAGE method used.

A distinct chlorophyll *a*–protein complex, CPa, was also resolved which has a higher, variable chl *a*/chl *b* ratio, and resembles complex IV [9] which was enriched in PS 2 particles. CPa accounts for 25% of the total chlorophyll in the chl *b*-deficient barley mutant. The chl *b* present in variable amounts in barley and spinach CPa is probably due to partial dissociation of $LHCP^1$ and the very unstable $LHCP^2$. Since CPa is present in PS2 particles and barley mutant, it may be the reaction centre complex of PS 2 and be analogous to CP1 of PS 1. However, if so, CPa must be partly dissociated, as it has no trace of 695 nm fluorescence emission thought to come from the antenna chl *a* of PS 2 [18].

The oligomers of both CP1 and LHCP observed here may have structural significance as they more closely resemble the in vivo state than the monomers formed exclusively in the earlier gel methods [1–4]. Significantly, we find that all conditions, such as higher SDS/chl ratios, higher temperature and longer times for solubilization and PAGE, causing partial or complete dissociation of the oligomeric complexes, give increasing amounts of monomers. Further, no conditions have been found where either LHCP or CP1 reform oligomeric complexes. We believe that the harsher conditions of earlier methods led to the resolution of monomers because they favoured disruption of the quaternary structures of higher molecular weight aggregates. It is significant that CP1a has its fluorescence emission peak further to the far-red than CP1.

In contrast to photosynthetic bacteria, little is known about the organization of carotenoids in higher plants. Our method shows that most of the carotenoids are also complexed to protein. Fluorescence excitation spectra of all 6 complexes demonstrate that carotenoid(s) contribute to the chlorophyll *a* fluorescence emission, showing that carotenoids are integral components of each complex. We anticipate that all of the photosynthetic pigments of higher plants are complexed to protein in vivo.

References

[1] Thornber, J. P. (1975) Annu. Rev. Plant Physiol. 26, 127–158.

[2] Anderson, J. M. (1975) Biochim. Biophys. Acta 416, 191–235.
[3] Thornber, J. P., Alberte, R. S., Hunter, F. A., Shiozawa, J. A. and Kan, K-S. (1977) Brookhaven Symp. Biol. 28, 132–148.
[4] Boardman, N. K., Anderson, J. M. and Goodchild, D. J. (1978) in: Current Topics in Bioenergetics (Sanadi, D. R. and Vernon, L. P. eds) vol. 8, in press.
[5] Herrmann, F. and Meister, A. (1972) Photosynthetica 6, 177–182.
[6] Hiller, R. G., Genge, S. and Pilger, D. (1974) Plant Sci. Lett. 2, 239–242.
[7] Genge, S., Pilger, D. and Hiller, R. G. (1974) Biochim. Biophys. Acta 347, 22–30.
[8] Remy, R., Hoarau, J. and Leclerc, J. C. (1977) Photochem. Photobiol. 26 151–158.
[9] Hayden, D. B. and Hopkins, W. G. (1977) Can. J. Bot. 55, 2525–2529.
[10] Dunkley, P. R. and Anderson, J. M. (1978) Biochim. Biophys. Acta, in press.
[11] Anderson, J. M. and Boardman, N. K. (1966) Biochim. Biophys. Acta 112, 403–421.
[12] Highkin, H. R. (1950) Plant Physiol. 25, 294–306.
[13] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[14] Neville, D. M. (1971) J. Biol. Chem. 246, 6328–6334.
[15] Boardman, N. K. and Thorne, S. W. (1971) Biochim. Biophys. Acta 253, 222–231.
[16] Boardman, N. K. and Thorne, S. W. (1968) Biochim. Biophys. Acta 153, 448–458.
[17] Thornber, J. P. and Highkin, H. R. (1974) Eur. J. Biochem. 41, 109–116.
[18] Butler, W. L. and Kitajima, M. (1975) Biochim. Biophys. Acta 396, 72–85.
[19] Brown, J. S. (1977) Photochem. Photobiol. 26, 519–525.
[20] Boardman, N. K., Thorne, S. W. and Anderson, J. M. (1966) Proc. Natl. Acad. Sci. USA 56, 586–593.
[21] Chua, N. H., Matlin, K. and Bennoun, P. (1975) J. Cell Biol. 67, 361–377.
[22] Bar-Nun, S., Schantz, R. and Ohad, I. (1977) Biochim. Biophys. Acta 459, 451–467.