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THE RECONSTITUTION OF A PHOTOSYSTEM II PROTEIN COMPLEX, *P*-700-CHLOROPHYLL *a*-PROTEIN COMPLEX AND LIGHT-HARVESTING CHLOROPHYLL *a*/*b*-PROTEIN

A.W.D. LARKUM * and JAN M. ANDERSON

CSIRO, Division of Plant Industry, P.O. Box 1600, Canberra, ACT 2601 (Australia)

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A Photosystem II reaction centre protein complex was extracted from spinach chloroplasts using digitonin. This complex showed (i) high rates of dichloroindophenol and ferricyanide reduction in the presence of suitable donors, (ii) low-temperature fluorescence at 685 nm with a variable shoulder at 695 nm which increased as the complex aggregated due to depletion of digitonin and (iii) four major polypeptides of 47, 39, 31 and 6 kDa on dissociating polyacrylamide gels. The Photosystem II protein complex, together woth the *P*-700-chlorophyll *a* protein complex and light-harvesting chlorophyll *a*/*b*-protein complex (LHCP) also isolated using digitonin, were reconstituted with lipids from spinach chloroplasts to form proteoliposomes. The low-temperature (77 K) fluorescence properties of the various proteoliposomes were analysed. The F_{685}/F_{695} ratios of the Photosystem II reaction centre protein complex-liposomes decreased as the lipid to protein ratios were increased. The F_{681}/F_{697} ratios of LHCP-liposomes were found to behave similarly. Light excitation of chlorophyll *b* at 475 nm stimulated emission from both the Photosystem II protein complex (F_{685} and F_{695}) and the *P*-700-chlorophyll *a*-protein complex (F_{735}) when LHCP was reconstituted with either of these complexes, demonstrating energy transfer between LHCP and PS I or II complexes in liposomes. No evidence was found for energy transfer from the PS II complex to the *P*-700-chlorophyll *a*-protein complex reconstituted in the same proteoliposome preparation. Proteoliposome preparations containing all three chlorophyll-protein complexes showed fluorescence emission at 685, 700 and 735 nm.

Introduction

The conversion of solar energy into stored chemical energy by photosynthetic membranes depends critically on the molecular organization of the pigments and associated molecules in the membrane [1]. It is established that all the chlorophyll and carotenoids in the thylakoids of higher plants are non-covalently bound to polypeptides associated with intrinsic supramolecular complexes [2,3]. Three major Chl-protein complexes have been found: the *P*-700-Chl *a*-protein complex (PS I complex) which is part of the PS I assembly, the light-harvesting Chl *a*/*b*-protein complex (LHCP), which functions as a light-harvesting assembly principally for PS II, and a third Chl *a*-protein complex which is part of the PS II assembly [2,3] and which is hereafter referred to as the PS II complex.

Although the use of mutants and growth conditions has allowed some insight into the interactions

* Permanent address: School of Biological Sciences, Sydney University, Sydney, NSW 2006, Australia.

Abbreviations: CF_1, chloroplast coupling factor; Chl, chlorophyll; CPa, band from SDS-polyacrylamide gel electrophoresis with PS II characteristics; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; E_{436} (etc.), fluorescence excited at 436 nm; F_{685} (etc.), fluorescence with a maximum emission at 685 nm; LHCP, light-harvesting chlorophyll *a*/*b*-protein complex; PS, Photosystem; SDS, sodium dodecyl sulphate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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of the three major Chl-protein complexes [4–9] many features remain to be explained. Further, if there is an extreme lateral heterogeneity in the distribution of complexes along the plane of thylakoids in grana-containing chloroplasts, with PS I complex being located only in stroma-exposed thylakoids [10], we might expect that the reaction centre complexes do not interact. Clearly it is advantagenous to isolate each of these complexes in a purified, functional state in order to study their interactions in vitro. Since these complexes are intrinsic membrane proteins a useful procedure is to reconstitute purified complexes into artificial lipid membranes. Such an approach has recently led to advances with the *P*-700-Chl *a*-protein complex [6,11,12] and LHCP [13–15], but has not previously been attempted with the PS II complex, mainly because extraction procedures for this complex are difficult and the yields are low.

Although Triton X-100 has been used to isolate PS II fractions [16,17] digitonin extraction produces complexes with characteristics closer to the known in vivo properties [18,19] and particles with the reaction centre of PS II have been isolated from spinach chloroplasts. Some improvement to the procedure of Wessels [18], including isoelectric focusing, has recently been made by Satoh [19,20]. An independent study, also using long incubation in digitonin solutions, has been successful in obtaining a PS II complex from a blue-green alga [21].

In the present study we have adapted the procedure of Satoh [19,20] to obtain a PS II complex and have reconstituted this complex into liposomes prepared from the natural diacyl lipids obtained from spinach chloroplasts. In addition, we have made use of LHCP and the PS I complex which are byproducts of the PS II preparative procedure, to investigate the interaction of all three Chl-protein complexes in a reconstituted system.

Materials and Methods

Isolation of the Photosystem II complex

The PS II complex was isolated by the procedure of Satoh and Butler [19] with minor modifications. Chloroplasts were prepared from spinach (*Spinacia oleracea*) grown under water-culture [22]. Leaves (80 g) were blended in a Waring blender for 15 s in 200 ml isolation medium comprising 0.4 M Sorbitol/0.1 M Tricine (pH 7.8)/5 mM $MgCl_2$/10 mM NaCl. A chloroplast pellet was obtained by centrifugation at $3000 \times g$. Where chloroplasts deficient in CF_1 were prepared, the chloroplast pellet was washed with 0.75 mM EDTA (pH 8.0) according to McCarty [23] and the procedure was repeated twice. Normal or CF_1 depleted chloroplasts were resuspended at about 4 mg Chl/ml in 50 mM Tris-HCl (pH 7.8)/5 mM $MgCl_2$ and digitonin added (in the same medium) to give a final concentration of 0.5% digitonin (British Drug Houses, Analar grade) with a digitonin : Chl ratio of 6 (w/w). Digitonin treatment with 0.5% digitonin and then 1.6% digitonin followed by sucrose gradient centrifugation and enrichment for PSII activity (DPC → DCIP) on DEAE-cellulose (Whatman DE-52) columns was carried out essentially as described by Satoh [19, 20,24]. The PS II fraction from the column was concentrated and washed free of salts in an Amicon 50 ultrafiltration cell using a PM 10 membrane and an aqueous solution of 0.1% digitonin. The final suspension was concentrated in an Amicon 12 cell (approx. 0.2 ml) and 3 ml 5% ampholine (pH 4–6) solution (0.1% digitonin) was added. This suspension was added to gel taken from a prepared flat-bed isoelectric focusing gel (LKB preparative electrofocusing apparatus). The gel was prepared with 4 g LKB Ultrodex, and 100 ml 2% ampholine (pH 4–6) plus 0.1% digitonin and was dried to the recommended level (31–39% weight loss). The PS II fraction was added near the negative end of the slab gel and run for 14 h at 4°C in darkness. The developed gel was viewed under ultraviolet light which clearly indicated any LHCP which normally appeared as a distinct band with some tailing at p*I* 4.18, whereas PS II activity was associated with a clear green band at p*I* 4.46. A few very faint bands were occasionally present at higher p*I* levels, and the isolated PS I complex had a p*I* of 4.60.

The PS II band was eluted in an LKB elution column with distilled water and concentrated in an Amicon 12 cell (PM 10 membrane) taken to minimal volume and washed with 60–100 ml 50 mM Tris-HCl (pH 7.2) until a green precipitate formed in the medium and on the membrane. The precipitate was transferred to a small glass homogeniser (2 ml) and homogenised in 0.5–2 ml of the same medium. It was then sonicated (Branson sonicator, microtip) at 4°C 60 s prior to use. A similar sonication procedure was

used for PS I complex and LHCP. The complexes were stored at −20°C if necessary.

Preparation of chloroplast diacyl lipids

The main diacyl lipids were extracted from spinach chloroplasts according to Ryrie et al. [15]; the diacyl lipids were recovered from Kiesel gel columns and purified by thin-layer chromatography [15]. A mixture of the purified four diacyl lipids in the proportions found in chloroplast thylakoids (diacylgalactosyl glycerol : diacyldigalactosyl glycerol : diacylsulphoquinovosylglycerol : diacylglycerol phosphate of 53 : 26 : 15 : 6) was stored at 8 mg lipid/ml in chloroform containing 0.5 μg/ml butylated hydroxytoluene at −15°C under N_2.

Preparation of proteoliposomes containing the PS II preparation

Liposomes were prepared by first evaporating the diacyl lipid mixture to dryness under N_2, and dispersing the mixture at 8 mg lipid/ml in 5 mM Tricine buffer (pH 7.5)/50 mM sucrose by sonication (Branson sonicator, microtip) for 2–4 min under N_2 in a tube placed in an ice bucket. Similarly, the PS II fraction was routinely sonicated for 60 s before use. Proteoliposomes were prepared by adding the liposomes and the PS II fractions to give a lipid to protein ratio ranging from 1 to 17. The mixture was frozen in liquid N_2 for 60 s, thawed by agitation in water at 25°C and sonicated for 60 s at 4°C.

The mixture of proteoliposomes and unreconstituted components (1 ml) was placed on a 10–30% linear Ficoll 400 gradient (containing also 5 mM Tricine (pH 7.5) and 50 mM sucrose) and centrifuged in a Beckman SW 41 rotor at 34 500 rev./min (150 000 × *g*) for 14–17 h at 4°C.

Spectral analyses

Absorption spectra were measured at 25°C or 77 K (in the presence of 66% glycerol) on a Cary Model 14 or a Perkin Elmer Model 557 spectrophotometer. Fluorescence spectra were recorded at 77 K in 66% glycerol with a Perkin Elmer model MPF-44B spectrofluorometer calibrated in the red region with a calibrated tungsten lamp and corrected with a 'corrected spectra' unit (Perkin Elmer, DCSU-2).

SDS polyacrylamide gel electrophoresis

The PS II fraction was solubilized at 4°C with 0.3 M Tris-HCl (pH 8.8)/10% glycerol/0.375% SDS and a SDS/Chl weight ratio of 7.5 and discontinuous polyacrylamide gel electrophoresis run at 4°C in 10% polyacrylamide as described previously [22]. Concentration gradient slab gels based on O'Farrell's method were performed as before [22]. Standards and fractions were solubilized in an equal volume of Laemmli's solubilization buffer [25] containing 5% (v/v) mercaptoethanol. For strong dissociating conditions samples were heated at 75°C for 10 min and left for 1 h at 25°C prior to electrophoresis. Apparent molecular weights were obtained by comparison with known standards.

Photochemical assays

PS II activity was measured by the rate of dichloroindophenol (DCIP) reduction at 590 nm in a Cary model 14 spectrophotometer fitted with side-illumination of saturating red light on one cell. Samples (2–5 μg Chl) were suspended in 1.5 ml Tris-HCl (pH 7.2)/1 mM diphenylcarbazide (DPC)/33 μM DCIP/3% ethanol. The rate of reduction of 1 mM ferricyanide at 420 nm was measured similarly, using 1 mM diphenylsemicarbazide [21].

Chl concentrations and Chl *a*/Chl *b* ratios were determined in 80% acetone [26] or 99% ethanol [27], and protein was determined by the Lowry method [28].

Results

Characterization of the PS II reaction centre complex

The flat-bed isoelectric focusing step introduced in the current procedure resolved the PS II complex very well from some LHCP remaining even after the two DEAE-cellulose column separations according to Satoh and Butler [19]. Nevertheless, it was found necessary to retain the procedure of the two column treatments, since without this step prior to isoelectric focusing traces of LHCP were associated with the PS II band on the isoelectric focusing gel.

The PS II complex was found to have a DCIP reducing activity of between 170 and 311 μmol DCIP/mg Chl per h using DPC as electron donor. Some enrichment of activity was found compared to the activity of the 1.6% digitonin extract or the fraction passed through only one DEAE-cellulose column prior to isoelectric focusing (Table I). A higher rate

was found with ferricyanide reduction using diphenylsemicabazide as an electron donor (Table I) as found previously [21], although in terms of reducing equivalents the activity was lower than with DCIP. Inhibition by DCMU was relatively poor at low concentrations; higher concentrations caused greater inhibition (Table I).

The absorption spectra of the PS II complex at 77 K and 300 K were similar to those previously described [18,19,21]. The low temperature spectrum showed enhanced absorption at 672 nm as compared to the room temperature spectrum. β-Carotene and trace quantities of lutein were detected in the complex and these, together with small amounts of LHCP (Chl *b*, estimated according to Ref. 27, was less than one-twentieth the amount of Chl *a*), gave rise to minor bands at 465 nm and 490 nm.

The fluorescence emission spectrum of PS II complex at 77 K has a characteristic peak at 685 nm, accompanied by a secondary peak or shoulder of variable intensity at 695–696 nm (Fig. 1B). Indeed, in one preparation (out of six) the 695–696 nm band was equal in magnitude to the 685 nm band. The 695–696 nm emission band appeared to be enhanced by aggregation of the PS II complex. For instance, the band was somewhat reduced immediately upon elution from the isoelectric focusing gel, and it became more marked after washing with 5 mM Tris-HCl in an ultrafiltration cell. After extensive washing to reduce as much digitonin as possible, aggregation of the protein complex into visible particles occurred. (It should be noted that the complex was dispersed by sonication prior to fluorescence or other measurements.) The fluorescence excitation spectrum of the PS II complex (Fig. 1A) showed a major peak at 438 nm and a secondary peak at 418 nm (E_{418}/E_{438} of 0.85) with minor bands at 465 and 495 nm. The latter peaks may be ascribed to β-carotene mainly, although the small amount of Chl *b* (see above) may have contributed partially to the 465 nm band.

The Chl-protein complex content of the PS II fraction was determined by mild SDS-polyacrylamide gel electrophoresis [22]. Apart from some free chlorophyll at the gel front, only one green band was resolved (Fig. 2A2) which migrated with the same mobility as that of CPa, the PS II complex resolved from spinach thylakoids (Fig. 2A1). Since CPa is the most labile Chl-protein complex with this gel method, and CP1a, CP_1 or LHCP [1–3] may be re-electrophoresed without loss of chlorophyll, we would have been able to detect the latter complexes had they been present. We conclude that the PS II complex is not contaminated with either Chl-protein complexes of PS I or LHCP; the Chl *a*/Chl *b* ratio was usually

TABLE I

PHOTOSYNTHETIC RATES OF PS II FRACTION DURING PURIFICATION

DPC, diphenylcarbazide; DPSC, diphenylsemicarbazide; FeCy, ferricyanide

Isolation step	Photosynthetic rate (μmol/mg Chl per h)	
	DPC → DCIP	DPSC → FeCy
(a) Digitonin extract	150	320
(b) Single DEAE-cellulose column treatment followed by isoelectric focusing	221	–
(c) Second DEAE-cellulose column treatment followed by isoelectric focusing	311	598
(d) Inhibition	%	
2 μM DCMU	27	27
10 μM DCMU	49	45

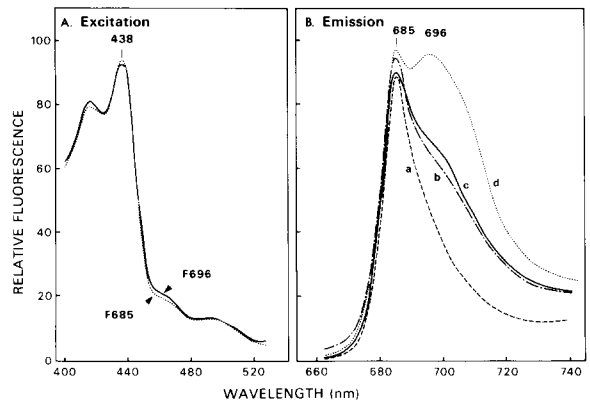

Fig. 1. Low-temperature (77 K) fluorescence excitation (A) and emission (B) spectra of PS II complex. The fluorescence emission spectra demonstrate the variable nature of F_{695} between different preparations (a, c, d) and the effect of the presence (a) and absence of digitonin (b) on the same preparation.

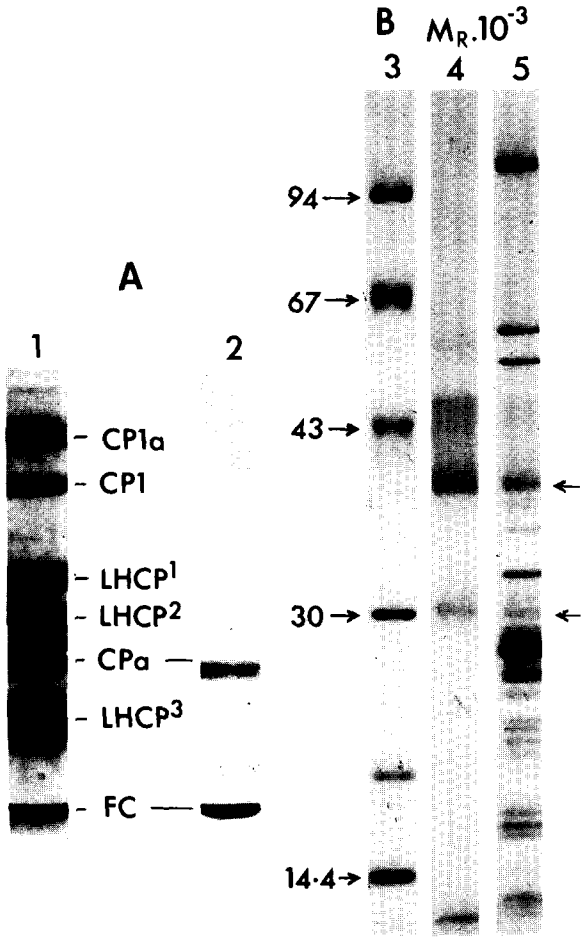

Fig. 2. SDS-polyacrylamide gels of spinach chloroplasts and the PS II complex. A. Non-dissociating conditions of spinach chloroplasts (1) and PS II complex (2). B. Polypeptide analysis of PS II complex (4) and a PS II complex from chloroplasts was washed with EDTA (5) shown alongside markers of known molecular weight (3). See Materials and Methods for details of the technique.

greater than 20 and the Chl-to-protein ratio was 0.55–0.63 (w/w).

The polypeptide compositions of the PS II fraction was broadly similar to that found for other PS II complexes [21,24,29]. The preparation from well-washed thylakoids depleted in CF_1, and four main bands, of M_r 47 000, 39 000, 31 000 and about 6000 (Fig. 2B4), under strongly reducing conditions for the solubilization of the sample prior to slab gel electrophoresis (see Methods). Under less reducing conditions, the M_r 39 000 band had an apparent molecular weight of 43 000 (not shown). If the PS II fraction was isolated from less extensively washed thylakoids, it had these four polypeptides as well as the five polypeptides of CF_1, and many other intrinsic thylakoid polypeptides of low molecular weight (Fig. 2B5). The 47 000 and 39 000 Da polypeptides are probably those carrying Chl [4,9,22,24,29], while the 31 000 Da polypeptide is a characteristic protein associated with PS II [4,9,22,29] and the low molecular weight polypeptide is probably that of cytochrome *b*-559 which is also present in this PS II fraction [18,21,29].

Reconstitution of photosystem II complex-liposomes

The PS II complex was incorporated into liposomes containing the four major, natural diacyl lipids of spinach thylakoids (diacylgalactosylglycerol : diacyldigalactosylglycerol : diacylsulphoquinovosylglycerol : diacylglycerol phosphate, 53 : 26 : 15 : 6; see Ref. 15). Proteoliposomes were easily prepared using a simple, one-step freeze-thaw reconstitution technique, and lipid-to-protein ratios between 1 and 17 were successfully used. This single-step freeze-thaw reconstitution method was previously shown to be better than cholate dialysis or direct incorporation by sonication for the isolated LHCP [15]. A Ficoll gradient technique was used to separate the proteoliposomes from liposomes and the PS II complex. The proteoliposomes formed a single discrete band on a developed gradient at a position which was related to the lipid-to-protein ratio used for their reconstitution. The lower the lipid-to-protein ratio, the lower the position on the gradient as might be expected (however, it may be noted that the PS II complex itself was found as a band near the top of the gradient). In most experiments, a portion of the Chl-protein complex and some liposomes did not reconstitute and remained in the upper part of the gradient which meant that it was not possible with the small amounts of material used to quantitate the degree of reconstitution.

The low yield of the purified PS II complex (less than 1 mg protein from 300 g spinach leaves) meant that only small amounts of proteoliposomes could be produced. As a result the present work was confined mainly to fluorescence characteristifcs at 77 K. The fluorescence characteristics of proteoliposomes reconstituted with the PS II complex are shown in Fig. 3.

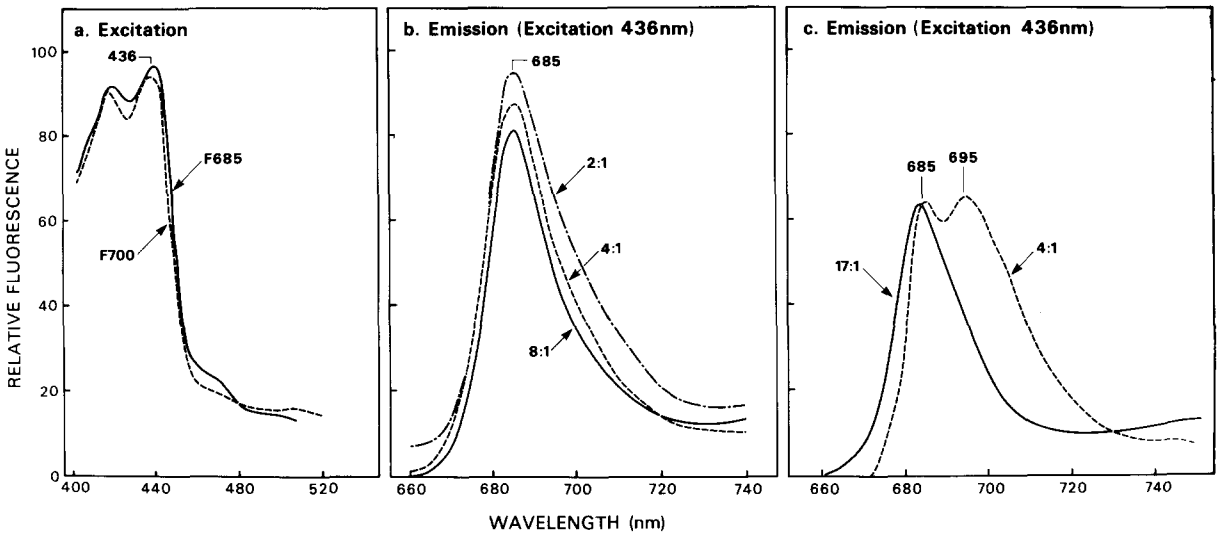

Fig. 3. Low-temperature fluorescence characteristics of proteoliposomes reconstituted with PS II complex. The fluorescence emission spectra shown are (b) for a preparation of a complex with low F_{695} at lipid-to-protein ratios of 8 : 1, 4 : 1 and 2 : 1 and (c) for a preparation of the complex with high F_{695} at lipid-to-protein ratios of 17 : 1 and 4 : 2.

The excitation spectrum of a typical preparation is similar to that of the complex in aqueous buffer, except that the minor bands at 465 nm and 495 nm are reduced (Fig. 3a). The F_{685}/F_{695} ratio varied according to the amount of lipid relative to protein used in the reconstitution. The effect of increasing the lipid-to-protein ratio was a narrowing of the F_{685} band, and there was a marked decrease in F_{695} (Fig. 3b). The reduction was particularly marked for the preparation with the large 695–696 nm band (Fig. 3c).

Reconstitution of PS II-LHCP-liposomes

The PS II complex was also reconstituted together with LHCP into liposomes. The LHCP was prepared as described by Satoh [20] (except for the details described in Materials and Methods) and had a Chl *a*/Chl *b* ratio of 1.2 (Chl-to-protein ratio, 0.38–0.43, w/w). The LHCP was depleted of digitonin in the same way as for PS II complex. Under such conditions, the LHCP exhibited a strong fluorescence emission band at 695–698 nm (F_{696}) as well as the major band with a maximum at 681 nm. F_{696} has been previously correlated with progressive aggregation of LHCP by Mg^{2+} [13,15]. When the LHCP was reconstituted alone into liposomes (Fig. 4) the 695–698 nm band was present at low lipid-to-protein ratios but it was decreased progressively in LHCP-proteoliposomes which had increasingly higher lipid-to-protein ratios.

When LHCP was reconstituted together with PS II complex, F_{681} was no longer apparent and the maximum emission was at 684–685 nm, the peak typical of PS II complex. This indicated that light energy absorbed by LHCP was being efficiently transferred to PS II complex (Fig. 5). The F_{685}/F_{695} ratios of PS

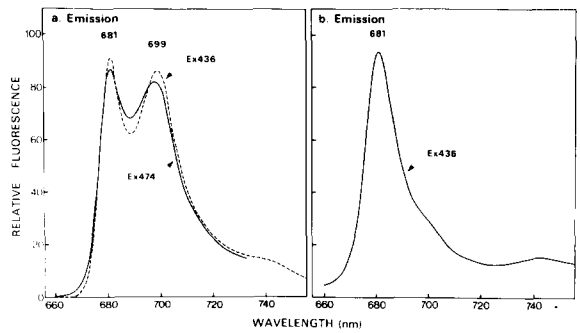

Fig. 4. Low-temperature fluorescence spectra of proteoliposomes reconstituted with LHCP at lipid-to-protein ratios of 2 : 1 (a) and 16 : 1 (b). E_{475}/E_{436} for F_{681}, (a) 0.77; (b) 0.76. Emission spectra normalised for F_{681}.

II-LHCP-proteoliposomes were also influenced by the lipid-to-protein ratio used for reconstitution, with the ratio decreasing as the lipid-to-protein ratio increased, as was found for each of the single component proteoliposomes (Figs. 3 and 4). In this particular experiment both PS II complex and LHCP preparations showed strong F_{695} or F_{696} emissions (F_{685}/F_{695} ratios: PS II, 1.1; LHCP, 1.2). Under such conditions, excitation at 470 nm (Chl *b*) gave a fluorescence emission spectrum identical to that for excitation with 436 nm. This also indicated that light energy absorbed by LHCP was being passed on to the PS II complex. However, the presence of the two peaks in the 695 nm region obscured the situation. In a further experiment, therefore, a PS II complex preparation with a low 695 nm band (F_{685}/F_{695} of 1.5) was reconstituted in the proportions of one to one with an LHCP preparation with a strong 695–698 nm band (F_{685}/F_{697} of 1.0) at a lipid-to-protein ratio of 4. Here nearly all the fluorescence emission of the PS II-LHCP-proteoliposomes was from the 684 nm band whether excitation was at 436 or at 475 nm (spectra not shown but very similar to Fig. 5d). Thus efficient energy transfer was taking place from LHCP to PS II. The presence of a strong fluorescence excitation band at 475 nm (E_{436}/E_{475}, 0.6 for F_{684}) also showed that LHCP had effectively transferred energy to Chl *a* of the PS II complex.

To test the possibility in the above experiment whether PS II complex was incorporated into one set of liposomes and LHCP into another (that is energy transfer occurred by interaction of the two types of liposome), proteoliposomes containing one or other of these complexes were formed separately and were then mixed (Fig. 6). The fluorescence emission properties of these mixtures were very different from those proteoliposomes where both complexes were incorporated together. In particular the fluorescence emission excited by Chl *b* absorption (470 nm) closely resembled the emission from LHCP alone, indicating that no transfer was occurring to the PS II

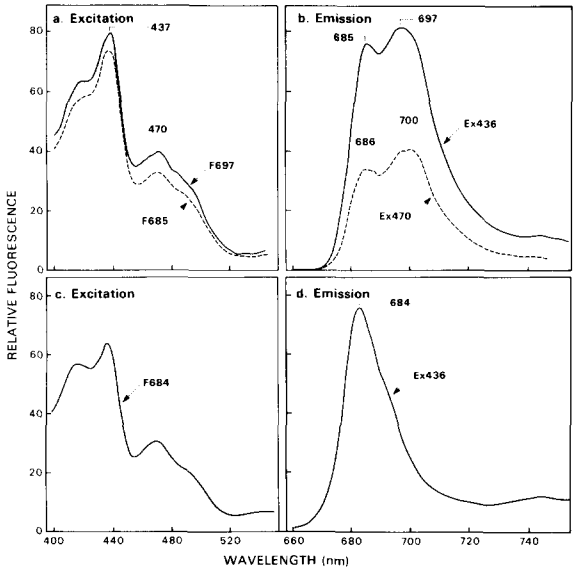

Fig. 5. Low-temperature fluorescence spectra of proteoliposomes reconstituted with LHCP and PS II complex (ratio 1 : 1 on a protein basis, at lipid-to-protein ratios of 4 : 1 (a, b) and 16 : 1 (c, d). E_{475}/E_{436} for F_{685}, (a) 0.45, (b) 0.48. Emission spectra not normalised.

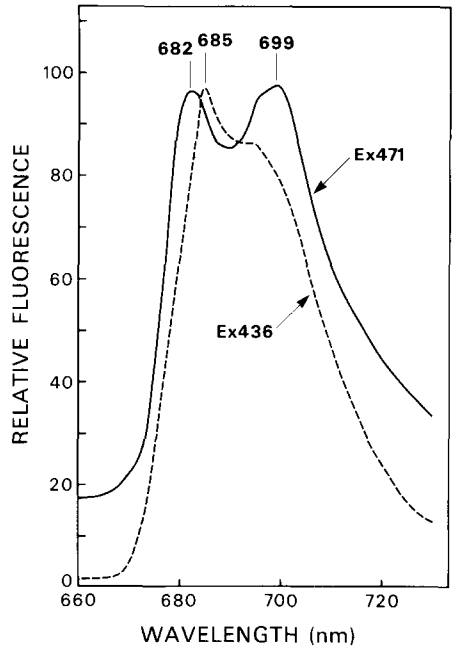

Fig. 6. Low-temperature fluorescence emission spectra of a mixture of proteoliposomes. One preparation of proteoliposomes was reconstituted with LHCP and the other preparation was reconstituted with PS II complex. The preparations were mixed (LHCP/PS II, 1 : 5 on a protein basis) after separation on a Ficoll density gradient as described in the text. The lipid-to-protein ratios were 4 : 1. E_{475}/E_{436}, 0.32 for F_{682}. Emission spectra were normalised for F_{682} or F_{685}.

complex. At relatively high ratios of PS II to LHCP (e.g., 5 : 1 for Fig. 6) the maximum emission for excitation at 436 nm was close to 685 nm, but at lower ratios the maximum was shifted to shorter wavelengths (for example, a ratio of 2 gave a maximum at 683 nm).

Reconstitution of varying mixtures of all three Chl-protein complexes

A preliminary attempt was also made to incorporate PS I complex into proteoliposomes containing either PS II or LHCP complex. The PS I complex was prepared according to Satoh [20]. This preparation contained some Chl *b* (estimated according to Ref. 27 to be about one-ninth the amount of Chl *a*). We assume that this was due to contamination by LHCP but it is interesting that it was apparently tightly bound to the PS I complex throughout the many isolation steps and was able to transfer excitation energy efficiently to PS I (low temperature fluorescence spectra of the preparation are not shown, but these were very similar to those shown in Fig. 7). This putative LHCP proved useful in that it 'labelled' the PS I complex (see below). The low-temperature fluorescence emission of the PS I complex-proteoliposomes occurred almost entirely at 735 nm, even when excited at 475 nm (Fig. 7). Thus, the putative LHCP was closely associated with the *P*-700-Chl *a*-protein complex even in proteoliposomes.

The fluorescence characteristics at 77 K of proteoliposomes in which the PS I complex was incorporated with PS II complex or LHCP complex, or both PS II plus LHCP complex are shown in Fig. 8. The PS II complex used had only a minor F_{695} emission (F_{685}/F_{695} of 1.5), whereas the LHCP complex had high F_{696} (F_{685}/F_{696} of 1.0). It should also be noted that the efficiency of incorporation of the PS I com-

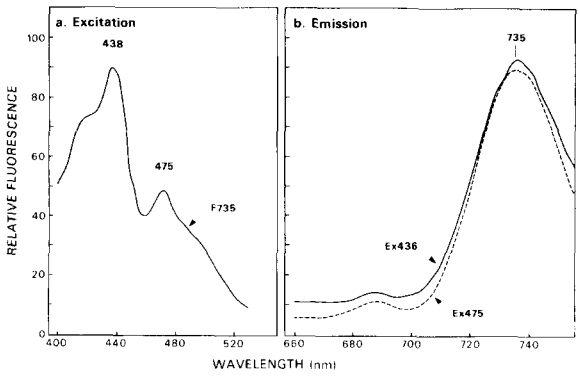

Fig. 7. Low-temperature fluorescence spectra of proteoliposomes reconstituted with *P*-700-Chl *a*-protein complex at a lipid-to-protein ratio of 4 : 1. E_{475}/E_{436}, 0.53 for F_{735}. Emission spectra were normalised for F_{735}.

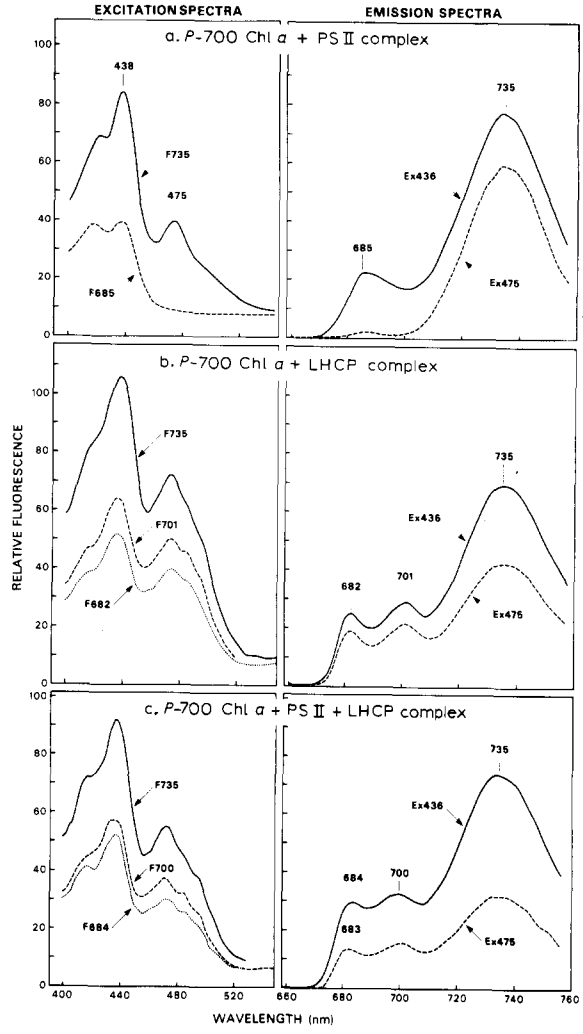

Fig. 8. Low-temperature fluorescence spectra of proteoliposomes reconstituted with (a) *P*-700-Chl *a*-protein complex plus PS II complex (ratio 1 : 1); (b) *P*-700-Chl *a*-protein complex plus LHCP (ratio 1 : 1); (c) *P*-700-Chl *a*-protein complex plus PS II complex plus LHCP (ratio 1 : 1). Lipid-to-protein ratios of 4 : 7 were used in all cases. E_{475}/E_{436}, 0.6 for F_{684} or F_{700} or F_{735}. Emission spectra were not normalised.

plex was apparently greater than that for the PS II or LHCP complex so that the proteoliposomes contained more PS I complex than either of the other two complexes (cf. Figs. 8 and 9).

Significantly, in the proteoliposomes with PS I complex and PS II complex the fluorescence emissions of the two complexes were clearly discernible (Fig. 8a). No energy transfer occurred from the PS I to the PS II complex as shown by the fluorescence emission spectrum for 475 nm exciting light and by the excitation spectra. Thus, it is possible that the two complexes were in different proteoliposomes. However, the occurrence of only a single discrete band on the Ficoll density gradient argues against this. It was also clear that the PS II complex incorporated in the proteoliposomes under these conditions had a significant shoulder in the 695–696 nm region (Fig. 8a) as evidenced by the high level of fluorescence in this region, which was not present in the control proteoliposomes with only the PS II protein complex (similar to Fig. 3b) or those with only the PS I complex (Fig. 7).

In the PS I complex-LHCP proteoliposomes, the fluorescence characteristics at 77 K indicated energy transfer from LHCP to the PS I complex (Fig. 8b). Inspection of the fluorescence emission and excitation curves and comparison with Fig. 7 indicate clearly that the separate LHCP as well as the LHCP closely associated with the PS I complex was involved in the transfer of energy. It is also to be noted that the LHCP bands were slightly red-shifted to 682 nm and 701 nm.

In the proteoliposome preparation with all three complexes present, PS I complex, PS II complex and LHCP, it is also apparent by comparison of Fig. 8c with Fig. 9 that energy transfer occurred from LHCP to the other two complexes, although the slight difference between the emission spectra excited at 436 nm or 475 nm and the presence of the 700 nm band indicates that some LHCP was not closely associated with either of the other two complexes and that some LHCP was therefore fluorescing independently. The blue-shift of the 685 nm band to 684 nm (436 nm excitation) or to 683 nm (475 nm excitation) also supports the latter conclusion.

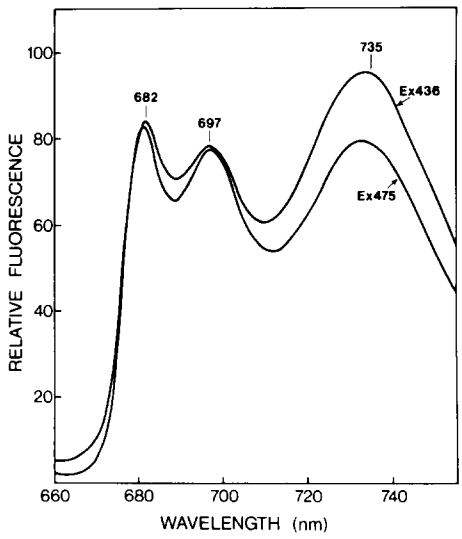

Fig. 9. Low-temperature fluorescence emission spectra of a mixture (1 : 1 : 1 on a protein basis) of PS I, PS II and LHCP complexes, prior to reconstitution. Excitation was at 436 nm and 475 nm; E_{475}/E_{436}, 0.70 for F_{682} or F_{697} or F_{735}. Emission spectra were normalised for F_{682}.

Discussion

The present results show clearly that a highly purified functional PS II complex has been isolated and reconstituted by a single step freeze-thaw method into liposomes formed from the four major diacyl lipids of spinach thylakoids. The presence of the 695 nm fluorescence emission band of variable intensity associated with a purified PS II complex has not been observed before with other digitonin-solubilized PS II complexes, although Satoh [30] observed that it could be induced by 1,10-phenanthroline in the presence of chaotropic agents. It is clearly a property of the PS II complex, since our preparations had no LHCP or only minute amounts. The F_{685}/F_{695} ratio decreased dramatically from 1.4–1.9 in fractions just eluted from the isoelectric focusing gel to 1.0–1.5 in samples which have undergone extensive dialysis to remove digitonin. This effect is due, at least in part, to aggregation of the PS II complex induced by the removal of digitonin. Thus, PS II complex, like other purified membrane intrinsic protein complexes such as LHCP [15], cytochrome reductase [31] or cytochrome oxidase [32], shows a strong tendency to form membranes upon removal of detergent. Interestingly, we have observed that the 695 nm band is

sometimes seen in the PS II complex, CPa, prepared by gel electrophoresis. In Newman and Sherman's preparations [21], a fraction with a large 695 nm band was isolated by zonal density centrifugation but was assumed to be less pure than the fraction with only a 685 nm band.

Reconstitution of PS II complex into vesicles demonstrated that the 685 nm fluorescence emission band is a stable, characteristic property of the complex. However, the magnitude of F_{695} compared to F_{685} in the PS II-proteoliposomes was influenced by the ratio of protein to lipid (Fig. 3), suggesting that the increased content of lipid in the vesicles interfered with the co-operative interactions of PS II complexes. No freeze-fracture study of the proteoliposomes was carried out during this study. Such studies in the future will be important for deciding whether the cooperative interactions of PS II complexes are accompanied by changes in membrane particle distribution.

The formation of a longer wavelength fluorescence band has also been observed with another isolated Chl-protein complex, LHCP. Isolated in Triton X-100 it has a characteristic fluorescence emission at 681 nm at 77 K. With the addition of Mg^{2+}, LHCP or LHCP-proteoliposomes tend to aggregate, and this aggregation is accompanied by the appearance of a 695 nm shoulder; the F_{681}/F_{695} ratio decreases markedly as aggregation proceeds [13,15]. Chloroplasts of most higher plants and green algae have fluorescence emission at 685 nm, 695 nm and 720–740 nm (cf. Refs. 2, 3). While there is agreement that F_{685} and F_{695} are associated mainly with PS II and F_{730} with PS I [33] there is speculation about which complexes emit the 685 and 695 nm fluorescence in vivo. Butler [34,35] assigned F_{685} to LHCP and F_{695} to the PS II complex; however, the Chl *b*-less barley mutant has both bands [36]. Rijgersberg et al. [37] suggest that both F_{685} and F_{695} are due to PS II complex. Our results show that the isolated PS II complex has both these emission bands but suggest that LHCP could contribute to F_{695}. In chloroplasts there is also evidence indicating that part of F_{695} comes from LHCP [13,15].

In proteoliposomes containing both PS II complex and LHCP, it is clearly seen that LHCP even when present in high concentrations transfers energy mainly to PS II complex, since the fluorescence emission is at 685 nm (PS II complex) rather than 681 nm (LHCP) (compare Figs. 5 and 6 with Fig. 4). This ability of LHCP to interact with PS II complex is expected since many developmental, structure and functional studies (cf. Ref. 36) show a close structural and functional association between PS II complex and LHCP. Further, the amounts of PS II and LHCP are comparable in appressed and stroma-exposed thylakoids [10]. LHCP was also shown to interact with the PS I complex (Fig. 8b) in proteoliposomes reconstituted with both these components. The question of how much LHCP is in direct contact with PS I complex in vivo and whether the tightly bound putative LHCP is really LHCP has not been resolved. Certainly, PS I fractions with Chl/P700 ratios of 200–150 contain some Chl *b* [33].

On the other hand, no interaction between PS I and PS II complexes was observed in proteoliposomes containing both these components. If it is assumed that the two complexes were reconstituted into the same proteoliposomes, which seems likely, then it is possible that either the complexes were associated, but their interaction was not detected by the present methods, or the complexes were not associated. Concerning the first possibility, the method used would detect only transfer of energy from PS I to PS II and not vice-versa. It has often been proposed that energy transfer in intact systems is unindirectional from PS II to PS I [34,35]. If this is true then it may prove very difficult to substantiate energy transfer between the two reconstituted complexes. Concerning the second possibility, non-interaction might occur because either reassembly is incomplete or there is a natural lack of association. Thus the present evidence on the interaction of the two reconstituted photosystems is inconclusive. Nevertheless, the following conclusions are still valid for the proteoliposome systems:

(1) energy transfer occurs from LHCP to the PS I complex;

(2) energy transfer occurs from LHCP to the PS II complex.

Therefore the simplest working hypothesis to explain the present results is that any energy transfer between the photosystems occurs via LHCP. Wollman and coworkers [38,39] have recently come to a similar conclusion for an intact system using a mutant of *Chlamydomonas*.

In the proteoliposomes reconstituted with all three complexes one might expect to find fluorescence emission characteristics similar to those of intact chloroplasts. The main differences between the reconstituted and intact system were the high emission of the PS I complex apparently due to greater efficiency of incorporation of this complex compared with the other two complexes (Figs. 8c and 9), and the presence of a 700 nm band compared to a band in the region of 695 nm in intact chloroplasts (Fig. 8). The latter difference was caused by the use of LHCP in an aggregated form, that already had a strong 695–699 nm fluorescence emission band, and the presence of some LHCP in the proteoliposomes which was not associated with either of the other two complexes. If a PS II complex had been used with a strong 695 nm band, and if the LHCP had had a weak 697–699 nm band, or if it had been fully associated with the other photosystems it seems very probable that a pattern of fluorescence emission very close to that of the intact system would have been found. However, until more is known of the interaction of the complexes in vivo and in artificial systems such as the one used here it would be unwise to draw too many comparisons between the two situations.

In conclusion, the present results which represent first attempts to reconstitute the major chlorophyll protein complexes of higher plants together, support a model in which LHCP interacts closely with and transfers energy to both the PS I complex and the PS II complex.

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