Biochimica et Biophysica Acta 848 (1986) 193–200
Elsevier

BBA 41914

A reevaluation of the fluorescence of the core chlorophylls of Photosystem I

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(Received August 27th, 1985)

Key words: Photosystem I; Chlorophyll-protein complex; Chlorophyll fluorescence; (Spinach, *Mastigocladus*)

Surfactants induced changes in the 77 K fluorescence spectra of thylakoid membranes and of various Photosystem I (PS I) core chlorophyll-protein complexes isolated from higher plants and a thermophilic cyanobacterium. Triton X-100, sodium dodecyl sulfate and, to a much lesser extent, dodecyl maltoside shift the 735 nm fluorescence of PS I in higher plant thylakoids to much shorter wavelengths, while in the cyanobacterium at the same ratios of surfactant/chlorophyll, a shift of only a few nanometers occurs. A higher plant PS I core complex, having a chlorophyll/P-700 of approx. 65, and a P-700-chlorophyll *a*-subunit I complex (chlorophyll/P-700 approx. 40) emit maximally in the 670–685 nm region in the presence of Triton or sodium dodecyl sulfate. However, when the surfactants associated with these complexes were exchanged for dodecyl maltoside, a reversible shift to 721–725 nm occurred in their emission maxima, but there was no change in their biochemical compositions. The unaltered wavelength maximum of the fluorescence of PS I core complex is likely to be close to 725 nm. Shifts of the emission spectrum caused by surfactants indicate the importance of controlling the type and amount of surfactant present to prevent an artifactual wavelength maximum being obtained for a complex. The data explain why different maxima have been reported for essentially identical PS I core chlorophyll-protein preparations, and indicate that the origin of PS I core complex fluorescence does not change as chlorophyll molecules are extracted from PS I core complex by Triton.

Introduction

Chlorophyll molecules are associated with several specific proteins in plants and bacteria. The plant pigment-proteins can be classified either as Photosystem (PS) I or II core components, i.e., those containing the reaction center and some closely associated antenna pigments, or light-harvesting complexes (LHC) that contain only antenna pigments (see Ref. 1 for a review). A fraction of the light absorbed during photosynthesis is reemitted as fluorescence by some of these pigment-proteins. In higher plant thylakoid membranes, this fluorescence occurs maximally at 685 nm at room temperatures, while at liquid nitrogen temperatures (77 K) three emission bands at about 685, 695 and 730 nm are usually seen [2]. The first two 77 K emissions come mainly from chlorophyll molecules associated with PS II, while the last originates from those in PS I [3–5]. It is important to identify the chlorophyll-protein(s) that is the source of each fluorescence band in both intact membranes and in thylakoid fractions to improve understanding of energy migration and trapping during photosynthesis (e.g., Refs. 2 and 6).

The emphasis in this article is on the 77 K fluorescence characteristics of the PS I chloro-

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Abbreviations: PS, Photosystem; LHC, light-harvesting complex.

phyll-proteins. Some previous work has indicated that the approx. 730 nm fluorescence at 77 K of a PS I particle prepared by using either Triton or sodium dodecyl sulfate as surfactants, arises from the LHC I component in it, and that after removal of LHC I, the residual material, PS I core complex, having a chlorophyll/P-700 of 65, now fluoresces at somewhat shorter wavelengths, 722 nm, while the fractionated LHC I emits at 717–730 nm [7–13]. More exhaustive treatment of thylakoid membranes or PS I particles with various surfactants yields a P-700-chlorophyll *a*-subunit I complex (sometimes called the P-700-chlorophyll *a*-protein or CP I [1,6] or the P-700 reaction center preparation [14,15] or PS I-40 [8]) having a greater enrichment of P-700 (chlorophyll/P-700 = 40) than occurs in PS I core complex. This chlorophyll-protein has been reported [6–8,16] to fluoresce maximally at wavelengths shorter than 700 nm at 77 K, i.e., 682–695 nm. Based on these data the 730 nm fluorescence of membranes or fractions thereof has been attributed to LHC I, the 722 nm to the PS I core component (PS I core complex) and the PS I fluorescence below 700 nm to those 40 chlorophyll *a* molecules most tightly associated with P-700 [7–12].

Not all fluorescence data are compatible with these assignments. For example, a cyanobacterial P-700-chlorophyll *a*-protein, essentially identical to its higher plant counterpart [6] and having 40 chlorophyll molecules/P-700, fluoresces at 722 nm [17–19], while some Triton-prepared complexes, having no chlorophyll *b* and hence no LHC I, fluoresce at 734 nm [5] and others having higher chlorophyll/P-700 ratios of 65–110 fluoresce maximally at 683 nm [20]. Furthermore, preparations from spinach of PS I components greatly enriched in P-700 (chlorophyll/P-700 = 15) and also having no chlorophyll *b* fluoresce at 730 nm [21,22]. Some researchers believe the PS I approx. 680 nm fluorescence arises from the core chlorophyll-protein having 40 or less chlorophylls per P-700 (e.g., 7), while others suggest that it does not arise from a chlorophyll-protein complex, but is due to surfactants extracting some of the chlorophyll from the particle making the surfactant-chlorophyll conjugate the major fluorescing species [5,20,21].

The work in this article was undertaken to explain the disparate fluorescence characteristics reported for apparently equivalent PS I fractions, to determine what are the correct characteristics for PS I and its fractions, particularly of its core component(s), to examine the effect of the concentration of some surfactants on the fluorescence of PS I particles of higher plants and a cyanobacterium, and to study whether the approx. 680 nm fluorescence arises from a chlorophyll-protein or from solubilized chlorophyll. To obtain unequivocal data it was important that pigment and P-700 concentrations and subunit compositions of the PS I fractions to be studied were known precisely. Therefore we used the well-characterized seven and four subunit-containing PS I reaction center preparations (chlorophyll/P-700 = 60) of spinach and *Mastigocladus laminosus*, respectively, as well as the single subunit preparation (chlorophyll/P-700 = 40) from both organisms [23].

Materials and Methods

Plant materials

Pea plants were grown in the greenhouse for two weeks and harvested immediately before use. Spinach leaves were obtained from local markets. *Mastigocladus laminosus* was grown at 50°C in medium D of Castenholz [24] at pH 8.2 with constant stirring [25].

Chemicals

Sodium dodecyl sulfate, octyl glucoside and Triton X-100 were from Sigma Chemical Company, and dodecyl-β-D-maltoside from Calbiochem Behring Diagnostics, La Jolla, CA.

Preparation of Photosystem I fractions

The so-called PS I reaction center preparations were isolated from spinach chloroplasts by the method of Nechushtai et al. [26], and from whole cells of *Mastigocladus laminosus* as described in Ref. 25; the former preparation contains seven subunits and the latter contains four. In both cases the final product was obtained in a buffer of 20 mM Tris-Cl + 0.2% Triton (pH 8.0). The PS I complexes, PS I-65 and PS I-40, described by Mullet et al. were purified from pea leaves as described in Ref. 8. PS I chlorophyll-protein complexes containing only subunit I (an approx. 60 kDa polypeptide) were obtained from the PS I

reaction center preparations described above, by sodium dodecyl sulfate treatment [14,15]: the preparations were incubated in 0.6% sodium dodecyl sulfate at 20°C for 15 min. After adding an equal volume of 20 mM Tris-Cl (pH 8.0) they were each applied to a 10–30% sucrose gradient containing 20 mM Tris-Cl (pH 8.0) + 0.2% Triton X-100, and centrifuged for 15 h at 160000 × *g*. Two fractions were collected from the resulting gradient: a bluish-green lower one containing the P-700-chlorophyll *a*-subunit I complex, and a yellow-green upper fraction containing subunits II–VII. These two fractions and the starting material from which they were derived, were each dialyzed for 72 h against 0.05 M sorbitol and 20 mM Tris-Cl (pH 8.0) before further analysis.

Exchange of surfactant and reduction of surfactant concentration in PS I fractions

The isolated spinach PS I reaction center preparation or the pea PS I-65 and PS I-40 fractions were absorbed onto a DEAE-cellulose column (0.6 × 10 cm), which was then washed with 20 ml 20 mM Tris-Cl (pH 8.0) containing 0.1% dodecyl maltoside. The complex was subsequently eluted by adding 0.2 M NaCl to the washing solution. The concentration of the dodecyl maltoside was then reduced by dialysis against a solution of 0.05 M sorbitol and 20 mM Tris-Cl, (pH 8.0) for 72 h. All fractions, including the P-700-chlorophyll *a*-subunit I, were each then centrifuged at 160000 × *g* for 15 h on a linear sucrose gradient (5–30% sucrose in 20 mM Tris-Cl (pH 8.0) layered above 2 ml of 60% sucrose). The green fraction migrating to a position between the 30% and 60% sucrose layers and representing essentially all of the chlorophyll loaded onto the gradient, was collected and dialyzed overnight against 0.05 M sorbitol in 20 mM Tris-Cl (pH 8.0).

Analytical methods

Chlorophyll concentrations were measured in 80% acetone using the equations of Arnon [27]. P-700 concentrations were determined as described in Ref. 28. Analytical electrophoresis was performed at 40°C in gels having a linear gradient of 10–15% acrylamide and containing a buffer of 375 mM Tris-Cl (pH 8.8) and 0.1% sodium dodecyl sulfate. Samples to be electrophoresed were dissociated in 2% sodium dodecyl sulfate and 2% β-mercaptoethanol for 1 h at 55°C.

Fluorescence

All samples, after diluting 1:10 with 20 mM Tris-Cl (pH 8.0) to give a final chlorophyll concentration of 10 μg/ml, were immediately cooled in liquid nitrogen as soon as they were available and their spectra were recorded with an Aminco SPF-500 spectrofluorometer; the excitation wavelength was 430 nm. The spectra shown are not corrected for the response of the phototube.

Results

The changes in the 77 K fluorescence of higher plant thylakoid membranes resulting from the addition of Triton X-100 are shown in Fig. 1. A relatively low Triton/chlorophyll of 4:1 (w/w)

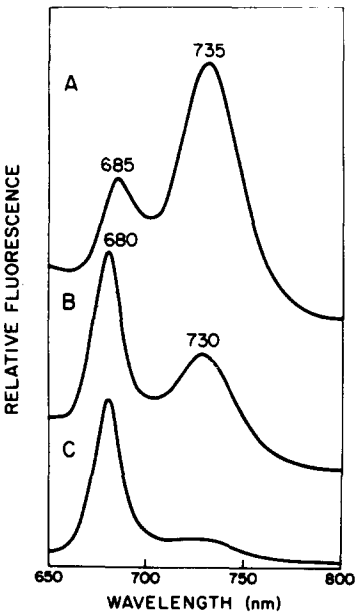

Fig. 1. Effect of different concentrations of Triton X-100 on the fluorescence characteristics of spinach thylakoid membranes. Membranes were exposed to Triton X-100 and immediately frozen in liquid nitrogen. The spectra are comparable, since the chlorophyll concentration was 10 μg/ml in each sample and the settings on the fluorometer were not changed for the different samples. Emission spectra (A) of membranes; (B) of membranes suspended in 0.004% Triton X-100 (Triton/chlorophyll = 4); and (C) of membranes in 0.02% Triton X-100 (Triton/chlorophyll = 20).

shifted the longest-wavelength fluorescence band from 735 to 730 nm, and decreased the proportion of this signal with respect to the shorter-wavelength emission(s). Increasing the surfactant/chlorophyll to 20:1, resulted in the virtual disappearance of the longest wavelength emission. Triton also increased the shorter wavelength fluorescence and shifted it to 680 nm (Fig. 1) (cf. Ref. 29). This increase has been attributed to physical dissociation of LHC II complexes from the PS II core (i.e., PS II core complex), so that they are unable to transfer energy to PS II core complex and therefore fluoresce [30]. We found that octyl glucoside gave much the same effect as Triton (cf. Ref. 29); however, a second, chemically similar surfactant, dodecyl maltoside, at a ratio of 10:1 (cf. Ref. 9) with respect to chlorophyll, caused only a 4 nm shift of the 735 nm emission without increasing the shorter wavelength peak (spectrum not shown).

PS I reaction center preparation from spinach, containing seven subunits [23] and having a ratio of 50–60 chlorophyll molecules per P-700, had a major emission at 681 nm and a relatively small emission at 721 nm (Fig. 2). Since the complex was suspended in Triton/chlorophyll = 20, which alters the fluorescence of thylakoid membranes extensively (Fig. 1), we exchanged the surfactant in the suspending solution to dodecyl maltoside (see Methods) which had had much less effect on the membrane's emission spectrum (see above and also Ref. 9). The complex's subunit composition and spectral properties were then reexamined. The subunit composition was identical to that of the starting PS I fraction (Fig. 2); however, its fluorescence spectrum was not (Fig. 2): the shorter wavelength fluorescence around 680 nm, observed in the starting material, was now virtually absent and there was a major longer-wavelength emission at 725 nm. A decrease in the dodecyl maltoside concentration by dialysis gave a product which fluoresced at a slightly longer wavelength (728 nm) (spectrum not shown). Antibodies against LHC I (kindly provided by E. Lam) were used to show that the PS I reaction center preparation did not contain LHC I; the polypeptide(s) around 21 kDa in the preparation (Fig. 2) is that known [14] as subunit II.

More vigorous treatment of PS I preparations

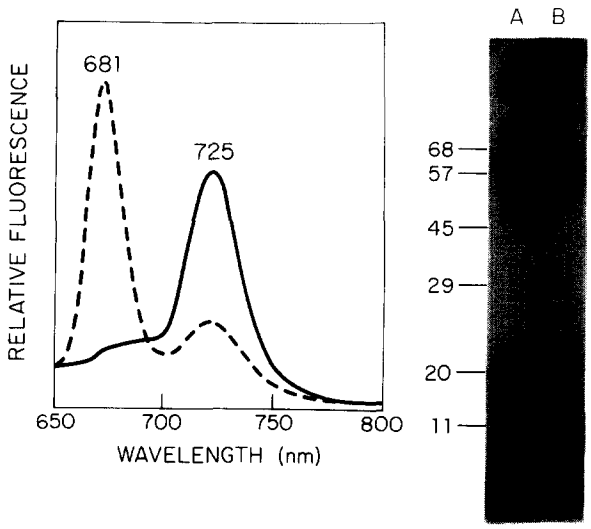

Fig. 2. Fluorescence emission spectra (left) and subunit compositions (right) of (A) spinach PS I reaction center preparation in buffered 0.02% Triton X-100 (Triton/chlorophyll = 20) (- - - - - -) and (B) of the same complex after the surfactant in which it was suspended had been changed to 0.01% dodecyl maltoside (dodecyl maltoside/chlorophyll = 10) (———). The chlorophyll concentration was 10 μg/ml in both cases.

with surfactants was used to strip most of the readily removable chlorophylls from the preparation, yielding a fraction having 40 or less chlorophyll molecules per P-700. The seven-subunit PS I preparation above was treated with sodium dodecyl sulfate to give such a product. Two green bands on a gradient (see Materials and Methods) were obtained from this treatment. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 right) showed that the lower fraction in the gradient contained only the approx. 60kDa polypeptide(s); antibodies against each of the seven subunits revealed that the aggregates above the approx. 60 kDa band contained only subunit I. The P-700-chlorophyll *a*-subunit I complex fluoresced at 671 nm (Fig. 3A). Subunits II–VII were in the upper fraction (Fig. 3 right) which had an approx. 673 nm fluorescence (Fig. 3B). Residual sodium dodecyl sulfate was removed from both fractions and replaced by Triton X-100. Subsequently, the Triton X-100 was exchanged for dodecyl maltoside, and then the latter's concentration was decreased to provide materials, the fluorescence characteristics of which should be little

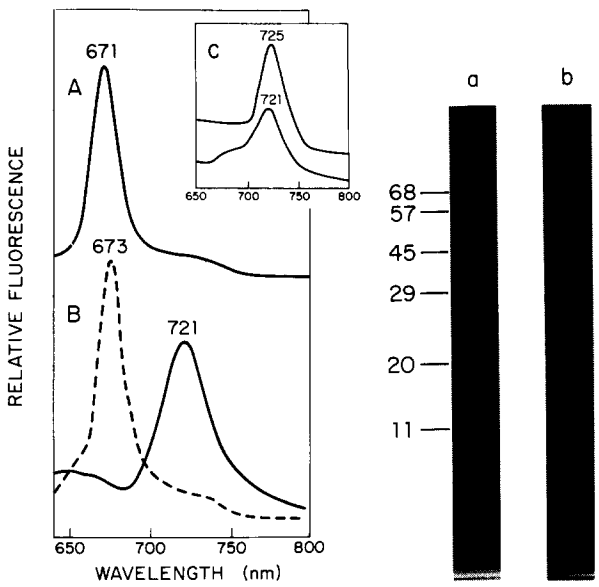

Fig. 3. Left: the fluorescence characteristics of PS I chlorophyll-protein fractions: (A) The P-700-chlorophyll *a*-subunit I complex in sodium dodecyl sulfate; sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this material is shown on the right under (a). (B) The two sucrose gradient fractions obtained after treating the PS I reaction center preparation with sodium dodecyl sulfate and then exchanging the sodium dodecyl sulfate for dodecyl maltoside: the P-700-chlorophyll *a*-subunit I complex (———); subunits II–VII (- - - - - -) (C) (Inset) Fluorescence spectrum of pea PS I-65 (upper trace) and PS I-40 (lower trace) prepared according to Mullet et al. [8] and after exchanging the surfactants associated with the complexes for dodecyl maltoside. Right: polypeptide compositions of sucrose gradient fractions, (a) P-700-chlorophyll *a*-subunit I complex, and (b) subunits II–VII.

affected by the surfactants present. The resulting material (P-700-chlorophyll *a*-subunit I complex) fluoresced maximally at 721 nm (Fig. 3B), while the chlorophyll associated with subunits II–VII still fluoresced at 673 nm. This 4–7 nm difference between the emission maxima of the seven-subunit and one-subunit preparations suspended in the same buffer and surfactant concentration might be due to some residual sodium dodecyl sulfate in the P-700-chlorophyll *a*-subunit I complex. Nevertheless, its fluorescence maximum is quite different from the 694 nm reported for other PS I core complexes having a similar enrichment of P-700 (e.g., the PS I-40 fraction of Mullet et al. [8]). We suspected this difference was due to the presence of surfactants in other equivalent preparations (e.g., 1% Triton, 1 mM octyl glucoside and 0.2% digitonin in the Mullet et al. [8] preparation). To test this idea we repeated their work, confirmed that PS I-65 fluoresced maximally at 725 nm (Fig. 3C) and that PS I-40 derived from it had a maximum emission at 696 nm (data not shown). We then removed the surfactants from PS I-40 by sucrose gradient centrifugation, and exchanged any remaining surfactant for dodecyl maltoside. The long-wavelength fluorescence maximum of the pea PS I-40 fraction treated in this manner was at 721 nm (Fig. 3C).

To detect whether another class of oxygen-evolving organisms also had a 77 K fluorescence in the 720–740 nm region from the core chlorophylls of PS I, we isolated a PS I reaction center preparation from the thermophilic cyanobacterium *Mastigocladus laminosus* [25]. This complex, having 50–60 chlorophyll molecules per P-700 and containing four protein subunits, is particularly stable to heat and surfactant treatments. The fluorescence characteristics of *Mastigocladus* photosynthetic membranes and purified PS I fractions and their subunit compositions, are shown in fig.

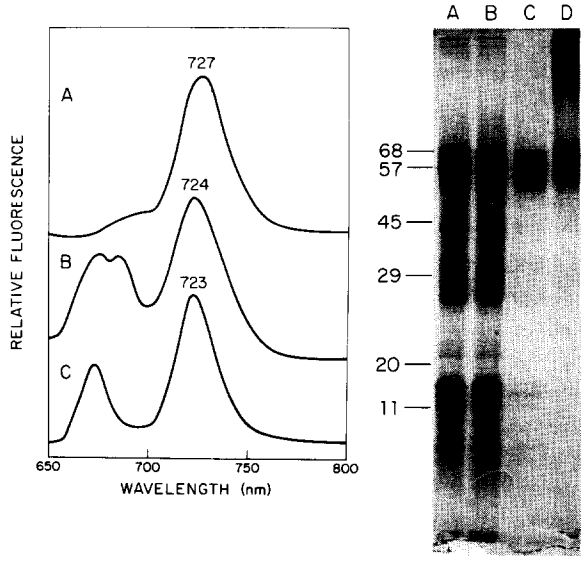

Fig. 4. The fluorescence characteristics (left) and the polypeptide compositions (right) of *Mastigocladus* membranes and a PS I fraction: (A) Intact membranes suspended in buffer with no detergent; (B) membranes suspended in 0.02% Triton X-100 (Triton/chlorophyll = 20); (C) the PS I reaction center preparation in 0.02% Triton X-100 (Triton/chlorophyll = 20); (D) *Mastigocladus* P-700-chlorophyll *a*-subunit I complex.

4. Whole membranes had a 77 K long-wavelength emission at 727 nm; Triton X-100 at a mass ratio of 20 with respect to chlorophyll shifted this maximum to 724 nm. The four subunit PS I reaction center preparation fluoresced at 723 nm even at a Triton/chlorophyll of 20 (Fig 4), and the P-700-chlorophyll *a*-subunit I complex had an identical fluorescence spectrum (data not shown). The shorter wavelength fluorescence emission around 680 nm observed in membranes is almost certainly derived from PS II chlorophyll molecules and it only became substantial after the membranes were exposed to Triton (Fig. 4B). We attribute the difference in sensitivity to the surfactant to the greater stability of this cyanobacterium's pigment-protein complexes at higher temperatures (cf. Ref. 25).

In all of our observations we noted that changes in the PS I fluorescence spectrum are greater as the surfactant/chlorophyll is increased, and that the emission(s) do not become larger when the concentration of surfactant is increased, provided that its ratio with respect to chlorophyll is kept constant. For example, with *Mastigocladus* membranes, at a Triton concentration of 0.2% the emission maximum is 726 nm at a Triton/chlorophyll ratio of 4, and at 724 nm at a ratio of 20. For the PS I fraction in 0.2% Triton it is 725 nm at a ratio of 4 and 723 nm at a ratio of 20 (data not shown).

Discussion

Addition of surfactants to the thylakoid membrane results either in the incorporation of surfactant molecules into the membrane, or in the formation of lipid-protein-surfactant micelles, depending on the critical micelle concentration of the surfactant [31]. In both events a direct interaction of the surfactant with chlorophyll or protein molecules of the chlorophyll-protein complexes might change, among other things, the fluorescence characteristics of the thylakoid membrane and fractions thereof (cf. Refs. 21 and 32). That this occurs for thylakoid membranes and PS I chlorophyll-proteins treated with some surfactants is shown in Figs. 1–4; Triton and sodium dodecyl sulfate shifted their emissions substantially, and essentially reversibly.

To define which complexes give rise to fluorescence maxima observed in vivo, the consequences of a surfactant interacting with specific chlorophyll-binding proteins must first be understood. For example, if the surfactants studied in the present work had directly interacted with the chlorophyll moiety then every chlorophyll-containing fraction should have fluoresced maximally at 720–730 nm in the presence of low concentrations of dodecyl maltoside. However, fluorescence of the chlorophyll associated with subunits II–VII and with LHC II (data not shown) under such conditions demonstrates that such a view is incorrect (Fig. 3). Another consequence could be that surfactants extract chlorophyll molecules from their apo-proteins producing chlorophyll-surfactant micelles which are thought to fluoresce at 670–685 nm [5,20,21]. However, such short-wavelength fluorescence of chlorophyll is not always indicative of free chlorophyll. For example, in our preparative procedure, surfactant-solubilized chlorophyll sedimented at a different rate than the P-700-chlorophyll *a*-subunit I complex which could therefore be separated from free chlorophyll, and yet the complex still fluoresces at 671 nm (Fig. 2). Thus in our own case, and possibly in several others, the fluorescence shift of PS I particles more likely arises from the surfactant interacting with the protein moiety of the complexes to disrupt inter-complex energy migration rather than extracting chlorophyll molecules from their protein environment.

We suggest that the longest (as opposed to the shortest) wavelength observed for the emission of a complex in the presence of surfactants more nearly represents the unaltered fluorescence of the complex. This view is supported by two observations: first, a thermophilic cyanobacterium's PS I complex, which has a greater stability to denaturation than its higher plant counterpart (cf. Ref. 25), emitted at 723 nm in a Triton/chlorophyll of 20 and not at approx. 680 nm as observed for the higher plant components (Fig. 4C). Secondly, Triton and sodium dodecyl sulfate shift the fluorescence maximum of higher plant PS I complexes to increasingly shorter wavelengths as the ratio of surfactant to chlorophyll is raised, and their replacement by dodecyl maltoside returns the maximum to that observed at very low concentrations

of the other two surfactants.

Our observations suggest that the discrepancy about the wavelength of maximum fluorescence of PS I core particles, which has been reported by some investigators [7,8,20] to be below 700 nm and by others [5,12,17–19] to be above 720 nm, is almost certainly explained by the nature and amount of the surfactant present in the particular preparation examined. Specifically, the 680–694 nm emission reported for the PS I core fractions [7,8,20] is an artifact caused by the presence of certain surfactants which may directly alter the conformation of PS I subunit I polypeptides of higher plants. Thus, one must control not only the surfactant type present but also its ratio with respect to chlorophyll in order not to obtain artifactual emission spectra of chloroplast fractions. It seems very likely from our work that the fluorescence of the core complex chlorophylls (e.g., the P-700-chlorophyll *a*-subunit I preparation or PS I-40) is at the same wavelength, and probably arises from the same chlorophyll molecule(s), as PS I core complex preparations with higher chlorophyll/P-700 ratios (e.g., PS I reaction center preparation or PS I-65). Furthermore, the fluorescence from the PS I core must be located quite close to those chlorophyll molecules that form the donor and acceptor(s) in the primary charge separation since the fluorescence of PS I in fractions having only 15 chlorophylls per P-700 also occurs at approx. 720 nm [21,22]. With a rigidly controlled surfactant environment, the task now becomes one of identifying more exactly the location of the emitting chlorophyll molecule(s).

Considerable evidence exists that in higher plants some, perhaps all, of the longest-wavelength fluorescence (approx. 735 nm) of intact thylakoids comes from chlorophylls in LHC I, which, with PS I core complex are the chlorophyll-proteins constituting PS I (cf. Ref. 1). The suggestion that LHC II also adds to this fluorescence band [33] may be incorrect since the LHC II preparation examined could well have contained LHC I (cf. Refs. 12 and 34). Our data show that isolated PS I core complex, devoid of LHC I, has a wavelength maximum for its essentially unaltered emission spectrum at a slightly shorter wavelength than LHC I's. This difference in wavelength maxima is compatible with (and explains why) the fluorescence of the two electrophoretically isolated PS I complexes, termed CP Ia(= LHC I + PS I core complex) and CP I(= PS I core complex) is maximal at two slightly different wavelengths, 730 and 723 nm respectively, in higher plants [7,12,35]. While in *Chlamydomonas*, which has a LHC I (= CP0) fluorescing at a wavelength (708–712 nm) somewhat shorter than its higher plant counterpart, the equivalent fractions fluoresce at 714 and 718–720 nm [36]. It also explains why barley, wheat and clover mutants lacking chlorophyll *b* and hence presumably LHC I, but containing PS I core complex, have a 77 K longest wavelength fluorescence at 10–15 nm shorter wavelengths than their wild types [8,37,38], and it would explain why cyanobacterial thylakoids, which are also presumed to lack LHC I (cf. Refs. 39), have a PS I emission peak very close in wavelength (i.e., 727 nm) to that of the isolated higher plant and cyanobacterial PS I core complex complexes (Figs. 2 and 4). If PS I structures in the intact membranes are of two forms, one having LHCI + PS I core complex (i.e., a CP Ia-like structure) and one having PS I core complex only (CP I-like structure) as is indicated by some electrophoretic studies (e.g., Ref. 12) and if both forms fluoresce, then it would explain why the higher plant PS I 77 K emission peak at approx. 730 nm has been reported to be composed of two overlapping bands [40,41]. Lastly, the possibility remains that PS I core complex in situ (in the absence of detergent) might fluoresce at a wavelength as long as 735 nm, and, if so, this component's fluorescence, and not LHC I's, could be that seen in whole membranes at liquid nitrogen temperatures.

Acknowledgements

This work was supported by NSF Grant DMB 84-17720. Rachel Nechushtai was supported by Dr. Chaim Weizmann postdoctoral fellowships for scientific research. We wish to thank Mr. P.L. Schneider for his technical assistance, Drs. E. Lam and R. Malkin for helpful discussions and Ms. Camille Peterson and Mr. Gary Peter for their help in preparing the manuscript. We also acknowledge Dr. W.E. Dietrich's seminal observation, made in our laboratory in 1983, that sodium dodecyl sulfate reversibly affected fluorescence spectra of cyanobacterial chlorophyll-proteins.

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