ENERGY TRANSFER IN REAL AND ARTIFICIAL PHOTOSYNTHETIC SYSTEMS

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† Dedicated to the memory of Gerhard L. Closs
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Fluorescence emission from the photosynthetic organisms *Tribonema aequale*, *Anacystis nidulans*, and *Chlorella vulgaris* and from some chlorophyll model systems have been recorded as a function of excitation wavelength and temperature. Considerable similarity was observed in the effects of excitation wavelength and temperature on the fluorescence from intact photosynthetic organisms and the model systems. The parallelism in behavior suggest that self-assembly processes may occur in both the *in vivo* and *in vitro* systems that give rise to chlorophyll species at low temperature that may differ significantly from those present at ambient temperatures.

**Introduction**

Despite major advances in the understanding of the primary light conversion events in photosynthesis resulting from the successful crystallization of the photoreaction center of photosynthetic bacteria, many aspects of the primary processes of photosynthesis, particularly in green plants, remain obscure. The current consensus has it that charge oxidation and reduction capacity in green plant photosynthesis takes place by serial operation of two photoreaction centers (PSI and PSII), which, like the light-harvesting apparatus, consist of chlorophyll-protein complexes. It is also widely assumed that the anomalous optical properties of *in vivo* chlorophyll can be attributed to chlorophyll-protein interactions. Further, it is generally accepted that fluorescence originating in the reaction centers or the antenna chlorophyll is an accurate indicator of energy flow. All of these statements may in fact be correct. However, in our view, the experimental bases for these postulates, though voluminous, are nevertheless, in our view, not commensurate with the importance of the propositions. We have therefore undertaken a comparison of the fluorescence emissions of three intact photosynthetic organisms with those of some model chlorophyll systems to address some issues that we feel are still unresolved.

Most of what is known or surmised about chlorophyll function in the primary light conversion events in green plants is derived from optical spectroscopy on intact living organisms or on (partially) functional fragments extracted from them. Even though such systems are considerably simplified relative to the intact organism, with the exception of crystalline reaction centers, they are still very complex. The precise relationships of the isolated thylakoid, reaction center preparation, or chlorophyll-protein complex to what is actually present in the intact
organism is for the most part enigmatic. While optical spectroscopy is a powerful tool for characterizing excited state energy levels, the amount of structural information that can be extracted from optical data is distressingly small, and the assignment of the spectra is not at all a straightforward matter. In this communication, we have attempted to increase the information content of optical experiments by conducting parallel experiments on intact or fragmented photosynthetic systems, and on simple laboratory systems of well-defined composition and structure.

Model Systems

Porphyrins in various configurations have traditionally served as the models of choice for in vivo chlorophyll. This is not at all surprising, considering the important role porphyrins and porphyrin derivatives play in respiration, electron transfer agents, and enzymes. The biosynthesis of chlorophyll proceeds along the same pathway as that of the porphyrins present in hemoglobin, cytochrome c, and peroxidase. As chlorophyll once extracted from photosynthetic organisms is rather fragile, the much sturdier porphyrins have been used to study optical and other spectroscopic properties, in the expectation that information acquired on porphyrin systems will be applicable to the chlorophylls.

The spectroscopy of monomeric porphyrins and metalloporphyrins has received much attention, and the results have made important contributions to the spectroscopy of the chlorophylls. However, the applicability of monomeric porphyrins as models for chlorophyll is open to serious question. Monomeric porphyrins have optical, redox, solubility, and aggregation properties markedly different from those of the chlorophylls, and thus have only limited utility. However, model systems based on porphyrins covalently linked to other porphyrins or to electron acceptors have played an important role in the study of electron transfer in photosynthesis. Particularly useful have been the models in which porphyrins have been linked to electron acceptors through rigid spacer molecules that position the donor and acceptor molecules at known fixed distances. Several excellent reviews of these model systems are cited in Ref. 4.

There are good reasons to suppose that the photosynthetic apparatus, both in green plants and bacteria, contains very little monomeric chlorophyll, and that aggregated chlorophyll is implicated in photoreaction center function. The aggregation properties of porphyrins are very different from those of the chlorophylls, and therefore
porphyrin models are at best rough approximations. Consequently, models in which covalently linked chlorophyll macrorcycles are used have been developed, which more closely mimic the in vivo photoreaction center. These models consist of two pyrochlorophyllide, or better, chlorophyllide macrorcycles, covalently linked through the hydroxyl groups of ethylene glycol (or longer chain diol) through the two propionic acid side chains of the macrorcycles. The basic factors involved in the self-assembly process have been identified from IR and NMR spectroscopic studies on chlorophyll by Closs, Katz, and co-workers.

We have considered model systems for photosynthesis research elsewhere and will not discuss these further here. For our present purposes, we have used model systems that are self-assembled, that is, we have used Chla under conditions where self-assembly occurs to mimic both the primary electron donor in photosynthesis and the light harvesting antenna. For the antenna, we have used a chlorophyll a (Chla) oligomer, (Chla), which is formed at ambient temperatures by a keto carbonyl interaction of one macrorcycle and the central magnesium atom of another, i.e., keto C=O···Mg. In the absence of other nucleophiles, as in a nonpolar solvent such as n-octane, or methyl cyclohexane, aggregates containing twenty or more chlorophyll macrorcycles are formed. This oligomer absorbs maximally at 680nm. For organisms that contain Chl as well as Chla, such as Chlorella vulgaris, an antenna model containing only Chla is obviously deficient. We have therefore also chosen for study two photosynthetic organisms that contain only Chla but which nevertheless evolve oxygen in photosynthesis. The modest red shift relative to monomeric Chla is indicative of only small π-π interactions in the oligomer, despite its large size. A reasonable presumption is that the chlorophyll macrorcycles in (Chl)b are orthogonal to each other. This chlorophyll species appears to have a strongly diminished, if, in fact, any capacity at all, for fluorescence at room temperature.

To mimic the P700 of PSI we use in this paper a self-assembled Chla-ethanol/methyl cyclohexane system contains significant amounts of 700 nm-absorbing species at low temperatures. Self-assembled systems can be formed that contain monomeric Chl a (λmax ~ 665 nm), oligomeric (Chl a), (λmax ~ 678 nm), and species very similar to those formed by linked pairs in the folded configuration (λmax = 698 nm), depending on the temperature and the presence or absence of extraneous nucleophiles. These model systems, by virtue of the differences in the optical maxima, provide an excellent opportunity to study energy transfer by selective photoexcitation under well-defined conditions.
Photosynthetic Organisms

We have chosen three photosynthetic organisms for our comparative fluorescence study. *Chlorella vulgaris* may be taken as the exemplar of higher green plants; its photosynthetic pigments consist of Chl$\alpha$ and Chl$\beta$, and carotenoids. *Tribonema aequale* is a yellow-green alga (*Xanthophyceae*) that contains Chl$\alpha$ and carotenoids. This organism evolves oxygen during photosynthesis; although it contains no Chl$\beta$, and no phycobilins. *Anacystis nidulans* is a cyanobacterium (blue-green alga) that contains Chl$\alpha$, the phycobilins phycocyanin and phycoerythrin, and carotenoids. Photoselective excitation permits the preferential excitation of particular pigments in specific absorption bands, and the various pigment mixes in these three organisms provide good opportunities for the application of photoselective excitation. The general techniques for recording the fluorescence spectra are described in detail elsewhere. Excitation was provided by a Molelectron UV1000 nitrogen laser at 337 nm or by the output of a dye laser pumped by the Molelectron.

Results

Figure 1A shows composite room temperature absorption spectra of the various Chl$\alpha$ species present in the self-assembled systems, and also spectra of β-carotene and the phycobilins, phycocyanin and phycoerythrin. Figure 1B shows the absorption spectrum of *Anacystis nidulans*. Indicated by Roman numerals are (approximate) regions for light absorption by different pigment systems. The selection of excitation wavelengths was partially based on the wavelength regions where the different pigments absorb, but primarily on the wavelengths of absorption of specific chlorophyll entities *in vitro*. Reference 10 describes the parameters for selective excitation in detail. As indicated in Figure 1A, excitation in the 430-440 nm and 650-670 nm regions preferentially excites monomer chlorophyll. Excitation near 450 and 690-700 nm can be used to preferentially excite chlorophyll species having the spectral properties of special pair chlorophyll (P700). In addition, if the in vitro systems contain species with the properties of chlorophyll oligomers, these can be excited by 678-681 nm light.

Figure 2 shows the fluorescence spectra of the different pigment systems. Indicated are the emission wavelength maxima assigned to components of the *in vivo* photosystems PSI and PSII. As can be seen in this figure, of the auxiliary pigments only phycocyanin shows significant emission in the PSI and PSII regions.
Obviously, under some conditions emission from this (and perhaps related pigments) could complicate interpretation of \textit{in vivo} fluorescence observations in cyanobacteria. The most important aspect of the data in Figure 2 is the significant emission in the PSI and PSII regions from a Chl-ethanol/methylecyclohexane self-assembled system both at room temperature and at 150 K. For example, the band head near 683-685 nm is in the self-assembled system at the wavelength that has been assigned to the light harvesting Chl$\alpha\beta$ protein complex (LHC) in PSII. We also note the red shift of the Chl $\alpha$ emission in the PSI region that occurs with decreasing temperature. We have previously suggested that this phenomenon may reflect temperature dependent configurational changes in the self-assembled special pair type molecules that emit in this region\textsuperscript{10}.

Figure 3 illustrates the effect of temperature and selective excitation on the fluorescence excited in the photosynthetic organisms we have used. In Table 1 we summarize in more detail the experimental observations, and in Table 2 we list the absorption and emission bands that have been assigned to components of the photosynthetic unit by various authors. These data, then, provide a basis for a discussion of possible relationships between \textit{in vitro} and \textit{in vivo} systems.

Discussion

Room temperature \textit{in vivo} fluorescence emissions are generally very broad and ill-defined as can be judged from Figure 2. If we were to deconvolute these spectral bands into Gaussian components, the bands would clearly appear to be compound. This has been noted previously (cf. Litvin and Sineshchekov, ref. 19). What is important about this observation is the strong implication that there may be a large number of different species or different configurations of the same species that contribute to the emission in a particular wavelength region. It is, therefore, an oversimplification to talk about a limited number of \textit{in vivo} states (i.e., PSI, PSII, etc.) as implied in the breakdown compiled from the literature in Table 2. Lowering the temperature does not necessarily result in the appearance of sharply defined fluorescence spectra that can be associated with a limited number of states, although Rijgersberg and Amesz\textsuperscript{20} show clearly defined 685 and 695 nm bands in the 77 K fluorescence spectrum of \textit{Anacystis nidulans} excited with either 442 or 562 nm light. On the other hand, Satolf\textsuperscript{3} has also pointed out that it is often very difficult to detect a distinct F695 in the 77 K emission from the chloroplasts of higher plants and algae. It is clear
from an examination of Table I and Figure 3 that the nature of the observed emissions depends strongly on both temperature and excitation wavelength. Although we do see emission band heads at 685 and 695 nm under specific excitation conditions, generally our emission curves are broad, which indicates that there are a number of emission bands in this wavelength region.

A very important question arises in connection with the observed emissions: how specifically can the emissions in a wavelength region be assigned to a particular species? For example, as indicated in Table 2, there is a widely held view that the 685 nm fluorescence emanates from the light-harvesting Chlαβ protein complex (LHC or CPII) of PSII. Sato21 challenges this assignment with the observation that a prominent emission near this wavelength is observed in Tribonema and Anacystis under a variety of excitation conditions. Neither of these algae contains Chlβ. Further, as indicated earlier and illustrated in Table 2, the emission wavelength for isolated light-harvesting Chl αβ protein complexes (LHC) tends to be closer to 681-682 nm than to 685 nm (see also reference 21). Moreover, the isolated LHC's have absorption maxima near 671-672 nm and not at 680 nm as is characteristically observed for in vivo absorption (Table 2, note both PSI and PSII fractions). Although such small differences in absorption maxima are not necessarily highly significant, they must be taken into account in arriving at an assignment of the 685 nm emission to an emitting species of specific composition. Finally, we note that fluorescence maxima in the 680-690 nm spectral region can be observed under a number of conditions. For example, as indicated in Figure 2, fluorescence maxima near 685 nm can be obtained from Chlα in a methylcyclohexane/ethanol solution under appropriate conditions of excitation wavelength and temperature. It has been suggested that in this case, the emitting species may be a (Chl)a oligomer coordinated to ethanol9. We have also seen weak emissions at 668 and 685 nm in Nujol mulls of H2O-saturated octane. We observe a red shift of the emission wavelength for monomeric Chlα in pyridine as the Chlα concentration increases11, a phenomenon that can be related to self-absorption in concentrated solutions. These various observations suggest that it is, at present, not possible to make a definitive attribution to the source of the in vivo 680-690 nm fluorescence.

Questions also arise about the origins of both the 695 nm and 710-730 nm fluorescence. One view is that these arise from antenna chlorophylls in PSI and PSII, respectively13,14. It has been suggested15 that F695 arises from a species of chlorophyll in PSII, which forms on cooling to temperatures below 113 K and that the 710-730 nm
fluorescence also arises from a species formed at low temperature in PSI. The latter species is designated by Satoh and Butler as C705\textsuperscript{15}. These authors consider that this species competes with P700 for the excitation energy in PSI. This interpretation is based on the assumption that there is no significant temperature effect on any of the fundamental rate constants for deexcitation by any pathway including nonradiative decay. The factor responsible for the large increase in fluorescence with decreasing temperature is presumed to be an increase in the concentration of C705 traps\textsuperscript{15,16}. Butler et al.\textsuperscript{17} suggested that this species may be present at all temperatures but becomes more fluorescent at low temperatures. It would appear equally reasonable to assume that the increase in the fluorescence quantum yield could be associated with the formation of concentrations of P700 species in excess of those required to carry out the photochemistry. Thus, it appears unnecessary to assume that C705 is qualitatively different from P700. Both the increase in fluorescence yield and the increase in fluorescence lifetime\textsuperscript{8,22} of PSI emitting fractions at low temperature are in accord with this view. We note that the in vitro experiments on self-assembly suggest the formation of a number of species or configurations that have P700 or special pair-like properties\textsuperscript{9}.

Implicit in the preceding discussion is the presumption that the low temperature PSI fluorescence arises from special pair type molecules, i.e. nucleophile-containing chlorophyll species. We know of no experimental observations indicating significant fluorescence from any chlorophyll species that are not monomeric or oriented by bifunctional nucleophiles. If, therefore, the antennae are oligomeric chlorophyll species, as is suggested by the absorption spectra\textsuperscript{8,10,12} then no antenna fluorescence should be observed. Thus, the observed fluorescence associated with PSII, F685 and F695, would appear to also involve chlorophyll molecules coordinated to a ligand.

As indicated in Table 1, excitation of intact Tribonema at 337 nm suspended in their growth medium at room temperature results in a broad fluorescence emission with maxima at 690 nm and 740 nm. Cooling to 77 K narrows the fluorescence emission band and shifts the principal emission band to approximately 720 nm, with a distinct shoulder in the 690 nm region. Warming the solutions to room temperature results in reversion to the original fluorescence band shapes and maxima.

When 450 nm radiation is used for excitation at room temperature the primary emission band from Tribonema is at 686 nm with a broad tail to the red. The primary band has a shoulder at approximately 673 nm, near the wavelength expected for monomer Chl \textit{a} fluorescence. This is also the case for \textit{Anacystis} (see Figure 3A).
As indicated earlier, this emission cannot originate from a Chl/b protein complex since these algae contain no Chl b. Cooling to 77 K results in spectral changes similar to those observed with 337 nm excitation, and the red maximum is near 715 nm.

The results obtained with 672 or 681 nm radiation on Tribonema are particularly interesting. At room temperature the primary emission band is now observed at 683 nm (Figure 3D). The only other condition under which we see a band at this wavelength is at low temperature. When the excitation wavelength is 672 nm, there is evidence for a shoulder in the 685 nm region. The primary emission band (695 nm) is relatively narrow, suggesting that the species responsible for the emission may be well defined in a structural sense. On cooling, the fluorescence emission is red shifted and broadened and at 148 K, the primary emission is observed near 705 nm. Obviously, the observations in this case are not consistent with the assumption that the F695 forms on cooling or that F695 is present, but nonfluorescent at room temperature (see prior discussion).

An important aspect of the present observations is that both the emission wavelengths and the shape of the emission bands depend on the excitation wavelength as well as on temperature. For example, in Figure 3A and 3C we show room temperature fluorescence spectra for Anacystis and Chlorella excited by 450 nm radiation. At room temperature, the principal emission for both algae is at 685 nm. The major difference is the shoulder near 650 nm in Anacystis resulting from phycocyanin emission. We contrast these results with those of Figure 3B, where Anacystis is excited by 627 nm radiation, i.e., in the absorption region of phycocyanin. In this case, at room temperature the principal emission is at 651 nm with a secondary maximum at 708 nm. This suggests that the fluorescence is dominated by emission from phycocyanin. Such an assignment agrees with the observations of Dale and Teale who found that c-phycocyanin isolated from Anacystis has a fluorescence maximum at 651 nm with a secondary maximum near 710 nm. The fact that we see such a strong phycocyanin emission at 651 nm but not the Chl a maximum at 682-685 nm suggests that energy transfer from phycocyanin to Chl a may be slow. If the pigment composition were homogeneous and the energy transfer fast, no dependence of the fluorescence on the excitation wavelength would be observed.

The fluorescence intensity of phycocyanin at wavelengths longer than 670 nm is much less than that of Chl
when the pure pigments are excited at their respective absorption maxima (Figure 2A). A comparison of these spectra with the room temperature spectra of Figure 3A and 3B shows that there must be a significant contribution by Chla to the Anacystis fluorescence at wavelengths longer than 670 nm. At low temperature this comparison strongly suggests that the fluorescence maxima at 682 and 715 nm as well as the shoulder in the 690-700 nm region (Figure 3B) are contributed by Chlz species. The marked difference in the fluorescence spectra of Anacystis obtained with different excitation wavelengths suggests that in addition to possibly slow energy transfer steps there may be a significant heterogeneity in the pigment distribution. Such a heterogeneity, coupled with a slow rate of excitation energy transfer between the different structures would be consistent with the observed dependence of the fluorescence wavelengths on excitation frequency. This interpretation is the same as that advanced to explain a similar phenomenon in vitro chlorophyll solutions containing a mixture of species.

Concluding Remarks

Fluorescence emission from the algae Tribonema aestuarii, Anacystis nidulans, and Chlorella vulgaris have been recorded as a function of excitation wavelength and temperature. Light emission from the algae are found to depend on both wavelength and temperature. The fluorescence data suggest that there are slow steps in energy transfer between pigment systems in these algae. We observe considerable similarity in the effects of excitation frequency and temperature on the fluorescence of intact algae and that of suitably chosen in vitro chlorophyll systems. The parallelism in behavior between in vivo and in vitro systems suggest that self-assembly processes result in the formation of new chlorophyll species not present at room temperature in photosynthetic organisms as a result of lowering the temperature, and that as a consequence, interpretation of low temperature observations in terms of the photosynthetic species present at ordinary temperatures may be open to question.

Acknowledgement

References


Figure Legends

Figure 1. Absorption spectra.

A. Pigments

... Chl a in ethanol-methylcyclohexane at 298 K. Chl a in ethanol-methylcyclohexane at 150 K. (Self assembled special pair.)

Chl a = 2.25 x 10^{-3} M.

Ethanol = 3.6 x 10^{-2} M.

x—x β-carotene in octane (Absn. scale not comparable with Chl a).

—— Phycoerythrin in 0.1 M phosphate buffer.

pH = 5.9 (Absorption scale not comparable with Chl a).

.... Phycocyanin in 0.1 M Na phosphate buffer.

pH = 5.9 (Absorption scale not comparable with Chl a).

B. *Anacystis nidulans*

*Anacystis* (H_{2}O), suspended in glycerol, 298 K.

↓ Wavelengths for selective excitation.

450, 696 nm, "self-assembled" special pairs.

620 nm, phycocyanin.

660 nm, monomer Chl a.

670, 680 nm, oligomer (antenna) Chl a.

I. Chlorophyll a Soret.

II,III. Auxiliary pigments.

IV. Chlorophyll a, Q_{y} region.
Figure 2. Fluorescence spectra of pigments.

A. 298 K, fluorescence scales for different pigments not comparable.

... Phycoerythrin in 0.1 M Na phosphate buffer.

pH = 5.9, λ_{exc} = 337 nm.

— Phycocyanin in 0.1 M Na phosphate buffer.

pH = 5.9, λ_{exc} = 627 nm (Note Figure 1B).

x—x Chlorophyll a in ethanol/methylcyclohexane.

λ_{exc} = 672 nm, similar spectra obtained with 681 nm excitation.

PSII, PSI, ↓ Fluorescence regions assigned to Photosystems I and II in vivo, see text.

B. Phycocyanin in 0.1 M Na phosphate buffer.

pH = 5.9, 130 K, λ_{exc} = 627 nm.

x—x Chlorophyll a in ethanol/methylcyclohexane, 150 K, λ_{exc} = 672 nm. Similar spectrum obtained with 681 nm excitation.

Figure 3. Fluorescence spectra of algae. S indicates a shoulder.

A. *Anacystis* (H_{2}O) suspended in growth medium.

λ_{exc} = 448-451 nm.

... 290 K.

... 77 K.

B. *Anacystis* (H_{2}O), growth medium.

λ_{exc} = 627 nm.

... 298 K.

... 77 K.

C. *Chlorella* (H_{2}O), glycerol suspension.

λ_{exc} = 450 nm.

... 298 K.

... 77 K.
D. *Tribonema* (*H₂O*), culture medium.

\[ \lambda_{\text{exc}} = 672 \text{ nm} \ (\lambda_{\text{exc}} = 680 \text{ nm}, \text{ also shows } 693.4 \text{ nm band}) \]

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298 K

77 K
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Footnotes for Table 1

a\textsuperscript{β}-carotene absorption max 468 broad with S445, 496. No fluorescence when excited at 430-50.

b Xanthophylls (oxycarotenes), like carotenes, absorption 430-500 nm, weakly fluorescent.

c According to Rijgersberg and Amesz,\textsuperscript{20} the 650 nm emission in Anacystis involves two bands contributed by C-phycocyanin and allophycocyanin. Bands at 650 nm and 680 nm obtained from isolated phycobilisomes are ascribed to allophycocyanin and allophycocyanin B, respectively.

d Very little room temperature fluorescence when pumped at 680-85 and 695 nm.

e The Chlorella used for the fluorescence studies were suspended in glycerol.

f The 694 and 706 nm emissions represent band heads in a very broad emission spectrum with a shallow minimum at 700 nm.
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<td>CPII chlorophyllin$^e$</td>
<td>298</td>
<td>673-77,490$^d$</td>
<td>685,725,735(sh)$^e$</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td></td>
<td>685,695,705,720,735</td>
</tr>
<tr>
<td>PSI Fraction$^g$</td>
<td>298</td>
<td>437,680</td>
<td>682(730-740)$^b$</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td></td>
<td>730,735</td>
</tr>
<tr>
<td>PSI, Antenna and Reaction Center$^g$</td>
<td>77</td>
<td>681</td>
<td>730-58,$^h$</td>
</tr>
<tr>
<td>PSII Fraction</td>
<td>298</td>
<td>436,471</td>
<td>682(730-740)</td>
</tr>
<tr>
<td>LHC, Chl $a/b$ Protein$^a$</td>
<td>77</td>
<td>653,680</td>
<td>683-4,695-6</td>
</tr>
<tr>
<td>Antenna, trap$^g$</td>
<td>77</td>
<td>650,677</td>
<td>694-5,$^h$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>670(sh)</td>
<td></td>
</tr>
<tr>
<td>CPII chlorophyllin$^i$</td>
<td>298</td>
<td>652,672</td>
<td>681</td>
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<tr>
<td>Chl $a/b$ chlorophyllin</td>
<td></td>
<td>475</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Searle et al.$^{18}$

$^b$Very weak emission.

$^c$Thornber et al.$^{24}$

$^d$β-carotene-chlorophyllin, Thornber et al.$^{24}$

$^e$shoulder, sh

$^f$Emission data from Brown$^{25}$, both 290 K and 77K. At 77 K, 685 nm band decreases relative to 695 nm band and amplitude bands 705, 720, 735 variable with different preparations.

$^g$Kitajima and Butler$^{26}$. Assignment of absorption wavelength based on excitation spectra.

$^h$Butler$^{16}$.

$^i$CPII chlorophyllin - light harvesting Chl $a/b$ protein complex in PSII. May be complex mixture including oligomeric forms. See reference (6).
Figure 2
Figure 3