

The Position of Carotene in the D-1/D-2 Sub-Core Complex of Photosystem II

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When the sub-core complex of photosystem II, D1/D2, is irradiated at 436 or 415 nm (absorption by chlorophyll and pheophytin and β -carotene) or 540 nm (absorption primarily by pheophytin), the low temperature fluorescence spectrum has two maxima, at 685 and 674 nm. This shows the existence of at least two different fluorescent forms of chlorophyll (chlorophyll *a* and perhaps pheophytin *a*). When carotene is irradiated at 485 nm (absorption primarily by β -carotene), only fluorescence at 685 nm is observed: this indicates that carotene is transferring energy to only the long-wavelength form of chlorophyll in the D1/D2 sub-core complex. The band of carotene (at 485 nm) does not appear in the fluorescence excitation spectrum, measured at 674 nm. The position of the carotene molecule relative to each of the fluorescent forms of chlorophyll was determined from the excitation spectra of each of the fluorescence bands.

Introduction

The photosynthetic reaction center that carries out the photolysis of water, photosystem-II (PS-II), is composed of three non-pigment extrinsic polypeptides [1] and six different pigment-protein complexes. The latter include CP-47 kDa and CP-43 kDa, two polypeptides of cytochrome *b*-559, and the polypeptides D-1 and D-2 (about 30 kDa) [2, 3], plus sixteen units of light harvester complex (LHC-II) CP-25 kDa. The site of primary charge separation in PS-II appears to be localized in the D-1 and D-2 polypeptides [4]. The spectral properties of PS-II prepared according to Berthold *et al.* [5] and referred to as BBY particles, were reported by Dorssen *et al.* [6].

The carotenes in PS-II are organized in pigment-protein complexes. The presence and number of β -carotene in intact PS-II particles have been established (for a review see [7]). The β -carotene in thylakoid membranes are about 80% *all-trans*- β -carotene and about 20% of various *cis* isomers [8]. The presence and identity of isomers in PS-II and effect of light on the carotenes were reported by Ashikawa *et al.* [9]. Based on the abundance of carotenoids in thylakoid membranes it is suggested that they serve some role in photosynthetic reactions or in stabilizing the photosynthetic apparatus [7].

The D-1/D-2 sub-core complex of photosystem II contains one β -carotene molecule, four molecules of

chlorophyll *a* and two of pheophytin *a* [4, 10]. The function of β -carotene and its position in the complex, relative to chlorophyll, is unknown. When irradiating at 436 nm, the low temperature fluorescence spectrum has two maxima indicating the existence of at least two different fluorescent forms of chlorophyll (including pheophytin). To obtain information as to the position of the β -carotene molecule relative to the fluorescent forms of chlorophyll, excitation spectra are measured for each of the two (chlorophyll) fluorescence maxima.

Materials and Methods

PS-II particles were prepared as described by Kuwabara and Murata [1, 11]. The D-1/D-2 sub-core particles were prepared from the PS-II particles as described by Nanba and Satoh [4]. PS-II particles (10 mg chlorophyll) were suspended in 10 ml of 50 mM Tris-HCl (pH 7.2) containing 4% Triton X-100, and stirred for 1 h, at 4 °C. The particles were centrifuged at 10,000 $\times g$ for 1 h, and the supernatant was recovered. The HPLC column was a TSKgel DEAE-5pw (18 cm \times 1.5 cm) which was equilibrated with 50 mM Tris-HCl (pH 7.2) containing 30 mM NaCl and 0.05% Triton X-100. The column was washed for 2 h with the same medium as used for equilibration to remove a large amount of LHC (light harvesting chlorophyll). The D-1/D-2 complex was then eluted with a NaCl gradient from 30 mM to 200 mM Tris-HCl (pH 7.2) and 0.05% Triton X-100

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for 60 min. The D-1/D-2 was eluted at about 150 mM NaCl. To clean the column it was washed with 500 mM NaCl, 50 mM Tris-HCl (pH 7.2) and 0.05% Triton X-100 for 30 min. A flow rate of 3 ml/min was used for all chromatographic procedures. Elution of the chlorophyll-containing complex was monitored at 670–674 nm. The column temperature was kept at 5 °C. The preparations were kept in total darkness and in an ice bath, until subjected to experimentation. The purity of the preparation was assayed by its absorption spectrum. The absorption spectra of the D-1/D-2 particles was identical to that reported by Nanba and Satoh [4]. Absorption spectra were measured with a Shimadzu double beam, difference/dual wavelength recording spectrophotometer UV-300.

The activity of the sub-core complex was measured using the same procedure described by Nanba and Satoh (using Na dithionite and methyl viologen). The maximum light induced change in absorbance measured between 450 and 422 nm was 0.055, compared to the 0.07 change reported by Nanba and Satoh [4]. This absorbance change includes a correction for the difference between the concentration of chlorophyll used in the present measurement (which was 1.1 μM), and that used by Nanba and Satoh (which was 4.8 μM).

The position of the emission maximum, from D-1/D-2, is very sensitive to the composition of the suspending media and presence or absence of the 33 kDa protein. When these variables are controlled the 90% confidence limits for the wavelengths of the emission maxima are ± 0.4 nm. For fluorescence measurements the particles were usually suspended in 50 mM Tris-HCl (pH 7.2), 120 mM NaCl, 0.05% Triton. Fluorescence and excitation spectra were measured with a Hitachi model 850 fluorescence spectrophotometer. Slit widths were adjusted to give a resolution of 2 nm when measuring fluorescence spectra and 3 nm for excitation spectra. Room temperature spectra were measured in a 1 cm cuvette, using a right angle between the excitation light and the fluorescence observation. Low temperature spectra were measured using front surface illumination in a 1 mm sample holder. To minimize self absorption the absorbance of the red absorption band was less than 0.05 (chlorophyll concentration 0.7 $\mu\text{g}/\text{ml}$) for all measurements of fluorescence. A red filter (HOYA Glass Co. R-61) was used for fluorescence measurements to eliminate second order effects from the exciting light.

Results and Discussion

Photosystem II particles: Excitation of fluorescence with blue light (436 nm), at 77 °K, results in a fluorescence spectrum having two bands, with maxima at about 695 and 685 nm (Fig. 1). However, when the exciting light is 485 nm (an absorption band of carotenoid) there is only a single fluorescence band, with maximum at 695 nm; there is almost no emission at 685 nm (Fig. 1). These observations indicate that a carotenoid is located in the vicinity and transfers energy preferentially to chlorophyll, F695 and not chlorophyll, F685. Of interest is the observation that the position of the maximum of the secondary peak also depends on the wavelength of the exciting light. When excitation is at 436 nm the maximum is at 732 nm, when exciting at 485 nm the maximum is at 742 nm (Fig. 1).

Fig. 1

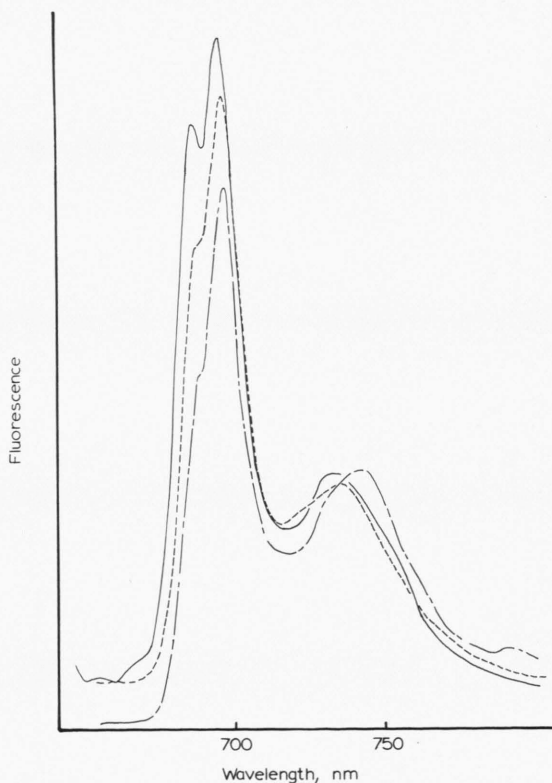


Fig. 1. Fluorescence spectra of photosystem II particles, at 77 °K, as a function of wavelength of exciting light. The spectrum when irradiating with light of wavelength 485 nm is shown by the curve with long dashes (---), irradiating with 470 nm the spectrum is shown by short dashes (-----), irradiating with 435 nm it is shown by a solid line (—).

D-1/D-2 sub-core complexes of photosystem II: The absorption bands at room temperature of D-1/D-2, of interest in this work, are identified as follows: chlorophyll *a* at 435 nm, carotenoid at 485 and 468 nm, pheophytin *a* at 540, 510 and 415 nm.

Room temperature: There appears to be several fluorescent forms of chlorophyll at room temperature. This is indicated by the observation that the wavelength of the fluorescence maximum, is dependent upon the wavelength of the exciting light. When excitation is at 438 or 540 nm the fluorescence maximum is at 681.0 ± 0.4 nm. When primarily the protein is excited at 285 nm, the fluorescence maximum is shifted toward the blue (to 679.0 ± 0.4 nm) (Fig. 2). On the other hand, when excitation is at 485 nm the fluorescence maximum is shifted to the red (to 682.0 ± 0.4 nm). When the 33 kDa protein is present in the medium the emission maxima from D-1/D-2 are shifted; when illuminating at 285, 438 or 485 nm results in maxima at 676.0 ± 0.5 , 677.5 ± 0.5 or 680.0 ± 0.5 nm, respectively. These spectral variations can be accounted for by the presence of at least two different species whose fluorescence may be sensitized by different accessory pigments. Illuminating primarily the protein results in emission mainly from the short wavelength form of chlorophyll. When β -

carotene is illuminated, fluorescence is primarily from the long wavelength emitting form of chlorophyll. Illuminating chlorophyll or pheophytin gives rise to a similar fluorescence spectrum, containing both forms of chlorophyll. The fact that irradiation of chlorophyll or pheophytin results in the same fluorescence spectrum, indicates that energy absorbed by pheophytin essentially transfers all its energy to chlorophyll. Since the absorption spectra of β -carotene, chlorophyll and pheophytin overlap each other in the blue region of the spectrum, one cannot easily evaluate the role of pheophytin in sensitizing chlorophyll or its own fluorescence. The possibility of self absorption was carefully considered (see below).

Low temperature: When D-1/D-2 is illuminated with 436 nm light, at low temperature (77 °K) there are two well resolved fluorescence maxima (at 672.5 and 682 nm). The fluorescence spectrum, at 77 °K, depends on the exciting wavelength; e.g. exciting with 452, 460, 470, 485, or 490 nm (wavelengths of strong absorption by β -carotene) gives a single emission maximum near 682 nm (Fig. 3). The relative intensities of the two emission bands varies somewhat; their relative intensities depend on the percent detergent, salt concentration, and presence of 33 kDa protein. The fluorescence spectrum, in the presence of the 33 kDa protein, when excited with light of wavelength 530, 460 and 440 nm is shown in Fig. 3; irradiating with either 440 or 415 nm gives the same fluorescence spectrum.

The excitation spectra for the emission band at 682 and the one near 674 nm (Fig. 4) are quite different in the spectral region where carotenoids are the major absorbing species. The excitation spectrum for fluorescence at near 674 nm has a minimum at 487 nm, while the excitation spectrum for fluorescence at 682 nm has a maximum at 487 nm.

The different fluorescence spectra observed both at room temperature and 77 °K indicates either that the chlorophylls in D-1/D-2 are not all in the same environment or that there is also emission from pheophytin. Based on these observations, in the D-1/D-2 sub-core complex, β -carotene appears to be located in the vicinity of the chlorophyll that emits at the longest wavelength (at 684 nm) and not the chlorophyll (or pheophytin) that emits around 674 nm. Likewise, in PS-II, β -carotene is in the vicinity of the chlorophyll that emits at the longest wavelength (at 695 nm).

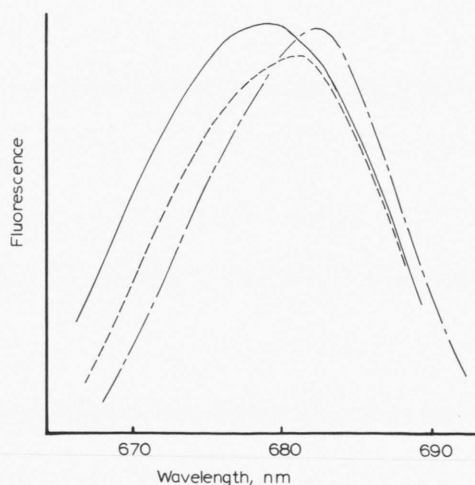


Fig. 2. Fluorescence spectra of D-1/D-2 sub-core complexes of photosystem II, at room temperature, as a function of exciting wavelength. The spectrum when irradiating with 285 nm is shown by a solid line, irradiating with 438 nm is shown by short dashes (-----), irradiating with 485 nm is shown by long and short dashes (-----).



Fig. 3. Fluorescence spectra of D-1/D-2 sub-core complexes of photosystem II, at 77 °K, as a function of both the wavelength of the exciting light and presence of the 33 kDa protein. The spectrum of D-1/D-2 when irradiating with 435 nm is shown by curve with open circles (—○—), when irradiating with 490 or 485 or 470 nm is shown by long dashes (---). The spectrum of D-1/D-2 in the presence of the 33 kDa protein, when irradiating with 530 nm is shown by short dashes (----), when irradiating with 460 nm it is shown by a solid line, when irradiating with 415 or 440 nm it is shown by long and short dashes (---).—).

The question arises whether or not the single chlorophyll fluorescence band observed when β -carotene is irradiated (at 452 or 460 nm) results from self absorption. Firstly, as pointed out in the Materials and Methods the absorbance was very low to minimize self absorption. As an experimental test for self absorption the fluorescence spectra were compared when excitation was at different wavelengths, but at wavelengths where the absorbance was similar. Chlorophyll fluorescence was excited by irradiat-

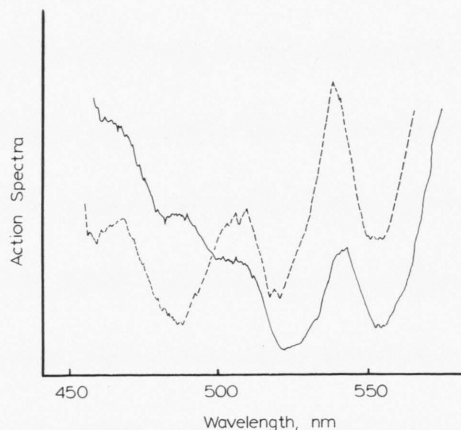


Fig. 4. Fluorescence excitation spectra of the D-1/D-2 sub-core complex of photosystem II, at 77 °K. The excitation spectrum for fluorescence near 672 nm is shown by the curve with a broken line (---), the spectrum for fluorescence at 682 nm is shown by the curve with a solid line.

ing in spectral region 500 to 600 nm (absorption by chlorophyll and pheophytin), where absorbencies are comparable to that of the carotenoid spectral region (450 to 490 nm). When exciting chlorophyll fluorescence at 510 or 540 nm, the emission spectrum has two maxima. While absorbance at 460 nm is about the same as at 510 and 540 nm, nevertheless irradiating at 460 nm results in only a single fluorescence band. On this basis we conclude that the single emission maximum observed when irradiating carotenoid (452 or 460 nm) is not an artifact arising from self absorption.

We have also considered the possibility that the emission at 674 nm arises from chlorophyll in β -carotene-free micelles and not D-1/D-2. The D-1/D-2 samples used for these measurements are used immediately after they are collected from the HPLC, so there is little possibility of contamination by chlorophyll in micelles. Furthermore, the same selective excitation by β -carotene of only one fluorescence chlorophyll band, is also observed with PS-II particles. So the spectral properties reported probably result from chlorophyll associated (in some manner) with the D-1/D-2 complex.

The fluorescence maximum at 674 nm might be comparable to what one observes from chlorophyll in solution or bound to protein [12]. Perhaps one or more of the chlorophyll's in D-1/D-2 may be exposed to chemical regions having an elevated dielectric con-

stant and/or index of refraction, so that there is a significant spectral shift. The presence of β -carotene near one or two molecules of chlorophyll might cause a (bathochromic) spectral shift of the chlorophyll molecules similar to that reported in compressed monomolecular films of chlorophyll and a carotene [13].

Danielius *et al.* [10] showed that the recovery of the flash-induced change in absorption at 680 nm, in D-1/D-2 sub-core complexes, occurs in nanoseconds and is at least biphasic. The fastest component (5 nsec) is ascribed to an "unconnected chlorophyll", which does not transfer energy to chlorophyll absorbing at 680 nm. The relative contribution of the 5 nsec component is larger when measured at 675 nm than at 680 nm. The fast, 5 nsec, unconnected chlorophyll component might be associated with the fluorescence maximum at 674 nm, at 77 °K, reported in the present study.

The fluorescence at 674 nm might originate from pheophytin. When fluorescence is sensitized by irradiating at 435 nm, light is absorbed by β -carotene,

chlorophyll and pheophytin *a*. When primarily β -carotene is irradiated, at 470 or 485 or 490 nm, only a single fluorescence band is observed around 685 nm. When primarily pheophytin is irradiated, at 530 nm, in addition to the peak around 685 nm one also observes the fluorescence band at 674 nm. The latter band could originate from pheophytin *a*.

It appears that some of the light energy absorbed by pheophytin can be transferred to chlorophyll *a*. In the excitation spectra, Fig. 4, the two maxima near 510 and 540 nm correspond to the absorption maximum of pheophytin *a*. The peak at 540 nm, in the excitation spectrum for fluorescence at 682 nm, indicates that some energy absorbed by pheophytin *a* is transferred to chlorophyll *a*.

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