Rebinding of the 33 kDalton Polypeptide of Photosystem II to the D-1/D-2 Sub-Core Complex

Seymour Steven Brody

Department of Biology, New York University New York, N.Y. 10003, U.S.A.

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Specific and stoichiometric binding is shown between the D-1/D-2 sub-core complex and the 33 kDa polypeptide of photosystem II. Fluorescence from chlorophyll is used as an endogenous probe. When binding occurs there is an increase in fluorescence yield, as well as changes in both the fluorescence spectrum and excitation spectrum.

Introduction

The photosystem II (PS-II) reaction center is composed of several polypeptides which contain carotenoids and chlorophyll. Three pigment-free extrinsic proteins, with masses 33, 24, 18 kDa, are involved in the oxygen evolving system [1]. One of the extrinsic proteins, a 33 kDa polypeptide, accelerates a dark step in the oxygen-evolving reaction [2] and preserves the binding of the Mn atoms to the oxygen-evolving complex [3]. The 33 kDa protein is readily detached from the PS-II reaction center. The 33 kDa polypeptide is necessary to both preserve binding of 2 of the 4 Mn atoms to PS-II [3] and maintain the conformation of the Mn cluster. The 33 kDa polypeptide also accelerates the dark step in evolution; removal of 33 kDa retards the S_3-S_0 transition. Other proteins in the oxygen evolution process presumably function in light harvesting, regulatory and ion concentrating [4].

The object of these experiments is to determine if the 33 kDa protein will rebind to the D-1/D-2 sub-core complex of photosystem II. Chlorophyll in the D-1/D-2 complex is used as an endogenous fluorescence probe to assay binding of the 33 kDa protein to the complex. Evidence for binding between D-1/D-2 and 33 kDa was obtained by observing the spectral changes induced upon addition of the 33 kDa polypeptide in: (1) room temperature, excitation spectra for chlorophyll fluorescence; (2) spectrum and yield of chlorophyll fluorescence; (3) low temperature, chlorophyll fluorescence spectra; (4) fluorescence yield as a function of the mole ratio of 33 kDa to D-1/D-2. The latter yields the stoichiometry, and evidence for specific binding between the 33 kDa polypeptide and the D-1/D-2 sub-core complex.

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Materials and Methods

The 33 kDa was prepared as described by Miyao *et al.* [2] and Kuwabara and Murata [1]. The 33 kDa was suspended in 30% (v/v) ethylene glycol, 200 mM NaCl, 300 mM sucrose, 25 mM Mes-NaOH, pH 6.5. The D-1/D-2 sub-core complex was prepared as described by Nanba and Satoh [5]. PS-II particles (10 mg chlorophyll) were suspended, at 4 °C, in 10 ml of 50 mM Tris-HCl (pH 7.2), 4% Triton X-100, and stirred for 1 h. The supernatant was recovered after the particles were centrifuged at $10{,}000 \times g$ for 1 h. The HPLC column was a TSKgel DEAE-5pw (18 cm × 1.5 cm) which was equilibrated with 50 mM Tris-HCl (pH 7.2), 30 mM NaCl and 0.05% Triton X-100. The column was washed for 2 h to remove a large amount of LHC (light harvesting chlorophyll). The D-1/D-2 complex was eluted with a NaCl gradient from 30 mM to 200 mM Tris-HCl (pH 7.2), 0.05% Triton X-100, for 60 min. The D-1/D-2 was eluted at about 150 mM NaCl. The column was cleaned by washing with 500 mM NaCl, 50 mM Tris-HCl (pH 7.2), 0.05% Triton X-100, for 30 min. A flow rate of 3 ml/min was used. 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 [5]. Activity of the D-1/D-2 sub-core complex was verified using the same reaction described by Nanba and Satoh [5]. In the presence of Na dithionite and methyl viologen the light induced

change of absorption by D-1/D-2, between 450 and 422 nm, was 0.055 (compared to 0.07 reported by Nanba and Satoh). This activity would indicate that denaturation of D-1/D-2 in our preparations was minimal.

For quantitative measurements of fluorescence it is necessary that the suspending media for the D-1/D-2 and 33 kDa preparations have identical compositions. The 33 kDa protein and D-1/D-2 sub-core complexes were each suspended in identical media by using dialysis to adjust the composition of their media. Unless otherwise noted the suspending medium for both the D-1/D-2 and 33 kDa preparations was 50 mM Tris, pH 7.2, 50 mM NaCl and 0.05% Triton.

Fluorescence and excitation spectra were measured with a Hitachi fluorescence spectrophotometer (model 850).

The concentration of D-1/D-2 was determined by spectrophotometrically assaying the total concentration of chlorophyll and pheophytin, and from the fact that there are four chlorophyll and two pheophytin molecules per D-1/D-2 [6]. The chlorophyll was extracted with acetone (80%) and its absorbance at 663 nm, A_{663}, was measured. The chlorophyll concentration (μg/ml) was set equal to $12.19 \times A_{663}$. Dividing by the molecular weight (of chlorophyll) to obtain the molar concentration and then dividing by six (the total number of chlorophyll's in D-1/D-2) gave the molar concentration of D-1/D-2.

The concentration of 33 kDa protein was determined from its absorbance at 276 nm and the molar absorption coefficient. The molar absorption coefficient used was 24.8 mM^{-1} cm^{-1} at 276 nm [7], which is based on the amino acid analysis of 33 kDa polypeptide that gives a molecular weight of 26,633.

Results and Discussion

The spectral properties and fluorescence yield of D-1/D-2 are markedly modified, when the medium is altered, *e.g.* salt concentration, addition of $MgCl_2$, or different Triton concentrations. The fluorescence yield of D-1/D-2 is greater in 0.05% Triton than in 0.01% (probably brought about by minor configurational changes induced by the detergent).

Room temperature fluorescence excitation spectra

Excitation of the UV band at 250–285 nm (absorbed both by chlorophyll and protein) of PS-II particles or D-1/D-2 sub-core complex can sensitize room temperature chlorophyll fluorescence. The fluorescence spectrum, excited at 285 nm, is different to that obtained when the pigments alone (chlorophylls and carotene) are excited at 438 nm. For the D-1/D-2 complex, the excitation spectrum for chlorophyll fluorescence at 685 nm, is shown in Fig. 1 (curve 1). The ratio of the excitation bands 280 nm to 436 nm (chlorophyll plus carotene) is equal to 0.56. When the 33 kDa protein is added to a suspension of D-1/D-2 complex the fluorescence excitation spectrum of chlorophyll is modified. The ratio of the excitation bands, 280/436, increases to a value of 0.65 (Fig. 1, curve 2). Addition of 33 kDa induces other changes in the shape of the excitation spectrum (see Fig. 1). These spectral changes show that 33 kDa protein (which has an absorption maximum at 280 nm) is binding to D-1/D-2 complex and efficiently transferring excitation energy to the chlorophyll molecule. To test for specific interaction between D-1/D-2 and 33 kDa, instead of 33 kDa, BSA (bovine serum albumin) is used. When BSA is added there are only minor changes in the spectral properties of D-1/D-2.

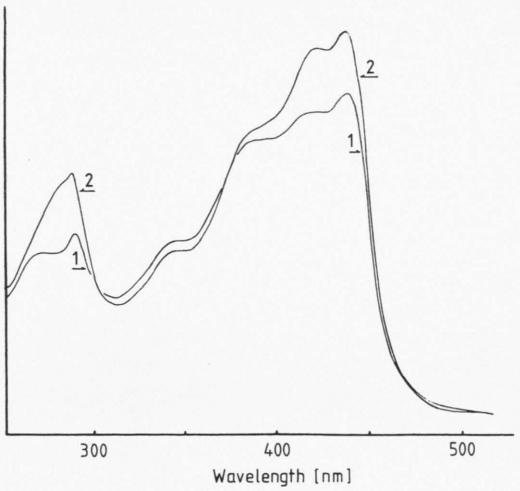

Fig. 1. Room temperature fluorescence excitation spectra, fluorescence wavelength is 685 nm. Curve 1 is a suspension of D-1/D-2 sub-core complex. Curve 2 is a suspension containing both 33 kDa protein and D-1/D-2 (mol ratio 2:3, respectively). The ratio of the protein band (278 nm) to the chlorophyll band (436) increases 15% when 33 kDa is added to the suspension containing D-1/D-2. The suspending media is 50 mM Tris, pH 7.2, 50 mM NaCl, 0.05% Triton.

Room temperature fluorescence spectra

The room temperature fluorescence maximum of D-1/D-2, is at 681 nm, when excitation is at 436, 415 or 540 nm. When energy is absorbed at 285 nm (absorption by chlorophyll and protein), the fluorescence is primarily from a short wavelength form of chlorophyll (Fig. 2, lower curves). Furthermore, the form of chlorophyll emitting at short wavelength does not transfer all its energy to the long wavelength form of chlorophyll.

Upon addition of 33 kDa to D-1/D-2 the fluorescence maximum is blue shifted (Fig. 2, upper curves). When excited by 438 nm the maximum is at 678 nm; when excited by 285 nm the maximum is at 676 nm. The fluorescence yield is increased about 20%, upon addition of the 33 kDa polypeptide.

Low temperature fluorescence spectra

Evidence for binding between 33 kDa protein and D-1/D-2 complex is also shown by the low temperature (77 °K) fluorescence spectrum of chlorophyll. The fluorescence yield increases upon addition of the 33 kDa polypeptide. When D-1/D-2 is irradiated at 440 nm the spectrum is shown in Fig. 3 (curve 1); the ratio of F682/F672 = 1.3. When 33 kDa is added there is a marked change in the spectrum; the ratio of F682/F672 decreases to 0.78 (Fig. 3, curve 2). The 33 kDa polypeptide promotes emission from the chlorophyll emitting at the 'short' wavelength.

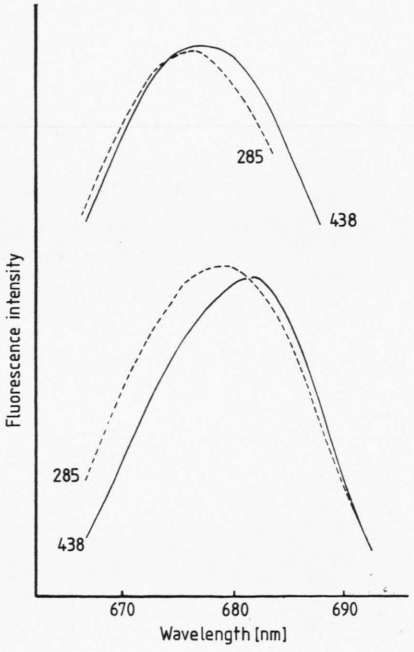

Fig. 2. Room temperature fluorescence spectra of D-1/D-2 sub-core complex. Lower curves are the fluorescence spectra of D-1/D-2 when irradiating at 438 nm (absorption by chlorophyll and carotene) (solid line), and when irradiating at 285 nm (absorption by chlorophyll and protein) (broken line). Upper curves are the fluorescence spectra of D-1/D-2 in the presence of 33 kDa. Irradiation with 438 nm and 285 nm are shown by solid and broken lines, respectively.

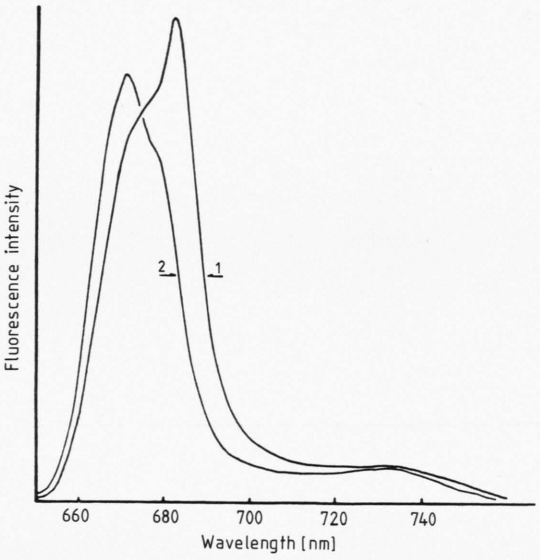

Fig. 3. Low temperature (77 °K) fluorescence spectrum of the D-1/D-2 sub-core complex (curve 1), in a suspending media as described in Fig. 1. When 33 kDa protein is added to the suspension the spectrum of the D-1/D-2 complex becomes as shown by curve 2. Fluorescence is sensitized by irradiation at 440 nm.

The fluorescence spectra reported here for D-1/D-2 are somewhat different from the spectra reported by van Dorssen *et al.* [8]. Their fluorescence spectra, at 77 °K, recorded maxima at 685 and 695 nm, a shift of 13 nm compared to the spectra shown in Fig. 3. The shift in the spectral properties undoubtedly reflect differences in the configurational state of D-1/D-2 complex used in this work and by van Dorssen *et al.* The origin of this configurational difference might arise from differences in the preparation and or suspension media for the D-1/D-2, used in the two investigations. The preparation of D-1/D-2 used in the present work serves our purpose very well. The relative intensities of the two fluorescence bands in our D-1/D-2 core complexes, are very sensitive to exter-

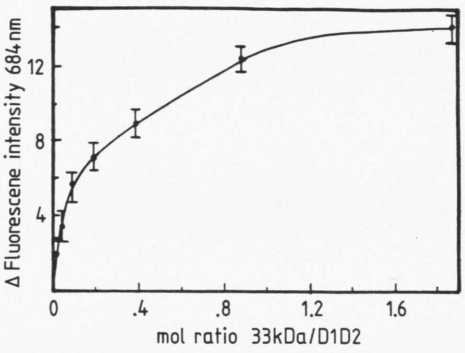

Fig. 4. Stoichiometry of binding between the D-1/D-2 sub-core complex and the 33 kDa protein. The change in fluorescence intensity of D-1/D-2 is plotted as a function of the mole ratio of 33 kDa to D-1/D-2. Suspending media is as described in Fig. 1. Fluorescence is sensitized by irradiation at 440 nm.

nal factors such as triton concentration and the presence or binding of 33 kDa protein (which is the subject of this paper).

Stoichiometry of binding between D-1/D-2 and 33 kDa

While the data in Fig. 1, 2, and 3 show there is interaction or rebinding between the 33 kDa polypeptide and the D-1/D-2 complex, additional evidence is necessary to prove that specific binding between the two has occurred. To obtain information as to whether or not the binding is specific the stoichiometry of binding is measured by quantitatively assaying the change in fluorescence yield as a function of the mole ratio of 33 kDa to D-1/D-2. Titration of the change in fluorescence yield of chlorophyll upon addition of 33 kDa protein is shown in Fig. 4. Addition of 33 kDa to D-1/D-2 complex increases the fluorescence yield of chlorophyll as the concentration of 33 kDa is increased (Fig. 4). The maximum relative increase in fluorescence yield is 26%.

From the limiting value of the fluorescence yield the binding ratio is between one and two molecules of 33 kDa to one D-1/D-2 complex.

While this data shows specific, stoichiometry rebinding of 33 kDa to D-1/D-2, it does not necessarily follow that the 33 kDa – D-1/D-2 complex has exactly the same conformation state as *in vivo*. Even in the unlikely condition, that the sub-core complex might be partially denatured, these experiments still show a selective binding of the 33 kDa by the D-1/D-2 sub-core complex.

The changes in the fluorescence and excitation spectra of D-1/D-2 upon addition of 33 kDa not only shows binding of the 33 kDa, but also that it induces configurational changes in the D-1/D-2 sub-core complex that effect the spectral properties of chlorophyll (which acts as an endogenous probe) and the efficiency of energy transfer between pigments within the complex.

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