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A Stepanov relation analysis of steady-state absorption and fluorescence spectra in the isolated D1/D2/cytochrome *b*-559 complex

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Abstract

The relation between room-temperature absorption and fluorescence spectra of the freshly prepared D1/D2/cyt*b*-559 complex was analysed according to the Stepanov equation (Stepanov, B.I. (1957) Sov. Phys. Dokl. 2, 81–84). It is shown that there is a close correspondence between the emission spectrum calculated from the absorption spectrum and the measured emission spectrum in the wavelength range 665–695 nm. This correspondence was not found for samples which had either been aged at room temperature or subjected to a brief illumination with high intensity light. The data are interpreted to indicate that in freshly prepared D1/D2/cyt*b*-559: (i) excited states are thermally equilibrated between all chromophores including P680, (ii) the fluorescence yield of all chromophores is similar, (iii) the freshly prepared complex does not contain a significant amount of uncoupled pigment. This latter conclusion is supported by straight line Stern-Volmer fluorescence quenching kinetics using the quinone quencher of chlorophyll fluorescence dibromothymoquinone.

Keywords: Absorption spectrum; Fluorescence emission spectrum; Fluorescence yield; Photosystem II reaction center; Thermal equilibration

1. Introduction

The pigment system of Photosystem II contains a large number of antenna pigments which absorb light energy and transfer singlet excitation to the primary RC electron donor, known as P680. Whilst there is no exact estimation of the exciton diffusion time from the antenna to P680, on the basis of Coulombic coupling energies in isolated LHCII, using the nearest neighbour Chl distance suggested by Kühlbrandt and Wang [1] and considering between two to three nearest neighbours for each Chl site [1] one can calculate with the Förster equation an average transfer rate of the order of 0.3–0.5 ps [2]. This suggests an overall transfer time from antenna to reaction centres of the order of 50–100 ps in PS II (about 250 antenna sites). The charge separation time for PS II, defined as the time between photon absorption and charge separation, is of the order of 300 ps [3,4]. Thus it is expected that energy flows in and out of P680 a number of times before charge separation occurs and trapping dynamics are expected to be in part limited by the rate of charge separation at PS II RCs. This idea is central to the extreme trap-limited model developed by Holzwarth and co-workers for PS II [5], known as the exciton equilibration model. Fundamental to this idea is the concept that energy rapidly equilibrates between the antenna pigments and P680, essentially according to the Boltzmann factor. This requires that the P680 excited state does not undergo any electronic rearrangements which might modify the energy transfer rate back to nearby pigments. It has, however, been proposed recently that an excitonic coupling may be broken in the excited state of P680 [6].

It is extremely difficult to study energy transfer between P680 and antenna pigments in PS II as the primary donor is red shifted by less than 0.5 k_BT with respect to the core and outer antenna. In principle the same problem also exists for the isolated RC complex (D1/D2/cyt*b*-559),

Abbreviations: Chl, chlorophyll; Pheo, pheophytin; LHCII, light harvesting chlorophyll *a*/*b* protein-complex II; PS II, Photosystem II; RC, reaction centre; P680, primary electron donor of RC II; FWHM, full width at half maximum; DBMIB, dibromothymoquinone.

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though in this case the situation is simplified as the P680 contribution to total absorption is expected to be between 0.13–0.26, based on generally accepted pigment binding stoichiometries [7,8]. One way therefore to examine this problem is to investigate whether singlet excitation equilibration occurs between P680 and the accessory pigments of the isolated RC complex according to the Boltzmann factor. This has been achieved in the present study by use of the Stepanov relation [9] which connects the absorption and fluorescence spectra associated with the $Q_{y(0,0)}$ transition under conditions in which (i) Boltzmann equilibration occurs between all singlet excited states in the system prior to fluorescence, (ii) no significant electronic rearrangement occurs in the excited state and (iii) the fluorescence yield is constant across the absorption band. In this way it is demonstrated that relative energy transfer rates into and out of P680 are according to the Boltzmann factor in freshly prepared D1/D2/cyt*b*-559. This is not the case in samples subjected to aging or briefly irradiated with light, probably due to the uncoupling of accessory pigments. In addition the results indicate that the fluorescence yields of all chromophores in the freshly prepared complex, including P680, are similar.

2. Materials and methods

The D1/D2/cyt*b*-559 complex was obtained according to Chapman et al. [10], starting from PS II membranes prepared from maize mesophyll chloroplasts [11] by the method of Berthold et al. [12] as modified by Dunahay et al. [13]. The polypeptide composition was determined by SDS polyacrylamide gel electrophoresis on a 12–17% gel containing 6 M urea and stained with Coomassie blue. The preparation was judged to be pure by comparison of the polypeptide pattern thus obtained with that reported by Barber et al. [14]. The absorption maximum at room temperature was around 676 nm, thus indicating that it had not undergone degradation [15].

For determination of the chlorophyll/pheophytin ratio, pigments were extracted with 90% acetone. After vortexing for two minutes, the suspension was cooled on ice and centrifuged at $14000 \times g$. 50 μl of the supernatant was injected into a C_{18} column (Beckman, 250 mm × 4.6 mm) of a high pressure liquid chromagraphy apparatus which consisted of a Programmable Solvent Module 126 high pressure pump (Beckman), a Diode Array Detector Module 168 (Beckman) and a Model 7725i Sample Injector (Rheodyne) equipped with a 500 μl loop. Pigment separation was carried out by gradient separation [16] and monitored at 431 nm and 425 nm. The Chl/Pheo ratio was calculated from the peak area ratios after calibration with standard solutions of chlorophyll *a* and pheophytin *a*. In this way the Chl/Pheo ratio was determined to be almost 3.0.

Both absorption and fluorescence spectra of the complex were measured at room temperature using an EG and G OMAIII (model 1460) with an intensified diode array (model 1420) mounted on a spectrograph (Jobin-Yvon HR320) with a 150 groove mm^{-1} grating. The wavelength scale of the instrument was calibrated using a neon spectral calibration source (Cathodeon). The wavelength spacing between pixels is about 0.5 nm. Absorption was measured using light from an halogen lamp, attenuated by neutral filters (Balzers). The light path was 3 mm. Fluorescence was excited at 440 nm, FWHM 2 nm, and measured at 90° after filtering through an OG 530 (Schott). A 3 × 3 mm cuvette was used. For absorption or fluorescence analysis the samples were diluted respectively to 0.15 or 0.008 $A\ mm^{-1}$ in a buffer containing Hepes 10 mM (pH 7.6), dodecyl maltoside 0.03%. Under these conditions no detectable reabsorption of fluorescence occurred. The emission spectra were corrected for instrumental distortion using an intensity calibrated source (ISCO Spectroradiometer Calibration). Absorption spectra were not corrected for light scattering, as this was judged not to be significant, since spectra go to zero outside the absorption band.

Fluorescence yield was measured in a home-built apparatus upon successive additions of 0.7 μM dibromothymoquinone. Excitation was at 440 nm and emission at 690 nm (FWHM 10 nm).

All experiments were performed with two separate D1/D2/cyt*b*-559 preparations which gave almost identical results.

3. Results

Fig. 1 shows the measured absorption and fluorescence spectra of a freshly prepared D1/D2/cyt*b*-559 sample

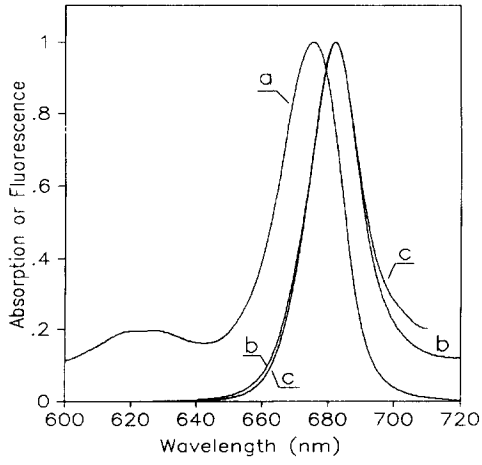

Fig. 1. The connection between absorption and fluorescence emission spectra according to the Stepanov relation in freshly prepared D1/D2/cyt*b*-559 complex. (a) Measured absorption spectrum; (b) measured fluorescence spectrum; (c) calculated fluorescence spectrum. For experimental details see Materials and Methods.

determined with the same sample-detector setup. The absorption maximum is at 675.8 nm, thus indicating that the complex is not degraded [15]. The fluorescence maximum is at 682.5 nm. Curve c represents the calculated room-temperature fluorescence spectrum according to the Stepanov relation (Eq. 1) [9], utilising the absorption spectrum (curve a):

$$\frac{F(\nu)}{A(\nu)\nu^3} = C(T)\exp(-(h\nu/k_BT)) \quad (1)$$

where $A(\nu)$ is the $Q_{y(0,0)}$ absorption spectrum, $F(\nu)$ is the emission spectrum, ν is the frequency, C is a function of the temperature, k_B is the Boltzmann constant, T is the absolute temperature of the surrounding medium (here 300 K) and h is the Planck constant.

It can be seen that the correspondence between the calculated and measured spectra is rather good between 665 and 695 nm, thus suggesting the following interpretation for the $Q_{y(0,0)}$ transition: (i) the fluorescence yield of all pigments is approximately equal, (ii) singlet excitation energy is equilibrated between all pigments of the complex according to the Boltzmann factor. This means that the amount of uncoupled pigments in this preparation is not great.

To give an idea of the resolution of this approach the results of calculations are presented in Fig. 2. We have assumed that uncoupled pigments absorb at either 660 nm or 670 nm, coincident with gaussian decomposition bands found at these wavelengths [17]. In each case calculations were performed assuming the uncoupling of one chlorophyll per complex, equivalent to about 15% of the total Q_y absorption oscillator strength. The fluorescence yields of uncoupled pigments were assumed equal to those of coupled pigments. From Fig. 2 it is clear that this pigment uncoupling leads to easily detectable blue shifting of the fluorescence spectrum. This would have been even greater if we had assumed higher quantum yields of fluorescence for uncoupled pigments. On the basis of calculations of this kind, we believe that we are able to detect upwards of 10% of fluoresence from uncoupled pigments. As this latter conclusion is not in agreement with the suggestion [15] that significant (about 40%) room-temperature fluorescence derives from dissociated Chl monomers, energetically uncoupled from the other pigments, we have further checked this point by analysing fluorescence yield as a function of the concentration of the fluorescence quencher dibromothymoquinone. Substituted quinones are thought to quench Chl singlet excited states by the formation of a charge transfer complex [18]. In photosystem antenna and isolated Chl-protein complexes the quenching efficiency is high and displays typical Stern-Volmer behaviour due to energy transfer between energetically coupled pigments [19–21]. Data are presented in Fig. 3, where it can be seen that a straight line Stern-Volmer plot was obtained for up to 80% fluorescence quenching. We believe that this method is capable of discriminating upwards of 10% of fluorescence from uncoupled pigments. This was shown by performing Stern-Volmer model calculations assuming two separate populations of pigments, one energetically coupled and the other uncoupled, both with equal fluorescence yields. The Stern-Volmer quenching constant for uncoupled pigments (k_{qu}) was taken as $k_{qu} = A\,k_{qc}$, where k_{qc} is the Stern-Volmer constant for coupled pigments and A is the fraction of uncoupled pigments. This is equivalent to assuming that the diffusion cross-section be linear with the number of coupled pigments, which is in line with the fact

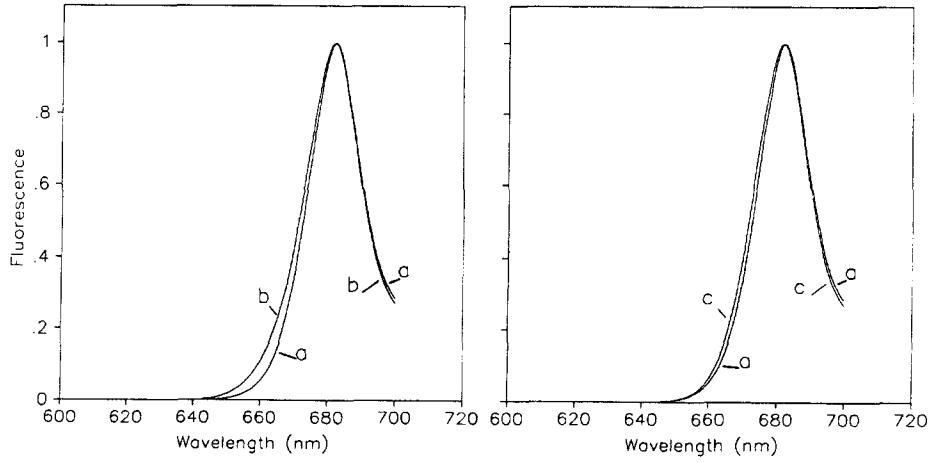

Fig. 2. Simulation of the effect of detergent-induced pigment uncoupling on the fluorescence emission spectra of the isolated D1/D2/cyt*b*-559 complex. All spectra were calculated from the $Q_{y(0,0)}$ absorption band using the Stepanov expression. Curves a are the thermally equilibrated emission spectra in which all pigments were assumed coupled. Curves b and c were calculated assuming the energetic uncoupling of pigments absorbing at 660 nm and 670 nm respectively. In both cases the uncoupled pigments were assumed to carry 15% of the total $Q_{y(0,0)}$ absorption intensity and to have the same fluorescence yield as the coupled pigments.

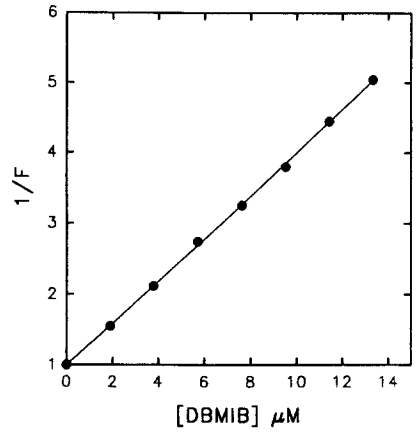

Fig. 3. Stern-Volmer titration with dibromothymoquinone of the fluorescence yield of freshly prepared D1/D2/cyt*b*-559. For experimental details see Materials and Methods.

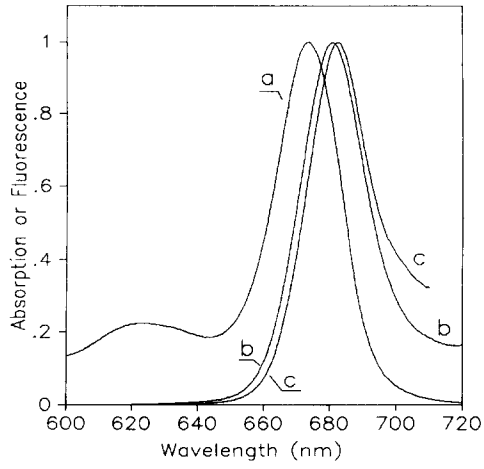

Fig. 5. The connection between absorption and fluorescence emission spectra according to the Stepanov relation in preilluminated D1/D2/cyt*b*-559. (a) Measured absorption spectrum; (b) measured fluorescence spectrum; (c) calculated fluorescence spectrum. Preillumination of samples was for 30 s with white light (500 W m^{-2}). For further experimental details see Materials and Methods.

that in coupled pigment-protein complexes it is the excited state which "diffuses". This observation is therefore supportive of the interpretation based on the Stepanov analysis that there is no significant emission from uncoupled pigments in our preparations.

The relationship between the absorption and fluorescence spectra was examined after aging of samples at room temperature and after a brief irradiation with intense light under aerobic conditions. These treatments are known to lead to pigment modifications [15,22,23] accompanied by absorption blue shifts, interpreted as being due to pigment uncoupling. From Figs. 4 and 5 it is apparent that the absorption/fluorescence connection relation via the Stepanov equation breaks down. This is in agreement with the interpretation that pigment molecules become uncoupled as a consequence of these treatments.

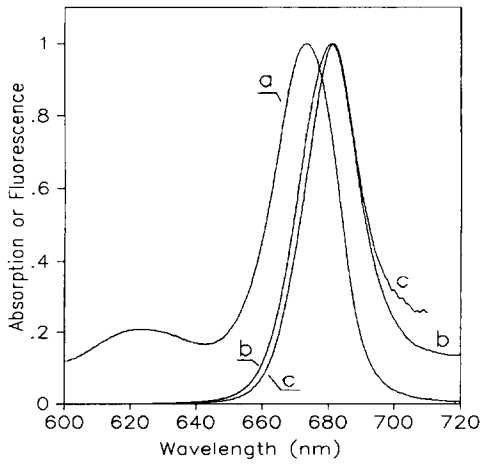

Fig. 4. The connection between absorption and fluorescence emission spectra according to the Stepanov relation of aged D1/D2/cyt*b*-559. (a) Measured absorption spectrum; (b) measured fluorescence spectrum; (c) calculated fluorescence spectrum. Samples were aged by incubation at room temperature for 24 h in the dark in the presence of 0.03% dodecyl maltoside. For further experimental details see Materials and Methods.

4. Discussion

In the present paper it is demonstrated that for the $Q_{y(0,0)}$ transition fluorescence and absorption spectra are well connected by the Stepanov relation in freshly prepared D1/D2/cyt*b*-559. This connection breaks down in samples which have been either aged at room temperature or illuminated with high light fluxes. Thus it is concluded that excitation energy is thermally equilibrated amongst all excited states in the D1/D2 system prior to fluorescence emission in freshly prepared samples. This requires that all pigments be energetically coupled at room temperature. Biochemical evidence indicates that the average pigment composition per complex is six Chl and two pheophytin molecules [7,8]. Five different homogeneously and inhomogeneously broadened absorption and fluorescence bands have been suggested from spectral decomposition studies [17]. The present analysis shows that energy is distributed between these transitions approximately according to the Boltzmann factor. Thus, the simplified equilibrium relation associating P680 with the accessory pigments (AP) may be represented by

$$P680^* + AP \underset{k_1}{\overset{k_{-1}}{\leftrightharpoons}} P680 + AP^*$$

where the equilibrium constant (k_1/k_{-1}) is given by the population-weighted Boltzmann distribution. This means that significant excited state electronic rearrangements in P680 do not occur. The data are therefore supportive of

simple thermal excited state equilibration between P680 and antenna pigments, as previously assumed [5]. On the other hand, the present analysis is not in agreement with the suggestion [6] that an excitonic coupling may be broken in the excited state of P680.

The Stepanov expression as used here requires that the fluorescence yield is constant across the absorption band. If this is not the case, a linear fluorescence yield term must be included in Eq. 1 [24]. In the context of the present study, the close correspondence between calculated and measured fluorescence spectra implies that most D1/D2 chromophores have an approximately similar fluorescence yield. Fluorescence from the primary donor P680 is expected in the 685–700 nm interval [17] where deviation between measured and calculated values is less than 0.1. Thus it is concluded that the primary donor P680 has a similar fluorescence yield to the accessory pigments. This conclusion is not in contradiction with the presence of a number of different kinetic components. The different lifetime values detected [15,25–28] are dominated by energy transfer and/or charge recombination dynamics and do not represent internal decay processes which determine fluorescence yield.

Fluorescence of the isolated D1/D2/cyt*b*-559 complex has been shown to be extremely heterogeneous kinetically. Thus Roelofs et al. [25,26] have detected six exponential decay components in the 3 ps–35 ns lifetime range. The sub-nanosecond components are however not expected to contribute significantly to the steady-state fluorescence which is dominated by a number of nanosecond components in the 5–80 ns range [15,27]. The longest lifetime components (20–80 ns) are generally thought to be associated with primary charge recombination between the pheophytin anion and the P680 cation, while the 5–6 ns component has been suggested to be due to uncoupled chromophores [15,25]. This component is expected to have a steady-state yield of about 0.3–0.4 and should therefore have been easily detected in our measurements. The conclusion that freshly prepared samples do not contain a significant amount of uncoupled pigments, based on both the Stepanov and Stern-Volmer analysis, may therefore be in disagreement with this assignment of the 5–6 ns component to free chromophores. This is in agreement with recent conclusions of Roelofs et al. [26] and Freiberg et al. [28], who suggest that at least part of this component is due to a recombination component.

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