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Dynamic fluorescence properties of D1-D2-cytochrome *b*-559 complex isolated from spinach chloroplasts: analysis by means of the time-resolved fluorescence spectra in picosecond time range

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Dynamic fluorescence properties of D1-D2-cytochrome *b*-559 complex isolated from spinach chloroplasts were analyzed with time-resolved fluorescence spectra and deconvolution of the spectra. The changes in the spectra at 4°C were fitted by the changes in the relative contribution of at least three fluorescence components whose emissions peak at 670, 676 and 682 nm (F_{670}, F_{676} and F_{682}). The F_{670} and F_{676} are interpreted to arise from accessory chlorophyll *a* and the F_{682} from the primary donor of reaction center II (P-680), based on the kinetic response and the energy level. The decay kinetics of F_{682} is composed of two components of lifetimes about 25 ps and 35 ns. The former lifetime corresponds probably to the kinetics of charge separation while the latter, to the kinetics of charge recombination between P-680 and pheophytin. The spectra at −196°C were fitted by four components, including the above three and an additional band peaking at 693 nm (F_{693}). The F_{683}, which corresponds to the F_{682} at 4°C, was the main band throughout the measuring time up to about 80 ns. Its decay kinetics had two components with its slow phase of about 40 ns. This corresponds to the time for the charge recombination at the low temperature. The rate constant and the absolute yield of the F_{683} slowest component (charge recombination) were almost independent of temperature, while the fluorescence yield of the other decay components was temperature dependent. This gave rise to the changes in the time-resolved fluorescence spectra under two temperature conditions.

Introduction

Recently, several lines of evidence are accumulating for the idea that the D1-D2 hetero-dimer constitutes the reaction center (RC) of Photosystem (PS) II in higher plants [1–4]. Although the hetero-dimer is actually isolated with the complex with cytochrome *b*-559 [3], this idea is supported based on the following results: a high homology of the primary structures of D1 and D2 polypeptides to those of L and M subunits of bacterial RC [1,2]; reversible absorbance changes of pheophytin [3]; and the formation of spin polarized triplet [4]. Comparison of the properties of this preparation with those of bacterial RC's enables us to elucidate an essential property of PS II RC. Char-

Abbreviations: F_{670}, a representative expression of the component band whose fluorescence maximum is located at 670 nm; FWHM, full width at half maximum; P-680, primary electron donor of reaction center II; PS, Photosystem; RC, reaction center; Chl, chlorophyll; Pheo, pheophytin.

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acterization of optical properties thus far studied [5] indicates the presence of an uncoupled Chl *a*, which cannot transfer excitation energy to RC Chl *a*. This is not known in bacterial RC's [6–8].

Time-resolved fluorescence spectroscopy can give a direct information on the excitation energy transfer in antenna pigments including RC Chl *a* [9,10]. Since D1-D2-cytochrome *b*-559 complex contains only four to five Chl *a* per two pheophytin molecules [3], it is possible to assign the energy levels of individual pigments and determine the energy transfer sequence among them by this method. We analyzed the dynamic fluorescence properties of D1-D2-cytochrome *b*-559 complex. Results indicate the fluorescence decay kinetics corresponding to the charge separation and recombination between the primary electron donor (P-680) and primary electron acceptor (pheophytin), and further suggest the energy transfer between the pigments. The 683-nm fluorescence (F_{683}) most probably originates from P-680 itself in this preparation.

Materials and Methods

Preparation of D1-D2-cytochrome b-559 complex. The D1-D2-cytochrome *b*-559 complex was isolated from spinach chloroplasts by the method of Nanba and Satoh [3] after a minor modification; after samples were adsorbed to a DEAE-Toyopearl 650S equilibrated with 50 mM Tris-HCl (pH 7.2), 0.05% Triton X-100 and 30 mM NaCl, the column was washed with the buffer solution containing 60 mM NaCl. Then the column was washed again with the buffer containing 30 mM NaCl and developed with a gradient of NaCl from 30 to 200 mM (Nanba and Satoh, unpublished results). Chl *a* content relative to two pheophytin molecules was about 4.5.

Optical measurements. Time-resolved fluorescence spectra in the ps time range were measured with the apparatus reported previously [9–11]. The light source was composed of a synchronously pumped, cavity-dumped dye laser and a mode-locked Ar^+-laser. The excitation was done at 630 nm with a pulse width of 6 ps (FWHM). The repetition rate of pulses was 800 kHz and the intensity was in a range of 10^8–10^9 photons/cm^2 per pulse, which is low enough to avoid singlet–singlet annihilation. Fluorescence was detected by a time-correlated single-photon counting method. The time resolution of the system was improved by the use of a 6-μm type microchannel plate photomultiplier (R2809U-01, S-20 type photocathode, Hamamatsu Photonics, Japan) (cf. Ref. 11). The instrumental response function for the scattered laser light was observed with a 30–40 ps pulse width (FWHM) and time resolution was achieved to 9.92 ps in terms of the spectrum. In the time-resolved spectra, time zero was set to the time when the excitation pulse showed the maximum intensity, estimated by separate measurements in the same geometry as in the case of fluorescence measurements. Since excitation pulse (6 ps, FWHM) was observed as a pulse of 30–40 ps (FWHM), spectra before time zero can be obtainable (cf. Fig. 4C). The spectral sensitivity of the apparatus was not corrected.

Samples were suspended in the buffer (50 mM Tris-HCl, pH 7.2) containing 0.05% Triton X-100, 0.3 M sucrose and 20% glycerol. For the measurements at 4°C, samples were continuously circulated to avoid a damage due to a long time irradiation. A sample holder and reservoir were cooled by thermostated water. For the measurements at −196°C, the samples in the same buffer solution as that at 4°C were mixed with an equal volume of polyethylene glycol 4000 (final concentration; 15%) to obtain homogeneous ice [12]. Samples in a 2-mm lucite cuvette were always immersed into liquid nitrogen during measurements. The intensity of excitation pulse was reduced to avoid the damage to the samples. Chlorophyll concentration of the samples was set to in a range of 0.7–0.8 μg/ml (4°C) or of 3.5–4 μg/ml (−196°C).

Convolution of decay kinetics. Fluorescence lifetimes were determined by the simulation calculation of a non-linear least-square method based on the Marquardt algorithm [13,14]. As a profile of the excitation light, scattered light from an identical sample in the same geometry for the measurements was monitored through (an) appropriate filter(s) to avoid the damage to the photomultiplier. Each decay curve was simulated by a multi-exponential function. As criteria of the best fit, we used two parameters; χ^2 and the Durbin-Watson parameter [15].

Deconvolution of the time-resolved fluorescence spectra. Fluorescence components were resolved by deconvolution of time-resolved spectra. A simulation calculation was done with an HP 216 microcomputer based on the least-square method.

Results

Time-resolved fluorescence spectra at 4 °C

When the D1-D2-cytochrome *b*-559 complex was excited at 630 nm and fluorescence was measured in a short time range (0–2.3 ns and the time interval between individual spectra was 9.92 ps), the apparent fluorescence maximum was initially observed at 671 nm (Fig. 1A; −20 ps). At 0 ps, the time when the excitation pulse showed the maximum, a plateau was observed between 670 and 685 nm. At 20 ps, a fluorescence maximum was found at 676 nm. In a later time range, no more shift of the maximum was observed, while the band width became narrower, indicating substantial disappearance of one or several fluorescence components. In the measurements of a longer time range (0–80 ns and the time interval between spectra was 393 ps), the maximum was initially observed at 676 nm (Fig. 1B). After 1 ns, the spectra were essentially identical up to about 15 ns. Then one could notice clearly a small shift of the maximum with time and the maximum reached 681 nm at 50 ns. Though the spectra were slightly noisy, the shift of the maximum was clear.

To detect components responsible for the changes in the spectra, we deconvoluted each time-resolved fluorescence spectrum with a following assumption. The origin of the spectrum observed between 1 and 15 ns was regarded as a single component (called F_{676} hereafter), because the time-resolved spectra in this time range were almost identical. Since Chl *a* content per two pheophytin is about 4 in this preparation, it does not seem probable that plural components are responsible for this constant spectrum. Only when plural components have the same or very similar lifetimes, those can be realized. This possibility is

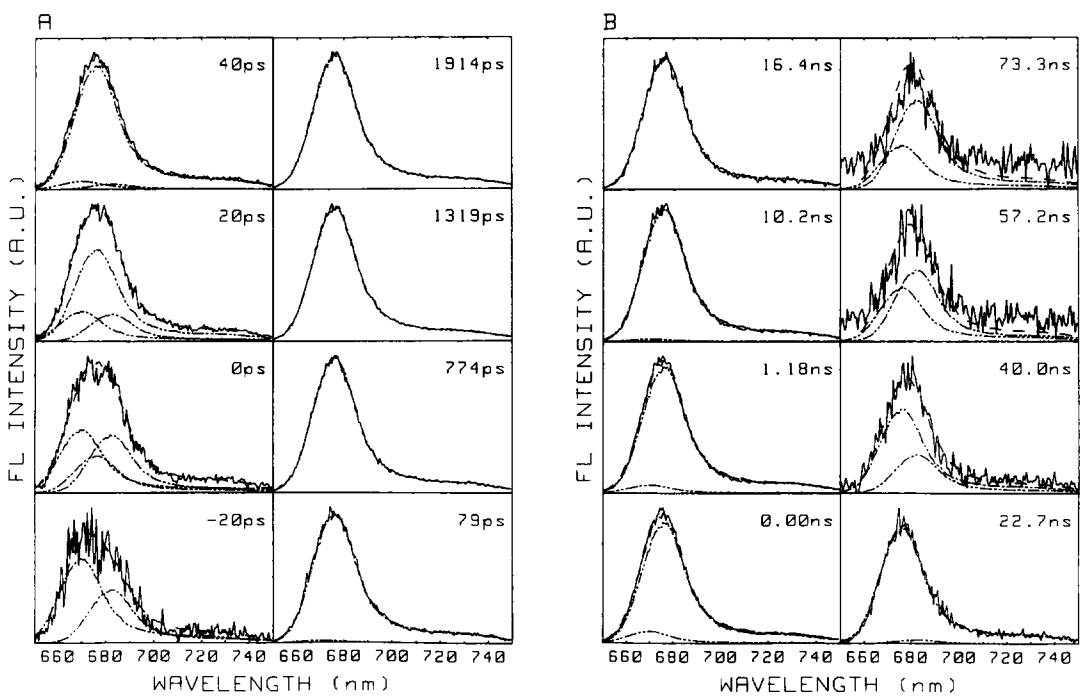

Fig. 1. Time-resolved fluorescence spectra of D1-D2-cytochrome *b*-559 complex at 4 °C. In (A), the time resolution of the time-to-amplitude converter was 9.92 ps per channel; and in (B), 393 ps per channel. Each spectrum was normalized to the maximum intensity. Numbers in the figures indicate the time after the excitation pulse. In each spectrum are shown the original data (———), the simulated curves of each component (– – –) and the sum of the simulated curves (------). For details, see the text.

very low, because of a low Chl *a* content. Individual spectra, therefore, were fitted by the sum of the F_{676} and a minimum number of additional component(s). The band shape of the F_{676} was used for estimating band shapes of other bands. The best fit was obtained when two more components (F_{670} and $_{682}$) were assumed.

Fluorescence components responsible for the changes in the time-resolved fluorescence spectra were thus F_{670}, F_{676} and F_{682} throughout the measuring time up to about 80 ns. In the initial time range, only the F_{670} and F_{682} were observed. Their fractions decreased very quickly (within 80 ps) with a concomitant increase of the F_{676} (Fig. 1A). After 20 ps, the F_{676} was dominant. On the other hand, the F_{682} substantially disappeared within 100 ps. From the deconvoluted spectra, we can estimate rise and decay kinetics of individual components (Fig. 2A). Time behaviour of the F_{670} and F_{682} was very similar, though the F_{682} decayed faster. The lifetimes of the F_{670} and F_{682} were estimated to be 40 and 25 ps, respectively, without convolution to the excitation pulse. The half-rise time of the F_{676} was about 45 ps, indicating a long lifetime compared with the other two components. In the measurements with a longer time range (Fig. 1B), the spectra were well fitted by the F_{676} and a small fraction of the F_{670} in the initial time span. The absence of the F_{682} may be due to a lower time resolution and to the faster decay of this component. In the intermediate time range (10–20 ns), the spectra were fitted only by the F_{676}. In a later time range, the spectra can be regarded as the sum of the F_{676} and F_{682}, as seen in Fig. 1B. The fraction of the F_{682} increased with time. This component was dominant at times later than 55 ns.

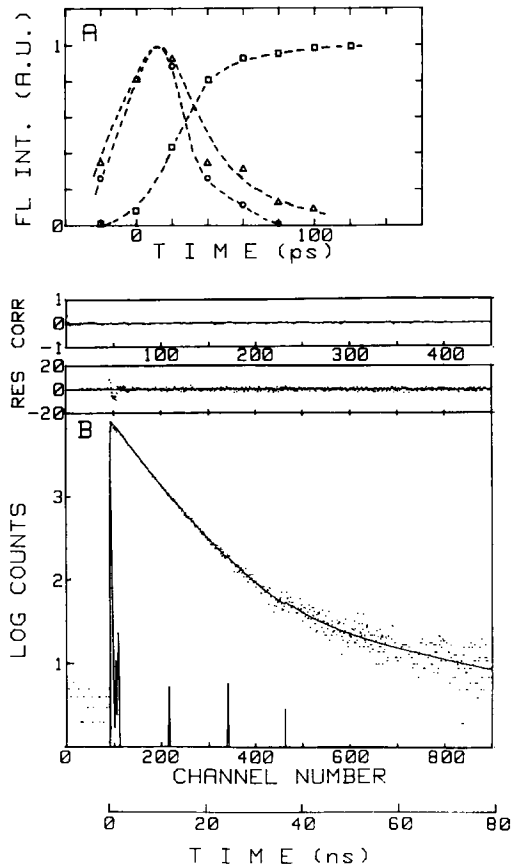

Fig. 2. Estimation of lifetime of fluorescence components in D1-D2-cytochrome *b*-559 complex at 4°C. (A) Normalized rise and decay curves of individual components based on the deconvoluted spectra (Fig. 1) and (B) decay curve at 680 m and simulated curve by convolution. In (A), the rise and the decay of F_{670} (△), of F_{676} (□) and of F_{682} (○). In (B), dots represent observed data and full line overlapping it shows a simulated curve. Full line in the lower part shows actual pulse shape. Small spikes following the initial pulse were due to a lower extinction ratio of a cavity dumper. Residual and correlation were also shown. Convolution was done on the decay curve with the assumption of exponential decay kinetics. For details, see the text and Table I.

Fluorescence decay curves at 4°C

For the analysis of the relationship between the fluorescence components and lifetimes, we measured decay curves at 670, 680 and 690 nm (Fig. 2B) and estimated lifetimes and amplitudes of individual components by convolution using multiexponential function (Table I). The time range of time-to-amplitude converter was set to 98.15 ps per channel. As criteria for the best fit, two parameters were used; χ^2 and the Durbin-Watson parameter, a parameter for serial correlation [15]. Of these, the Durbin-Watson parameter was mainly taken into account because the artificial minimum of χ^2 should be avoided. We estimated the lifetime of the slowest component by separate measurements in a longer time range. This was a reasonable treatment to estimate a long lifetime accurately. The average value we obtained was 35.5 ± 0.41 ns. This value was set to constant for the convolution calculation. When we assumed

TABLE I

FLUORESCENCE LIFETIMES OF INDIVIDUAL COMPONENTS IN D1-D2-CYTOCHROME *b*-559 COMPLEX UNDER TWO TEMPERATURE CONDITIONS

Convolution was done with the assumption of an exponential decay. Lifetimes (τ_i) were expressed in ns and amplitudes (A_i) were the relative values. DW means the Durbin-Watson parameter. χ^2 and the Durbin-Watson parameter are parameters for estimation of the best fit (cf. Ref. 15). For details, see the text.

λ (nm)	τ_1	A_1	τ_2	A_2	τ_3	A_3	τ_4	A_4	χ^2	DW
4°C										
670	0.0491	1541	5.85	738	35.5	0			4.812	1.829
680	0.0491	1524	6.00	765	35.5	1			4.961	1.888
690	0.0491	1657	6.00	690	35.5	2			4.996	2.052
−196°C										
670	0.050	1356	1.390	289	5.42	186	39.8	0	5.503	2.681
683	0.119	997	0.971	292	4.93	86	39.8	2	5.936	2.384
690	0.203	669	1.351	293	5.18	107	39.8	2	5.324	2.251

three components for the convolution, consistent lifetimes were obtained irrespective of the monitoring wavelengths; 49 ps, 5.95 ns and 35.5 ns (Table I). Of these the accuracy of the shortest lifetime was low due to the limited time resolution of time-to-amplitude converter (98.15 ps per channel) in these measurements. However, the convolution with only two longer lived components (5.95 and 35.5 ns) did not give a good fit. This suggests the presence of a faster decay process, probably corresponding to the decay of the F_{670} and F_{682}. In this sense, the convolution of the decay curves at fixed wavelength does not necessarily correspond to the decay kinetics obtained by deconvolution of the time-resolved fluorescence spectra. The lifetime of middle component (5.95 ns) is close to that of Chl *a*'s in organic solvent (5.1 ns in diethylether and 6.9 ns in methanol) [16]. The amplitudes were high in between 670 and 680 nm. These suggests that the fluorescence of middle component arises from the Chl *a*, most probably the F_{676}, which does not transfer the energy. The fraction of the longest lifetime component (35.5 ns) was higher in the decay kinetics in a longer wavelength region. This lifetime was very close to the rate constant of the charge recombination reaction between the primary electron donor (P680) and the primary electron acceptor (pheophytin), which was detected by the transient absorption spectroscopy on this preparation (32 ns [17]–36 ns [5]).

Time-resolved fluorescence spectra at −196°C

At −196°C, the spectra (Fig. 3A) showed significant differences from those at 4°C (Fig. 1A). The fluorescence maximum was observed at 680 nm with a shoulder around 670 nm upon the excitation at 630 nm. The maximum at 680 nm shifted to 683 nm within 150 ps, and no further shift was observed. The changes in the relative intensity at shorter wavelength of the maximum were complicated. In the initial time close to the excitation (−20 to 60 ps) where the fluorescence maximum shifted gradually, the intensity around 670 nm was significant and decreased with time. During the next time span (60–300 ps), the fluorescence band seemed symmetrical, indicating that the 683-nm component is predominant. The intensity around 675 nm increased after about 300 ps. This kind of time behaviour suggests that two different processes are involved in these changes. Increase in the intensity around 695 nm was also observed in a longer time range.

By the measurements with a longer time resolution (Fig. 3B), the time behaviour of each component was resolved more clearly. The relative intensity at 675 nm to that at 683 nm became higher with time after the excitation up to about 10 ns, and decreased after 15 ns. At times longer than 30 ns, this band (F_{675}) disappeared and the component peaking at 683 nm became dominant. Both phenomena were not observed at 4°C (cf. Fig. 1B). One additional fluorescence band showing

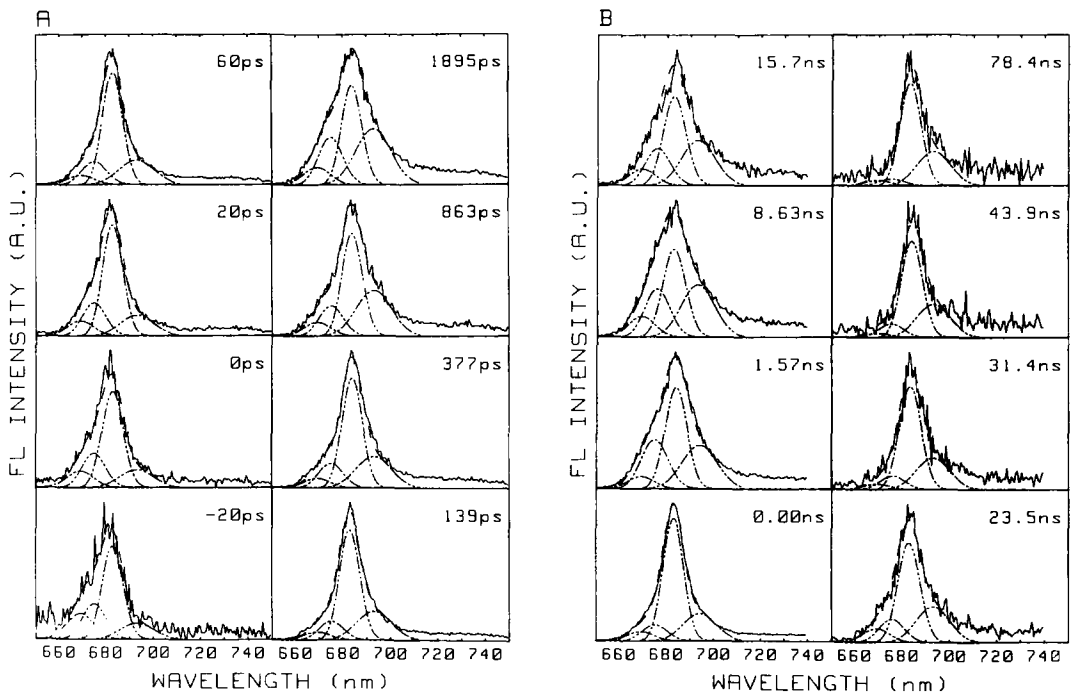

Fig. 3. Time-resolved fluorescence spectra of D1-D2-cytochrome *b*-559 complex at −196 °C. Fluorescence was monitored at 45° to the excitation beam with an appropriate filter. In (A), the spectrum in a fast time resolution (9.92 ps per channel of the time-to-amplitude converter) and in (B), in a slow time resolution (393 ps per channel). Other conditions were the same as in Fig. 1. For details, see the text.

similar time behaviour to that at 675 nm was found at the wavelength longer than that of the maximum. Compared with the spectrum at 0 ns, the band width of spectra between 1.57 to 15.7 ns was significantly wider. The difference spectrum suggests the presence of the component around 693 nm.

The time-resolved fluorescence spectra were deconvoluted to assess the changes in the various assumed components, as was done in the case at 4 °C. In the latter case, gaussian band shape was assumed for individual components, because the constant spectrum such as found at 4 °C was not observed under this temperature condition. In this procedure, the vibrational band is not taken into account, thus the fraction of the component at longer-wavelength side is overestimated. Four components were enough for the best fit; F_{670}, F_{675}, F_{683} and F_{693} with bandwidths of 8, 7.5, 6 and 10.5 nm, respectively. The F_{670}, F_{675} and F_{683} most probably correspond to the F_{670}, F_{676} and F_{682} at 4 °C. Thus, the spectra at −196 °C is regarded to consist of three common components and one additional component. These four components were always observed in each spectrum with different amplitudes. The shift of the maximum in the initial time range was due to the decrease in the fraction of the F_{670} with respect to the F_{675} and F_{693} (Fig. 3A). The fraction of the F_{675} and F_{693} increased in the intermediate time range (300 ps to 26 ns) and finally the F_{683} was the dominant component (Fig. 3A and B). The rise and decay curves of the individual components in the initial time range are shown in Fig. 4A after normalization. The F_{670} rose slightly faster than the F_{675}. The rise of the F_{683} was slower than those of the F_{670} and F_{675}. These rise kinetics do not necessarily give an indication of the sequential energy transfer among these three components.

Fluorescence decay curves at −196 °C

As in the case of the analysis at 4 °C, decay curves at 670, 683 and 690 nm were measured for the estimation of lifetimes with the time range of

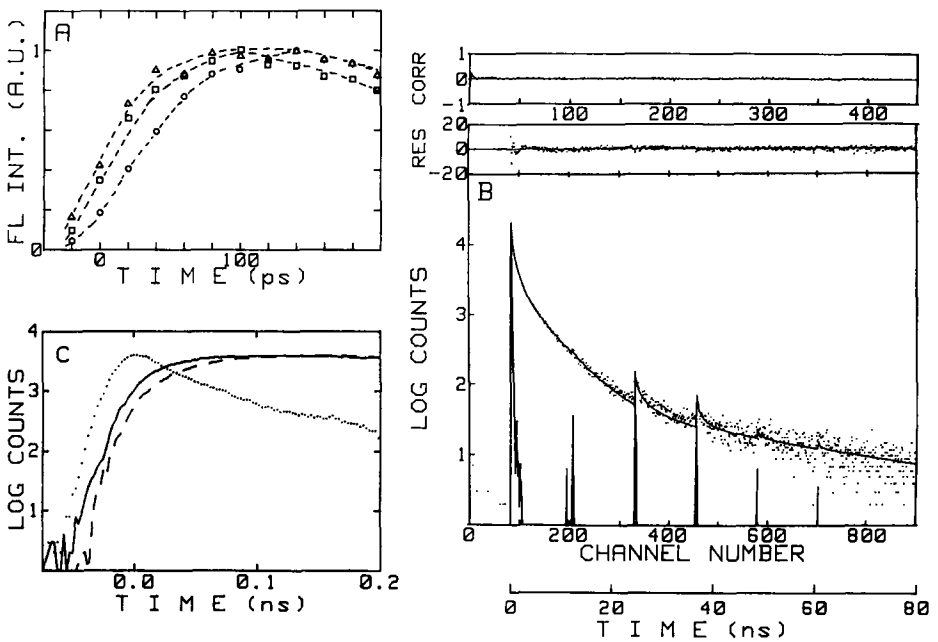

Fig. 4. Rise and decay kinetics and estimation of fluorescence lifetime in D1-D2-cytochrome *b*-559 complex at −196 °C. (A) Normalized rise and decay curves of individual components based on the deconvoluted spectra; F_{670} (△), F_{675} (□) and F_{683} (○). (B) decay curve at 683 nm and simulated curve by convolution. Time resolution of time-to-amplitude converter was 98.15 ps per channel. Dots represent the observed data, and full lines in a lower or upper part of the figure, excitation pulse and simulated curve, respectively. (C) Fluorescence decay curves at 670 nm (———) and 683 nm (– – –), and profile of the excitation pulse (· · · · ·) with the time range of 2.48 ps per channel in time-to-amplitude converter. The other conditions were the same as in Fig. 2. For details, see the text.

98.15 ps per channel in the time-to-amplitude converter (Fig. 4B). An exponential decay function was again assumed. The lifetime of the slowest component was estimated by a separate measurement, as in the case at 4 °C; it was 39.8 ± 0.53 ns. When three decay components were assumed, consistent lifetimes were not obtained by convolution. Therefore four components were assumed. The average lifetimes thus obtained were, except for the fastest component, 1.24 ns, 5.18 ns and 39.8 ns, respectively (Table I). The shortest lifetime varied from 50.3 ps (670 nm) to 203 ps (690 nm). Taken together with the time-resolved spectrum, the lifetime of 39.8 ns corresponds to the F_{683}. This long lifetime suggests that this fluorescence arises from the charge recombination, as in the case at 4 °C. This value (39.8 ns) agreed well to the rate constant of charge recombination measured by a transient absorption change at −196 °C (40 ns) [17]. The amplitude of the third lifetime component (5.18 ns in an average) was bigger at 670 and 690 nm compared with that at 683 nm. This lifetime seems to correspond to both of F_{675} and F_{693}, and indicates that their lifetimes are very close each other. The origin of the second lifetime component (1.24 ns in an average) is most probably the F_{683}. However, the dominant F_{683} throughout the observed time span cannot be explained only by these assignments. The F_{683} seems to show a relatively longer lifetime in addition to the 1.24 ns component, comparable to those of the F_{675} and F_{693}.

To investigate the energy transfer from the F_{670}, fluorescence decay curves were measured with a time range of 2.48 ps per channel in the time-to-amplitude converter (Fig. 4C). The rise at 670 nm was clearly faster than that at 683 nm. This indicates the presence of a short lifetime component. A probable reason for a short lifetime of accessory Chl *a* is the energy transfer. Therefore we can assume the energy transfer from the F_{670} to P-680. However, a corresponding rise was not clear in the

rise and decay curve at 683 nm. Convolution of the decay curves at 670 and 683 nm with a multi-exponential decay function did not give an indication of such transfer process (Table I). Contrary to this, the steady-state measurement of the action spectrum indicates that the energy is transferred from the component at the shorter wavelength to P-680 [18]. These results indicate that exponential function is not necessarily good enough to describe the transfer sequence from the F_{670} to P-680, as in the case of whole chloroplasts [19].

Discussion

The time-resolved fluorescence spectra measured at 4 and −196 °C were apparently different each other (Figs. 1 and 3). However, each spectrum can reasonably be fitted by common components (F_{670}, F_{675} and F_{683}) with an additional component (F_{693}) at −196 °C. This result indicates that the peak positions of essential components are independent of temperature. Contrary to this, the fluorescence yields of individual components are temperature dependent, which gives rise to changes in the spectra.

In the D1-D2-cytochrome *b*-559 complex used in this study, 4–5 molecules of Chl *a* were found per two pheophytin molecules, and the F_{693} was observed at −196 °C. These results suggest the contamination of the CP-47 in this preparation (cf. Ref. 18). However, the absorption band which has a peak close enough to emit the F_{693} was not observed in this preparation even at −269 °C [18]. The other possibility of contamination in this preparation is a long-lived F_{670}. In the time-resolved spectra at −196 °C, the relative intensity of the F_{670} was high in the initial time range and decreased once (around 139 ps, Fig. 3A). It again became higher in the middle time range (Fig. 3B). When only one component is present, a monotonous decrease in the relative intensity would be expected. These phenomena, thus, suggest the presence of two different F_{670}; one shows a fast rise and decay, and the other, a long-lived F_{670}. The two components, the F_{693} and a long-lived F_{670}, could be observed in the steady-state fluorescence spectrum (data not shown). However, when we used the other preparation which contains a less amount of contaminating Chl *a*'s, those two bands were not observed in the steady-state spectrum (Satoh, K. et al., unpublished data). These indicates that the F_{693} and a long-lived F_{670} are emitted by a minor, non-significant Chl *a* which accounts for a fraction exceeding four. Thus, the number of essential Chl *a* seems to be four in this preparation, as in the case of bacterial RC [6–8].

In the absorption spectrum of D1-D2-cytochrome *b*-559 complex, three components were detected at 670, 676 and 680 nm at −269 °C [18]. These absorption components most probably correspond to three fluorescence components which were shown in this study. In this complex, four Chl *a*'s are responsible for three fluorescence components. The F_{682}, which showed 25 ps and 35.5 ns lifetimes at 4 °C, are most probably the fluorescence from P-680, judging from the energy levels and time behaviour. The faster decay of the F_{682} may correspond to the charge separation and the slower decay, to the charge recombination, though both are indirect estimations. The rate constants of those reactions measured directly by the transient absorption changes are shorter than 25 ps [5] and 32–36 [5,17] ns, respectively. The proposal that the F_{695} arises from charge recombination [20] was not confirmed by the present study. Chlorophyll component emitting the F_{693} does not seem to exist in this preparation. P-680 is known to consist of Chl *a* dimer [21], thus the other two fluorescence components observed in this preparation correspond to remaining two Chl *a*'s. The F_{670} shows very fast decay at 4 °C. Though the energy transfer from this component to P-680 was not directly observed, the faster rise of the F_{670} at −196 °C suggests the occurrence of the energy transfer. The F_{676} originates from an uncoupled Chl *a* in terms of energy transfer, as shown by the other method [18].

The rate constants of charge separation (25 ps) and recombination (35.5 ns) at 4 °C can give an estimate for the energy difference between the states [P-680* Pheo] and [$P\text{-}680^+$ $Pheo^-$], when the charge recombination is the rate determining step of overall reactions. However, as suggested by a lower amplitude of charge recombination (Table I), the other pathway (triplet formation) is dominant. Therefore, the estimation of energy difference cannot be obtained by current measure-

ments. However, a low-temperature dependence of the rate constant for charge recombination (35.5 ns at 4°C and 39.8 ns at −196°C) is in a remarkable difference from that in the bacterial RC [22]. This small temperature dependency suggests that the contribution of entropy term is more significant in the free-energy changes in the RC II.

Some aspects of the optical properties (i.e., linear dichroism) of D1-D2-cytochrome *b*-559 complex vary depending on the isolation method (Breton, J., personal communication). This might be due to relatively unstable characteristics of this preparation at higher temperature. However, rate constants for charge separation and recombination obtained in this study are consistent with those reported by other groups [5,17], even though the methods are different from each other. In this sense, the fluorescence properties reported in this study most probably reflect precisely essential properties of this complex.

Acknowledgements

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