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Excitation energy transfer in *Rhodobacter sphaeroides* analyzed by the time-resolved fluorescence spectroscopy

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Excitation energy transfer in the photosynthetic bacterium, *Rhodobacter sphaeroides*, was analyzed using time-resolved fluorescence spectroscopy measured with a new type of microchannel-plate photomultiplier (S-1 type photocathode). Difference spectra and Gaussian deconvolution of fluorescence spectra of the bacterium at −196 °C clearly revealed three fluorescence components (F875, F911, F925). These were attributed to B850, B875 and an additional longer-wavelength antenna component (B890), respectively. Emission from B800 was not detected, indicating a very fast energy transfer from B800 to B850 with a transfer time shorter than 6 ps. Similar measurements also suggested the same upper limit for the time of energy transfer from B850 to B875. The rise and decay kinetics of the fluorescence components indicate a sequential energy flow in the order of B800, B850, B875, B890 and finally the reaction center. The three fluorescence components (F859, F889, F909) were also detected in spectra taken at 22 °C and were attributed to B850, B875 and B890, respectively. Changes in the fluorescence spectra were observed only in the initial 100 ps after the excitation pulse. The time needed for apparent energy transfer between components was estimated from the rise term of kinetics; 20 ps for B850 to B875 and 35 ps for B875 to B890. A rise in the relative intensity of F909 in the time-resolved spectra suggested a 50 ps excitation equilibration time between B890 and the reaction center. In a later time range, no spectral changes were observed, indicating equilibration of excitation transfer among the antenna components. After equilibration, the main fluorescence component decayed with the lifetime of 255 ± 10 ps. Based on the deconvoluted spectra, the absorption maximum of the longer-wavelength antenna was estimated to be 890 nm at 22 °C and 898 nm at −196 °C. The relative intensities of the deconvoluted fluorescence bands were used to estimate the content of B890 bacteriochlorophyll to be 7 ± 1 per reaction center.

Introduction

The light-harvesting system of the photosynthetic purple bacterium, *Rhodobacter sphaeroides*, consists of two major pigment-protein complexes, B800–850 and B875 [1–4]. The latter is postulated to be structurally associated with the reaction center (RC) in forming the RC-B875 complex in which there are about 24 molecules of B875 Bchl per RC [4]. These pigment-protein complexes are interconnected to form a large domain in which excitation energy can migrate until it is trapped by an RC in the open state [5,6]. Van Grondelle and co-workers determined the domain size in chromatophores of *Rb. sphaeroides, Rb. capsulatus* and *Rhodospirillum rubrum* as well as in isolated pigment-protein complexes by measuring rates of singlet-singlet annihilation [7–12]. In *Rb. sphaeroides*, the domain was shown to consist of approx. 30 RCs and up to 3000 light-harvesting Bchl molecules, the later being comparable to the total number of Bchl molecules in one chromatophore [10].

An additional longer-wavelength antenna component is suggested to have some function in energy transfer from B875 to RC [13–15]. It has tentatively been called B905 [14] or B896 [15]. Borisov et al. [14] have suggested the presence of 3–5 Bchl molecules of B905 per RC,

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Abbreviations: Bchl, bacteriochlorophyll; B800, B850 and B875, Bchl with absorption maxima are located at 800, 850 and 875 nm; RC, reaction center.

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and Kramer et al. [15] have proposed that about 15% of the Bchl molecules in the B875 light-harvesting complex correspond to B896. Measurements of polarized fluorescence, picosecond absorption recovery and deconvolution of absorption spectra also support the presence of the B896 [13–18].

Excitation energy transfer dynamics in photosynthetic bacteria have been studied by fluorescence lifetime measurements and recently by picosecond absorption recovery [19–27]. An overall trapping time of about 60 ps was reported for open RCs, and when RCs were oxidized, the time constant increased up to approx. 200 ps [7,14,16,23–25]. In B800–850, very fast (shorter than 2 ps) energy transfer from B800 to B850 was indicated [28–30]. Also, a very fast energy transfer from B800–850 to B875 was suggested by data taken using the multiple-frequency phase-modulation technique and on a picosecond pulse fluorometry [24–27]. Steady-state fluorescence measurements indicate, however, an equilibration of excitation energy between B800-850 and B875 [31–33]. Sundström et al. [16] interpreted the 35 ps decay for B850 and also for B875 as the time to equilibrate excitation densities between B800-850 and B875, and between B875 and B896, respectively.

The method of time-correlated single photon counting enables direct and precise measurement of the time behavior of fluorescence, reflecting the relaxation processes of pigments in the excited state [34]. In particular, time-resolved fluorescence spectra directly measure the energy transfer dynamics characterizing photosynthetic pigments in whole cells and isolated components [35–40]. An important problem in detecting fluorescence in the infrared region is thermally generated noise. Recently, an S-1 type microchannel-plate photomultiplier has been developed which has enabled studies on the fluorescence dynamics of bacterial photosynthetic pigments in the picosecond time range.

In the present study, we measured the time-resolved fluorescence spectra of intact cells of wild-type *Rb. sphaeroides* at physiological temperature (22° C) and at −196° C. The results show a very fast energy transfer from B850 to B875 and the presence of the longer wavelength antenna component (B890), even at physiological temperature, which corresponds to B896 [15] or B905 [14] found at low temperature.

Materials and Methods

Rb. sphaeroides 2.4.1. was grown photoheterotrophically at 30° C in a medium composed of 0.5% (w/v) polypeptone, 0.1% yeast extract and 0.4% sodium lactate (pH 7.0). Cells were harvested at the late log phase, washed and suspended in 10 mM Na-Mops (pH 7.0). The ratio of B800–850 to B875 in the cells was estimated to be about 2.5 on the basis of Bchl content determined by spectral analysis [41].

Time-resolved fluorescence spectra in the picosecond time range were measured with the apparatus reported previously [35–39,42]. Minor modifications in the optical apparatus were (1) a cooled (−30° C) microchannel-plate (12 μm) photomultiplier of an S-1 type photocathode (R1564U-05, Hamamatsu Photonics, Japan) and (2) a grating (600 lines/mm) with a blaze wavelength of 750 nm. The light source was composed of a mode-locked Ar^+-laser (Spectra Physics) and a synchronously pumped, cavity-dumped (800 kHz) dye laser (Spectra Physics). Excitation was at 590 nm with a pulse width of 6 ps (fwhm). The intensity was in a range of 10^8–10^9 photons/cm^2 per pulse, which was low enough to avoid singlet-singlet annihilation. Fluorescence was detected by the time-correlated single-photon counting method. The time resolution of the system was 6 ps. In the time-resolved spectra, time zero was set to the time when the excitation pulse was at its maximum intensity. The spectral sensitivity of the apparatus in the wavelength region used was almost flat (maximum difference, 8.5%), and therefore it was not corrected.

For the measurements at room temperature (22° C), the suspension of *Rb. sphaeroides* cells ($A_{850} = 0.05$) was continuously circulated to avoid damage caused by long periods of irradiation. In the −196° C measurement, the cell suspension ($A_{850} = 0.1$) was mixed with an equal volume of 30% poly(ethylene glycol) 4000 to obtain homogeneous ice, and set in a 2-mm lucite cuvette, frozen and immersed into liquid nitrogen during measurements. The intensity of excitation pulse was reduced to about 10^7–10^8 photons/cm^2 per pulse to avoid damaging the photosynthetic pigments. Convolution of decay kinetics and deconvolution of the time-resolved fluorescence spectra were carried out as described previously [35–39].

Results

Time-resolved fluorescence spectra at −196° C

In the present study, 590 nm excitation pulses were used to excite the Q_x band of all light-harvesting Bchl molecules. Considering the differences in absorption coefficients of B800-850 and B875 at 590 nm [15,28,29,43,44], more than 65% of excitation energy was expected to localize on B800-850 complex at the initial time of excitation. In the time following excitation, the spectral changes in fluorescence can be observed according to the energy flow among the components. To analyze these spectral changes, the number and the spectral properties of fluorescence components should be determined. Therefore, measurements at low temperature were carried out at first to obtain higher spectral resolution.

The time-resolved fluorescence spectra of *Rb. sphaeroides* normalized to the maximum intensity of each spectrum are shown in Fig. 1. The fluorescence

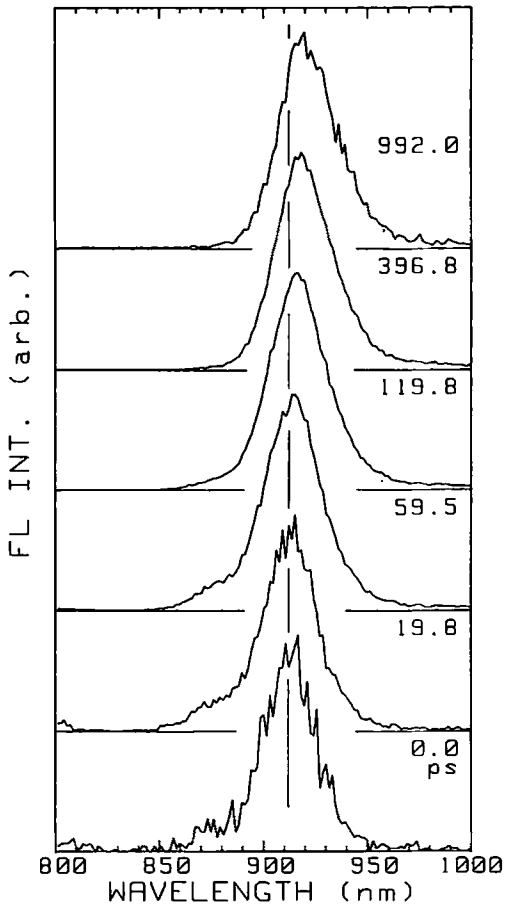

Fig. 1. Normalized time-resolved fluorescence spectra of intact cells of *Rb. sphaeroides* at −196°C. Numbers in the figure show the time after the excitation pulse. A vertical line represents location of the maximum of main component at 0 ps.

maximum was first observed around 910 nm even at 0 ps. Another fluorescence band was clearly detected around 875 nm. Apparently, the component around 875 nm disappeared within 100 ps and the maximum around 910 nm shifted gradually to about 920 nm within 1 ns, indicating heterogeneity of the major fluorescence band. No significant emission band arising from B800 was detected between 800 and 850 nm, even shortly after time zero.

Differences in the spectra at two different times indicated the presence of three components in the spectrum (Fig. 2A). The time-resolved fluorescence spectra were deconvoluted into Gaussian-shaped components based on the difference spectra. A good fit was obtained by three major components located at 875, 911 and 925 nm, and a minor one at 955 nm (Fig. 2B). The last component may be a vibrational band. In the time just after excitation, three components were clearly observed; however, in a later time range, F875 has substantially decreased. The three major components were readily interpreted as the fluorescence bands arising from B850, B875, and an additional longer-wavelength antenna component, respectively.

The location of the absorption maximum of the longer-wavelength antenna was estimated from the deconvoluted fluorescence spectrum according to Stepanov [45] using the following procedure. Corresponding to the observed fluorescence maxima at 875, 911 and 925 nm, the calculated peaks for B850, B875 and the longer-wavelength antenna were 855, 892 and 902 nm, respectively. The same treatment also gave maxima at 851, 879 and 894 nm at 22°C (see below). The maxima of 851 and 879 nm at 22°C, and 855 nm at −196°C are in good agreement with the previous reports; B850, at 851 nm [46], B875 at 879 nm [41] and B850 at 854 nm [46], with a maximum difference of 1 nm. Our estimate of 892 nm for the absorption peak for B875 at −196°C is 4 nm larger than another report of 888 nm for the maximum [47]. Thus we applied the same shift to the estimated location of the 894 and 902 nm bands which gives rise to the maxima at 890 nm at 22°C and 898 nm at −196°C for the longer-wavelength antenna. The latter estimate is very close to a previous report (896 nm [16,17] or about 12 nm longer than the location of B875 [15]). Thus, we call the longer-wavelength antenna B890 in this paper, according to its estimated absorption maximum at 22°C.

Energy flow among the antenna components, including B890, at −196°C was investigated by analysis of the decay kinetics of individual fluorescence components. The rise and decay kinetics of F875, F911 and

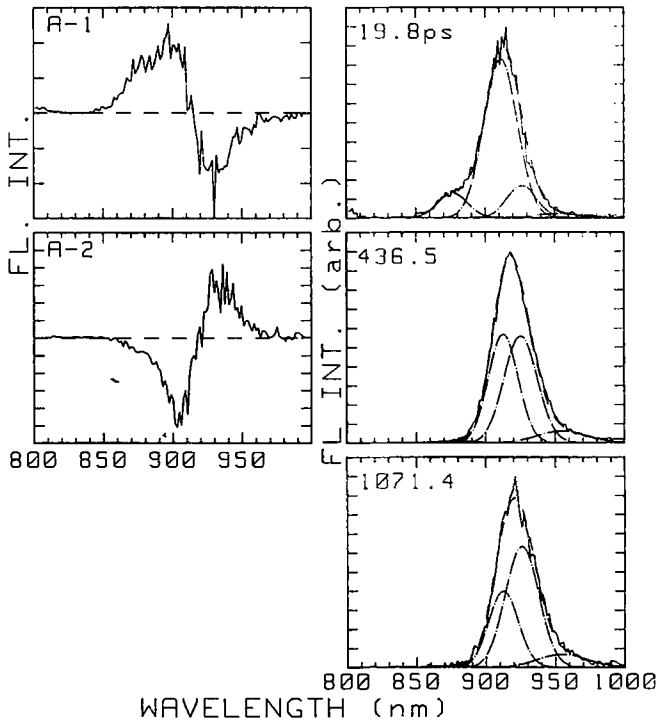

Fig. 2. Confirmation of the presence of three fluorescence components by the difference spectra of normalized time-resolved spectra at −196°C (A) and deconvolution of the time-resolved spectra (B). In (A), the differences between the spectra at 59.52 ps and that at 119.04 ps (A-1), and between 992 ps and 119.04 ps (A-2) were shown.

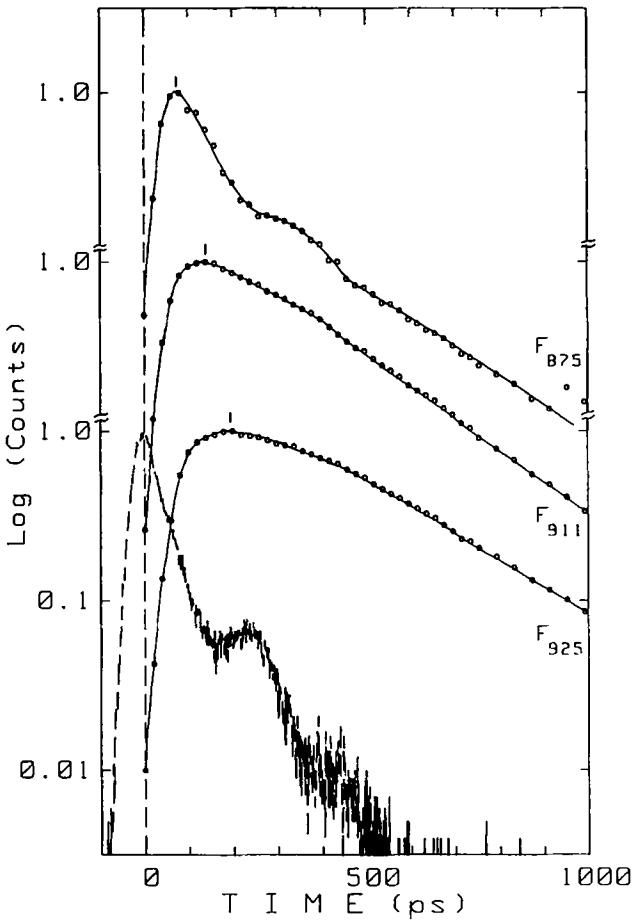

FIg. 3. Rise and decay kinetics of the three fluorescence components (F875, F911 and F925) at −196 °C. The broken line shows the pulse profile. Bars over the decay curves represent the times at which maximum intensity is reached.

F925 were obtained by deconvolution of the time-resolved spectra (Fig. 3). Maximum intensities were observed at 79, 139 and 198 ps after excitation for F875, F911 and F925, respectively. These results clearly show a sequential energy transfer from B850 to B875 and then to B890. The lifetimes of individual components, obtained by convolution with the excitation pulse, are about 50, 220 and 250 ps, for F875, F911 and F925 respectively. For F875, a contribution of a slow decay component was subtracted. In the decay of the F911 and F925, another type of slow decay component was found with a lifetime of about 1000 ps. The lifetimes of the B875 and B890 are in good agreement with other reported values (approx. 200 ps [7,14,16,23–25]), and shorter than those in isolated complexes [48].

At the initial time of excitation, the distribution of excited Bchl molecules among the antenna components is expected to be proportional to the absorption of photon by individual complexes. The absorption coefficient of B875 at 590 nm was estimated to be about 1.5-fold that of B800-850 in wild-type *Rb. sphaeroides* (Shimada et al., unpublished data; also see Refs. 15, 28, 29, 44). The molar ratio of B800–850 to B875 in the bacterium under our culture condition was about 2.5. It must also be considered that the transfer time from B800 to B850 was less than 2 ps [28–30]. Using the above results, we estimated that more than 60% of total excitation energy is initially on B850 after excitation. Contrary to this, the observed fluorescence spectrum indicates that most of fluorescence comes from B875, even at 0 ps. The relative emission from B850, F875/(F911 + F925), was estimated to be less than 10% by comparison of areas under individual fluorescence bands in wavenumber unit. Yet the ratio of F925 to F911 (about 0.18) in the fluorescence spectra during the initial time range appeared to reflect the actual number of Bchl in B890 and B875 [14,15]. These results suggest that significant amount of excitation energy on B850 is transferred to B875 within the time resolution of the instrument (less than 6 ps) and the decay time constant (50 ps) estimated above accounts for the residual part of excitation energy left on B850. Our results directly indicate a fast transfer of energy from B850 to B875, which has been suggested by other groups [25–27].

Time-resolved fluorescence spectra at room temperature

Fig. 4 shows the time-resolved fluorescence spectra of *Rb. sphaeroides* cells at 22 °C. A maximum around 895 nm and a shoulder around 860 nm were observed. The relative height of the shoulder decreased during the initial 100 ps after excitation. After this time, the fluorescence spectra remained virtually invariant. The red-shift of the maximum from 890 to 895 nm was observed only in the initial time after excitation; however, it was not so prominent as in the data taken at −196 °C. The difference in the spectra at two different times clearly indicates a decrease in the intensity of the component at 860 nm and an increase, around 910 nm (Fig. 5A). A small change was also detected around 888 nm. This indicates that the fluorescence spectra measured at room temperature were also composed of three major fluorescence components. Gaussian deconvolution showed a good fit with three major fluorescence bands, F859, F889 and F909, which most likely arise from B850, B875 and B890, respectively (Fig. 5B). This is, to our knowledge, the first demonstration of the presence of B890 at physiological temperature by fluorescence spectra.

The relative intensities of F859, F889 and F909 in the fluorescence spectra changed with time up to 100 ps (Fig. 4). Those of F859 and F889 decreased by 25% and 10%, respectively, while the relative intensity of F909 increased 60%. After 100 ps, the spectra become almost constant. Compared with the spectra at 335 ps after the excitation (dotted lines in Fig. 4), the spectrum at 99.2 ps was almost identical. This result clearly shows that, at room temperature, an equilibration of excitation transfer between the antenna pigments is attained within 100 ps after excitation.

Rise and decay curves of individual fluorescence components were obtained by the same treatment as in the case at −196 °C (Fig. 6). The three curves were almost the same except for the sequential shift of times at the maximum intensities. Note that the time differences between the maximum intensities were very little, compared with the case at −196 °C. The lifetimes of individual components were estimated after analysis by convolution. The main decay constant was 255 ± 10 ps in all cases, corresponding to the average trapping time by the RC with most of the RC closed [7,14,16,23–25]. In the decay of the F859, a fast decay of 12 ps was obtained. Clear rise terms were resolved in the kinetics of the F889 and F909: 20 and 35 ps, respectively. These can be interpreted as a generating process of the excited population in the case of closed trap, that is, the equilibration process among antenna molecules. The equilibration process between B890 and RC was not resolved as a rise term, simply because we could not identify the fluorescence from RC. When we plotted the relative intensity of the F909 in the spectra (cf. Fig. 5) as a function of time, a time constant of 50 ps was obtained (data not shown). This may correspond to the time for equilibration of excitations between B890 and RC.

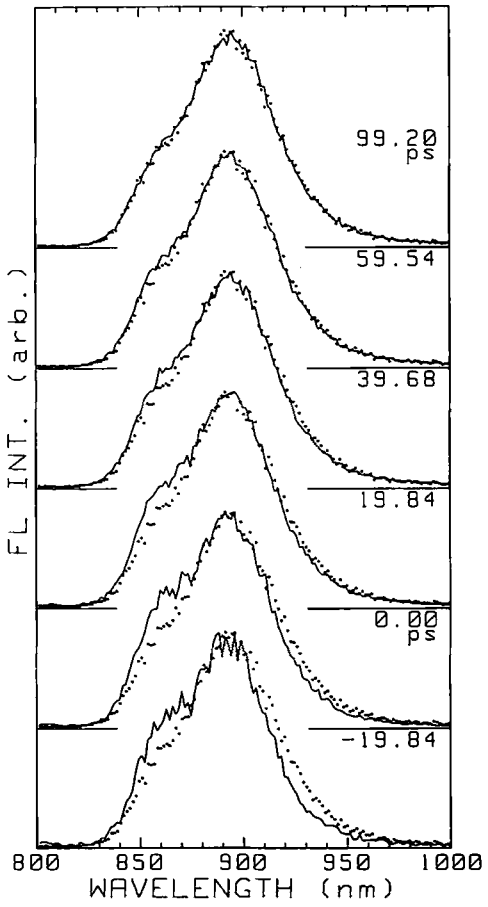

Fig. 4. Normalized time-resolved fluorescence spectra of intact cells of *Rb. sphaeroides* at 22 °C. Dotted lines show the spectra 335 ps after the excitation pulse.

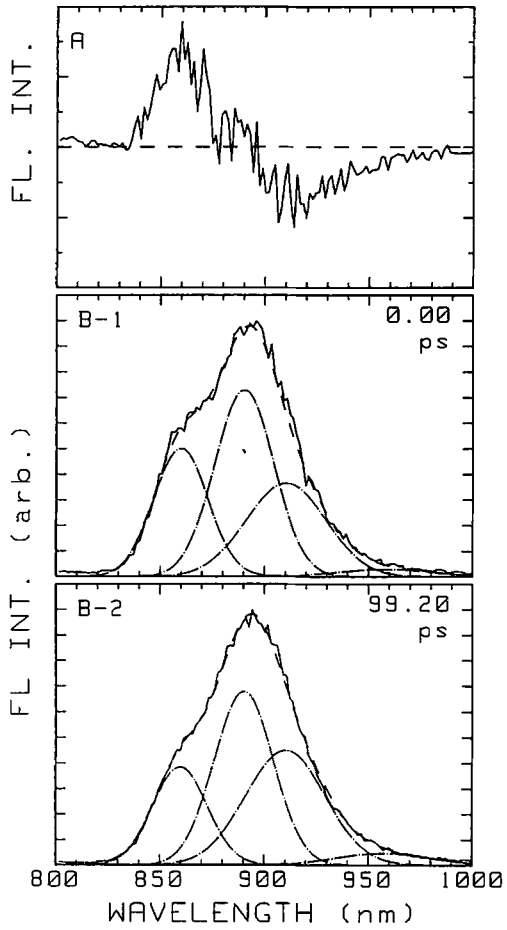

Fig. 5. Presence of three fluorescence bands at 22 °C detected by difference spectra (A) and deconvolution of the time-resolved spectra (B). In (A), difference between 0 ps and 133.92 ps is shown.

Discussion

Time-resolved fluorescence spectra measured with the time-correlated single-photon counting method have provided direct evidence and reliable information on the flow of excitation energy in a variety of photosynthetic light-harvesting systems [35–40]. In the present study, this method was applied to a bacterial light-harvesting system using a new type of microchannel-plate photomultiplier for improved detection in the near infrared region.

In the time-resolved fluorescence spectra measured at −196 °C, a maximum was found at about 910 nm in the 0 ps spectra. It shifted to about 920 nm at a later time (Figs. 1 and 2). The red-shift of this emission maximum clearly shows heterogeneity of the longer-wavelength light-harvesting complex, B875. The shift was also detected at room temperature (Fig. 4). These results clearly demonstrate the direct evidence for the presence of the longer wavelength antenna by fluores-

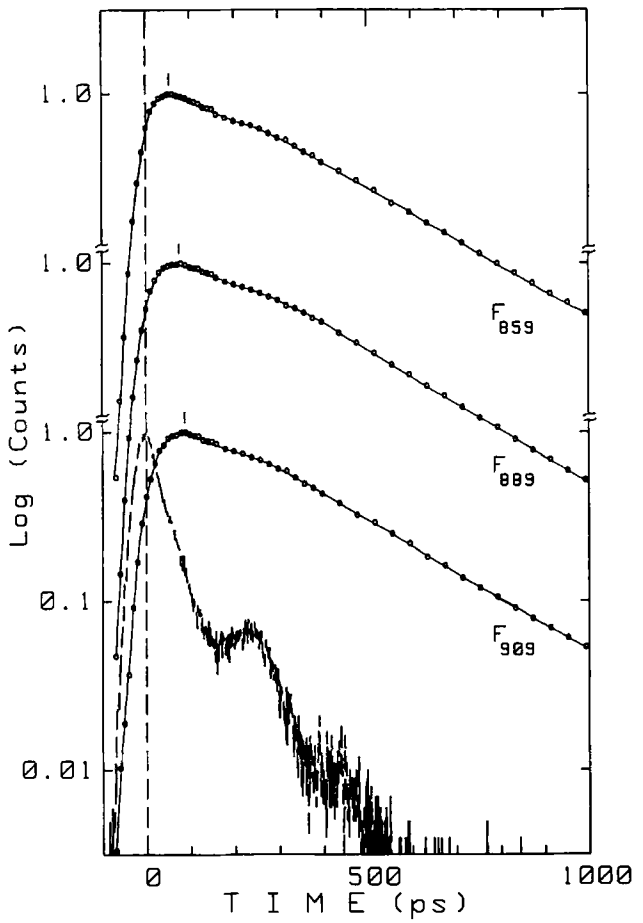

Fig. 6. Rise and decay kinetics of individual fluorescence components (F859, F889 and F909) at 22° C. Each point is estimated by deconvolution of spectra and actual fluorescence counts in each spectrum. The broken line shows the pulse profile. Bars over the decay curves represent the times at which maximum intensity is reached.

cence spectroscopy in *Rb. sphaeroides* at physiological temperature.

The longer-wavelength antenna has been suggested by various spectroscopic methods [13–18] and has been tentatively called B896 or B905. Deconvolution of the time-resolved fluorescence spectra revealed the fluorescence maximum of the longer wavelength antenna to be 909 nm at 22° C and 925 nm at −196° C. Corresponding absorption maximum of the longer wavelength antenna was estimated from our data using the Stepanov equation [45]. The values, 890 nm at room temperature and 898 nm at −196° C, indicate that the absorption maximum of this antenna component shifts depending on temperature, as do the other antenna components. Thus, we call this antenna component B890 based on the maximum absorption at room temperature. The heterogeneity of B875 or B880 was found both in *Rhodopseudomonas palustris* and *R. rubrum* as indicated by a red-shift of the fluorescence maximum in the time-resolved spectra (Shimada et al., unpublished data). This suggests that presence of the antenna with the energy level lower than that of RC is a general feature in purple non-sulfur photosynthetic bacteria.

In the all time-resolved fluorescence spectra measured in this study, we could not detect any fluorescence bands from B800 (Figs. 1 and 4). This can be interpreted as evidence for a very fast excitation transfer from B800 to B850 as indicated by other spectroscopic methods [28–30]. Its transfer time has been estimated to be shorter than 2 ps. Furthermore, a significant amount of the excitations in B850 seemed to be transferred to B875 within the time resolution of the instrument (6 ps). In the time-resolved fluorescence spectra at 0 ps, for both temperature conditions (Figs. 1 and 4), the intensity of F889 at 22° C or F911 at −196° C was very high. It appeared to be much higher than expected judging from the absorption spectrum of the *Rb. sphaeroides* cells used, even when the time resolution of the instrument was taken into account. Fast appearance of the F889 was also confirmed by the direct excitation of B800 at 22° C (Shimada et al., unpublished data). These results clearly point to the occurrence of a very fast excitation transfer from B850 to B875 as has been suggested by Sebban et al. [25], Borisov et al. [26] and Freiberg et al. [27].

The lifetime of B850 is estimated to be about 12 ps at 22° C (Fig. 3) or 50 ps at −196° C (Fig. 6) by convolution calculation. A major part of the energy on B850 is transferred to B875 with a transfer time shorter than 6 ps. Therefore, the above lifetimes do not correspond to rate constants for energy transfer. They may instead possibly reflect the succession of the equilibrium between B850 and B875. Changes in the time-resolved fluorescence spectra clearly indicate that equilibration of excitation energy transfer among B850, B875 and B890 is attained within 100 ps after the excitation pulse at room temperature (Fig. 5.). This indicates that the time constant for each single transfer, such as from B875 to B890 or back to B850, is much shorter than those described above. This may be consistent with the proposed fast transfer from B850 to B875 discussed above.

In the measurements at 22° C, rise terms were found in the decay kinetics of the F889 and F909. These reflect the process of equilibration of excitations between the antenna. The equilibration times are 20 ps between F875 and F889, and 35 ps between F875 and F909. A rise term in the decay kinetics indicates the generating process of the excited molecules; thus, it is a direct index for the shift of equilibrium. Our estimation is shorter than those proposed for these processes [16]. However, when we assume the transfer time from B850 to B875 as 6 ps, as stated above, the reverse or back transfer time is expected to be 12 ps, as suggested by Zankel (cf. Ref. 32). This values give rise to an equilibration time between these two components of 18 ps, the sum of both transfer times. This time is in good

agreement with our estimate (20 ps) and supports its reliability.

After equilibration, the three major fluorescence components decayed with the same lifetime (255 ± 10 ps, Fig. 6). This can be interpreted as the mean lifetime of the excitation energy which migrates among the three antenna components (B850, B875 and B890) and RC in the closed state, all of which are interconnected to form a large domain corresponding to one chromatophore [10,50]. The 255 ps decay time is consistent with that reported for total fluorescence decay times with closed traps [7,14,16,23–25], although our estimate is longer by about 50 ps. The 255 ps lifetime and a large domain size (up to 3000) suggest that the elementary step of energy transfer proceeds in less than 1 ps. This is another line of evidence that the observed lifetimes of individual fluorescence components represent the shift of the equilibrium.

In the equilibrium state of excitation transfer, the ratio of the fluorescence yield of a more energetic component to that of less energetic one is determined by three parameters; the ratio of the backward to the forward rates of energy flow, number of individual components and the energy difference between them [32]. Deconvolution of the time-resolved fluorescence spectra after equilibration showed that the ratio F859 : F889 : F909 was estimated to be 1.00 : 1.94 : 1.70 by integration of each spectrum on wavenumber basis (Fig. 4). When we assume the absorption maximum for each of the respective antenna components to be 851, 878 and 890 nm, the Bchl population giving the above ratio of fluorescence yield is estimated to be 1.00 : 0.33 : 0.14 for B850 : B875 : B890 on a Bchl basis. In the *Rb. sphaeroides* cells we used, the relative content of B800-850 to RC-B875(+B890) complex was estimated to be about 2.5 based on the amount of Bchl determined by a spectral analysis method [41]. In the B800-850 complex, B850/B800 was 2 on Bchl basis [29]. In RC-B875(+B890) complex, the molar ratio of Bchl to RC was estimated to be about 28, of which 4 belonged to RC and the rest (about 24), to B875 plus B890 [49]. The stoichiometry gives a value of 0.51 for (B875 + B890)/B850 in the cells, which is in good agreement with the value of 0.47 calculated from the deconvoluted fluorescence spectra.

From the fluorescence spectra, the ratio B875 : B890 was estimated to be 0.33 : 0.14 as described above. This indicates that, out of 28 Bchl molecules in a RC-B875(+B890) complex, 7 ± 1 Bchl molecules are attributed to B890 and 17 ± 1 to B875.

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