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Energy transfer within the bacteriochlorophyll antenna of purple bacteria at 77 K, studied by picosecond absorption recovery

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The dynamics of energy transfer within the bacteriochlorophyll antenna of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, with closed reaction centers, has been studied at 77 K using low-intensity infrared picosecond absorption recovery. Measurements of isotropic decay as well as decay of induced anisotropy yielded a detailed picture of the energy transfer pathways in the two antenna systems. The BChl antenna of *Rb. sphaeroides* seems to consist of at least four different BChl *a* species: BChl 800, BChl 850, BChl 875, and BChl 896. Upon excitation of the highest-energy pigment, a transfer sequence towards lower energy is initiated. The transfer steps between the different pigment pools are characterized by the following time constants: BChl 800 → BChl 850, $\tau = 2 \pm 1$ ps; BChl 850 → BChl 875, $\tau = 40 \pm 5$ ps; BChl 875 → BChl 896, $\tau = 20 \pm 5$ ps. Once the excitations are localized on B896 a slower quenching, $\tau = 190 \pm 10$ ps, by closed reaction centers (P^+) occurs. From measurements of decay of induced anisotropy it is further concluded that efficient transfer between BChl 800 molecules takes place on a time-scale comparable to the BChl 800 → BChl 850 transfer. A marked increase in anisotropy in the red wing of the absorption spectrum offers a clear evidence of the presence of the long-wavelength antenna component B896. The BChl antenna of *R. rubrum* is composed of two BChl *a* species, BChl 880, and BChl 896, and the energy transfer kinetics are observed to be very similar to the corresponding part of *Rb. sphaeroides*. Some evidence of further spectral inhomogeneity (apart from B896) or spectral shifts induced by excitation of the B880 antenna pigment was also obtained. Several possible models are discussed for the origin and organization of the B896 pigment.

Introduction

The primary processes in the antenna of purple bacteria are at present being intensively studied. A variety of spectroscopic techniques have been employed in this work, and both dynamic and structural information has been obtained. For a review of the energy transfer work, see Ref. 1. The spectroscopic techniques primarily yield information concerning the dynamics of these processes, but they also play an important role in obtaining structural information, since so far only one membrane-bound pigment-protein complex (the reaction center of *Rps. viridis*) has actually been crystallized and its three-dimensional structure determined [2]. For the process of energy transfer and structure of the light-harvesting antenna, the following general picture has evolved through these studies. In bacteriochlorophyll *a*- (BChl *a*-) con-

Abbreviations: BChl, bacteriochlorophyll; RC, reaction center.

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taining purple bacteria BChl 875 forms a long wavelength antenna that surrounds and interconnects the reaction centers (RC). In *R. rubrum* there are approx. 30–50 BChl molecules per RC and the B875 antenna seems to be organized as aggregates of basic units containing 6–8 BChl 875 molecules, and half this number of carotenoid molecules [1]. Upon excitation of the antenna energy is transferred to the RC with an efficiency of about 95%. The lifetime of the excitation in the B875 antenna was found to be dependent on the oxidation state of the primary donor, P870, of the RC [3,4]. Thus, for active RC (reduced P) an overall trapping time of about 60 ps was observed, and when P was oxidized, either chemically or by continuous illumination, this time constant lengthened to approx. 200 ps.

In *Rb. sphaeroides* the antenna contains an additional component B800-850, which was shown to be assembled of pigment-protein complexes holding at least six BChl (4 BChl 850 and 2 BChl 800) and three carotenoid molecules [1,5]. Steady-state fluorescence measurements [6,7] suggested an equilibration of the energy between the B800-850 and B875 antennas. However, phase fluorimetry measurements [8] gave no evidence of a decay component due to B800-850 → B875 transfer. This transfer step was consequently assumed to be very fast [8]. Transfer from B875 to the RC in *Rb. sphaeroides* is characterized by kinetics very similar to those observed for *R. rubrum* [3].

The polarization of fluorescence emission originating from the antenna was generally low ($p \approx 0.1$) [5,9–12], when measured on chromatophores and isolated light-harvesting complexes. Excitation in the long-wavelength wing of B875 constituted an exception; at low temperature (4 K) a maximum polarization of $p = 0.4$ was observed [5]. The low polarization observed through most of the spectrum led to the suggestion that the Q_y transitions of the BChl molecules of the repeating units of the B875 and B800-850 pigment-protein complexes lie in one plane or several parallel planes [5,11].

In a recent study [13] we employed low-intensity tunable infrared picosecond pulses to monitor the energy transfer dynamics throughout the absorption bands of *R. rubrum* and *Rb. sphaeroides* at room temperature. A detailed and in many respects new picture of the energy transfer dynamics was obtained. Upon excitation of the main antenna pigment B880 of *R. rubrum* a very rapid initial depolarization ($r(0) \approx 0.1$) within less than 1 ps was observed. This was interpreted as being due to very fast energy transfer between identical BChl 880 molecules, probably within a minimum unit of 6–8 BChl molecules [14,15]. A further decrease of the anisotropy to very low values, $r(\infty) \approx 0$, occurred with a time-constant of about 40 ps. A similar time constant was also observed in the isotropic absorption decay with P in the oxidized state, representing about 40% of the signal at 890 nm. This 40 ps decay component was tentatively assigned to the equilibration of energy between B880 and a long-wavelength absorbing antenna pigment, B896 [11]. From the equilibration rate an average jumping time between B880 minimum units of approx. 10 ps could be estimated. For open reaction centers the overall trapping time was found to be approx. 60 ps, in good agreement with fluorescence streak camera [3,16] and absorption recovery measurements [4]. Closed reaction centers were also observed to quench the antenna excitations quite efficiently ($\tau \approx 200$ ps). The fact that the overall trapping rate with open reaction centers is not too much different from the equilibration time between B880 and B896 led us to suggest that the diffusion of energy through the antenna towards the reaction center is the rate-limiting process in the overall trapping of excitation energy.

The results for *Rb. sphaeroides* [13] could be explained with a very similar model by taking into consideration the additional pigment-protein complex B800-850 present in this species. Upon excitation of BChl 800 a very fast transfer ($\tau \leqslant 1$ ps) to BChl 850 was observed, followed by an equilibration between BChl 850 and BChl 875 molecules. Anisotropy measurements suggested very fast, $\tau < 1$ ps, transfer among neighboring BChl 850 or BChl 875 molecules. Quenching by the reaction centers, either open or closed, was characterized by rate constants very similar to those observed for *R. rubrum*, under both open and closed reaction center conditions.

Although these room-temperature results [13] offered a quite detailed picture of the pathways of energy flow through the BChl antenna system,

there are a number of unanswered questions that we shall address in this paper. First, more conclusive evidence is required for the existence of the long-wavelength antenna component B896, in both *R. rubrum* and *Rb. sphaeroides*. Second, a better characterization of the BChl 800 → BChl 850 transfer is desirable. Finally, from the room-temperature measurements the extent of energy transfer among BChl 800 molecules and its relation to BChl 800 → BChl 850 transfer was not clear.

The rest of the paper is organized as follows. First, we will give a brief description of sample handling and the measurement technique. Then, the results of the isotropic kinetic measurements, at a number of wavelengths throughout the absorption bands of *R. rubrum* and *Rb. sphaeroides* are presented. These results are used to construct a model for the energy transfer which involves the sequence of pigments B800-850–B875–B896–RC for *Rb. sphaeroides* and B880–B896–RC in the case of *R. rubrum*. This is followed by a discussion of the time-resolved absorption anisotropy measurements in both species. We conclude by discussing possible models for the origin and organization of B896.

Materials and Methods

R. rubrum and *Rb. sphaeroides* were grown as described in Ref. 12. Chromatophores were prepared using a French press and were diluted in a buffer medium comprising 250 mM Tricine/5 mM KH_2PO_4/5 mM $MgCl_2$ (pH 8).

In order to obtain a good glass for the low temperature measurements, a concentrated chromatophore solution was diluted with glycerol to yield a solution of the desired optical absorbance (0.3–0.5/mm) with a glycerol-to-water ratio of about 3:1. Constant temperature, 77 K, was maintained by a liquid-nitrogen cryostat (Oxford Instruments). To achieve the conditions of closed reaction centers (P^+) the chromatophore solution was purged with nitrogen gas prior to cooling, and used in plain buffer or with the addition of small amounts of sodium ascorbate. Both methods were seen to yield identical results of the kinetic measurements. During cooling from room temperature to 77 K the sample was illuminated with white light from a slide projector, to accumulate the RCs in the state $P870^+$.

Picosecond absorption recovery and anisotropy kinetics, with closed RC, were measured as previously described elsewhere [13]. The recorded kinetic curves were analyzed as a sum of exponentials, and the lifetimes and amplitudes were extracted by a non-linear least-square fitting computer program employing the Marquardt-Levenberg algorithm [17]. In cases of very short-lived decay components a convolution of the calculated decay with a Gaussian pulse, having the width of the measured autocorrelation function, was performed. By this method, lifetimes which are considerably shorter than the width of the laser-pulse can be measured accurately. In order to evaluate the reliability of this method to measure short lifetimes, test experiments on dye molecules having lifetimes in the range 1–5 ps were performed. The results showed that lifetimes on the order of 1 ps can be accurately determined with the present method.

Results

Several absorption recovery curves for *Rb. sphaeroides* and *R. rubrum* measured at 77 K with isotropic polarization are shown in Figs. 1 and 2. The wavelengths of the displayed curves have been chosen to illustrate all the different types of kinetics observed, with respect to lifetimes, amplitude and sign of the decay components.

The measured kinetics are seen to be very rich in detail and display extensive variation with wavelength. Notice, however, that the measured kinetics appear to be relatively simple in the far red wing of the absorption spectrum ($\lambda > 900$ nm), and increase in complexity towards shorter wavelengths. This behavior is demonstrated by the kinetics of *Rb. sphaeroides* of Fig. 1. At $\lambda = 905$ nm the decay is well described by a single exponential decay having a time constant $\tau_1 \approx 190$ ps, very similar to the lifetime ascribed to the quenching by closed reaction centers at room temperature [8,13,16,18,19]. At shorter wavelengths the decays contain at least one additional, more short-lived component. Thus, in the wavelength interval 880–900 nm a negative component with time constant $\tau_2 \approx 20$ ps is observed, whereas in

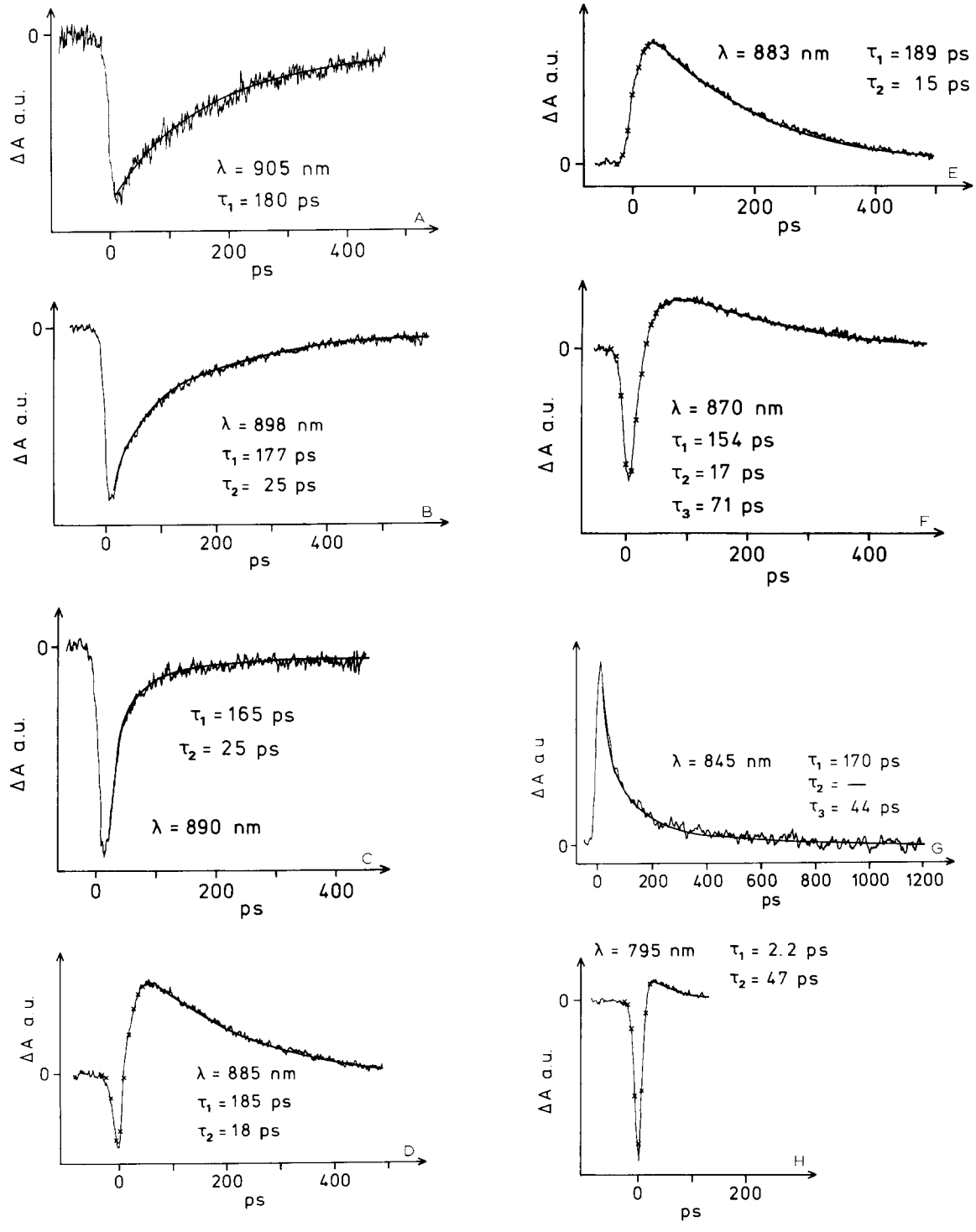


Fig. 1. Measured isotropic absorption recovery kinetics of *Rb. sphaeroides* chromatophores at 77 K with closed RC. A positive ΔA corresponds to increased absorption relative the initial state. Fitted kinetics are displayed with a full line or crosses, whichever stands out most clearly against the experimental curve. Maximum absolute bleaching (negative ΔA) of the kinetic curves are about $0.5 \cdot 10^{-3}$ at the peak of the absorption bands, and correspondingly lower at other wavelengths.

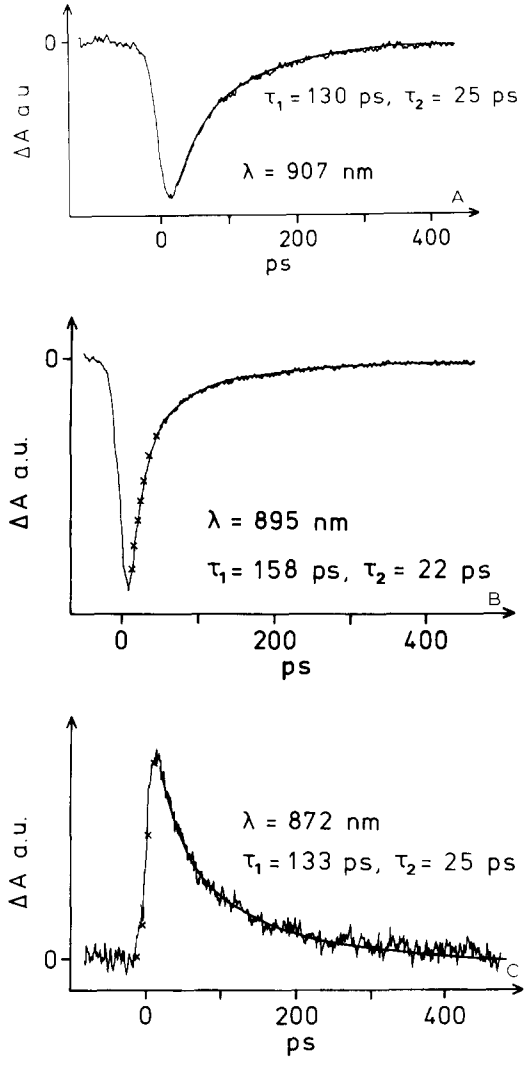


Fig. 2. Measured isotropic absorption recovery kinetics of *R. rubrum* chromatophores at 77 K with closed RC. Fitted kinetics are displayed as in Fig. 1.

the range 830–850 nm a component with positive amplitude and lifetime $\tau_3 \approx 40$ ps is observed. This 40 ps decay is also seen at around 800 nm.

In the wavelength region around 870 nm the decay is complex due to the coexistence of all three components, and the extracted lifetimes and amplitude values are associated with larger error bars as compared to those obtained at the other wavelengths. However, it is not only the complex three-lifetime decay that is responsible for these problems. The fact that the laser pulse is not a perfect Gaussian pulse (it carries more intensity in the wings than a truly Gaussian pulse) and the distortion of the decay curve around $t = 0$ due to the coherent coupling artifact (coherence spike) introduces errors when fitting on the rising part of the decay curve. There is still another possible reason for the larger deviations from average values at these wavelengths. Absorption spectra of chromatophores prepared from a mutant of *Rb. sphaeroides* lacking reaction centers and the B875 complex reveal a shoulder in the red wing of the BChl 850 band around 870 nm, that is absent in the spectrum of the isolated B800–850 complex, but probably present in the absorption spectrum of wild-type *Rb. sphaeroides* chromatophores (van Dorsen, R.J. et al., and Hunter, C.N. et al., unpublished data). This may be due to a previously unknown antenna component, which would then be expected to make the absorption recovery decay even more complex in the region around 870 nm.

As already mentioned, the influence on the fitted lifetimes of the finite pulse width (about 10 ps) was also checked by performing fits including a deconvolution procedure. It was concluded that for lifetimes longer than about 20 ps the fitted lifetimes are not significantly distorted by the laser pulse. There is, however, one exception to this conclusion: in order to resolve the fast $\tau_2 \approx 20$ ps component present as a risetime in the decay curves around 883 nm (see Fig. 1E), deconvolution was necessary. As discussed in detail below, a kinetic scheme involving three consecutive decays seems to be a reasonable description of the energy transfer kinetics of *Rb. sphaeroides.*

The kinetics of *Rb. sphaeroides* completely change character when exciting and probing in the BChl 800 band. An initial bleaching recovers very quickly, with close to pulse-limited response, to an absorbing state which recovers with a time-constant of about 45 ps. This slower lifetime appears to be identical to the lifetime τ_3 measured in the wavelength range 840–870 nm. The initial very fast decay is essentially limited by the pulse length. However, a close examination reveals that this decay appears to be approx. 10% broadened as compared to the pulse autocorrelation function. A deconvolution of the decay with a Gaussian pulse having the width of the pulse autocorrelation

TABLE I

SUMMARY OF ABSORPTION RECOVERY LIFETIMES AND ASSOCIATED RELATIVE AMPLITUDES ($R_{i/j}$) OF *RB. SPHAEROIDES* CHROMATOPHORES AT 77 K, WITH CLOSED RC

Indicated error limits are standard deviations of the evaluated lifetimes.

λ (nm)	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)	$R_{2/1}$	$R_{2/3}$	$R_{3/1}$	No. of meas.
794–800 [a]	$\tau_{800\text{-}850} = 2.2 \pm 1.0$		47 ± 5		-42		3
845	171	–	40 ± 5			1.6 ± 0.3	3
860	200 ± 20	25 ± 3	–	-5	–	–	4
866 [a]	190	15	40	-8	2.6	-3	2
870 [a]	180 ± 30	17	70	-2.3	3.3	-0.7	3
883 [a]	185 ± 5	27 ± 3	–	-0.8 ± 0.3			5
885 [a]	180 ± 5	18 ± 2	–	-2.3			3
889	150 ± 5	22 ± 3	–	≈ 10			3
890	165 ± 10	25 ± 5	–	≈ 10			3
895	170 ± 30	25 ± 5	–	1 ± 0.2			4
898	180 ± 15	25 ± 5	–	0.9 ± 0.1			3
900	200 ± 10	25 ± 5	–	0.7 ± 0.1			3
905	180 ± 15	–	–	< 0.1			2

[a] Deconvolution was used to obtain the kinetic parameters.

function yields a lifetime $\tau = 2.2 \pm 1.0$ ps.

From Fig. 2 it is seen that the absorption recovery kinetics of *R. rubrum* display a spectral variation which is similar to that observed for *Rb. sphaeroides*. However, the variations are less complicated; only one fast ($\tau_2 \approx 25$ ps) and one slow ($\tau_1 \approx 150$ ps) decay component are observed, which both change sign once. As will be seen from the analysis given below, this difference can be traced back to the difference in antenna pigment composition of the two species. *R. rubrum* is lacking the short wavelength antenna complex B800-850 present in *Rb. sphaeroides*.

TABLE II

SUMMARY OF ABSORPTION RECOVERY LIFETIMES AND AMPLITUDE RATIOS OF *R. RUBRUM* CHROMATOPHORES AT 77 K, WITH CLOSED RC

The indicated error limits were obtained as in Table I.

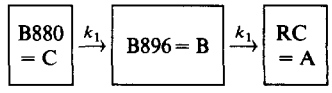
λ (nm)	τ_1 (ps)	τ_2 (ps)	$R_{2/1}$	No. of meas.
859–878 [a]	154 ± 40	25 ± 5	1.8 ± 0.5	12
880	155	32	4	2
895	158 ± 20	22 ± 2	≈ 10	4
900	135 ± 15	30 ± 5	3 ± 1	3
904	120	26	1.5	2
907	120	28	1	2

[a] Deconvolution was used to obtain the kinetic parameters.

The absorption recovery kinetics of *Rb. sphaeroides* and *R. rubrum* were measured at many more wavelengths than those displayed in Figs. 1 and 2. A summary of all the results is given in Tables I and II.

Interpretation and analysis of the decay kinetics

The bacteriochlorophyll antenna of *R. rubrum* has been considered to be composed of only one type of pigment-protein complex, B880 [1], and *Rb. sphaeroides* was believed to contain two spectrally (and structurally) distinct antenna pigments, B800-850 and B875 [1,20–22]. With such a pigment composition one would predict a single exponential decay (with lifetime 150 ps) of BChl excitations in *R. rubrum*, corresponding to the reaction center quenching of the B880 excitations. In the case of *Rb. sphaeroides* we would expect a double exponential decay, where, in addition to the decay associated with the RC quenching, another lifetime reflecting the B800-850 → B875 transfer should be observed (for the moment we ignore the very fast BChl 800 → BChl 850 transfer). The kinetic results presented above clearly demonstrate that these models cannot account for the spectral variation of the measured energy transfer kinetics in the two purple bacteria species investigated here. If we take into account our previous room-temperature absorption recovery



Scheme I. *R. rubrum.*

measurements on chromatophores [13] and isolated B800-850 complex [23] as well as low-temperature fluorescence depolarization measurements [11], the most reasonable explanation for the present results appears to be the existence of an additional long-wavelength antenna pigment, B896. This pigment appears to be present in both *R. rubrum* and *Rb. sphaeroides.* Considering the function of the bacteriochlorophyll antenna, i.e., to efficiently direct the excitation energy into the reaction centers, it appears reasonable to use the following kinetic model shown in Schemes I and II to describe the energy transfer of *R. rubrum* and *Rb. sphaeroides* at 77 K.

Assuming a 7 : 14 : 6 : 1 ratio of BChl 800 : BChl 850 : BChl 875 : BChl 896 in *Rb. sphaeroides* membranes and a 6 : 1 ratio of BChl 880 : BChl 896 in *R. rubrum* membranes [11] it is easily calculated that the backward rates are negligible in all cases. Only RC → B896 transfer may play a role, but we ignore this for the moment.

The two kinetic schemes allow us to calculate the number of excited BChl molecules of each pool as a function of time. Consider first *R. rubrum.* Allowing for direct excitation of BChl 880 and BChl 896, we obtain the following expression for the time-dependence of the recorded signal

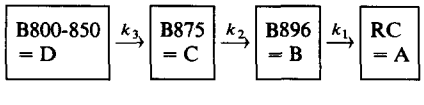
$$\Delta A(t) = \Psi_1 \cdot e^{-k_1 t} + \Psi_2 \cdot e^{-k_2 t} \tag{1}$$

The amplitudes Ψ_1 and Ψ_2 are functions of ground- and excited-state extinction coefficients (ε_i, ε_i^*), rate constants (k_i) and initial concentrations (B_0, C_0) of excited B and C.

$$\Psi_1 = (\varepsilon_B - \varepsilon_B^*) B_0 - (\varepsilon_C - \varepsilon_B^*) k_2 C_0 (k_1 - k_2)^{-1} \tag{2}$$

$$\Psi_2 = (\varepsilon_C - \varepsilon_C^*) C_0 + (\varepsilon_C - \varepsilon_B^*) k_2 C_0 (k_1 - k_2)^{-1} \tag{3}$$

In an analogous manner we obtain the time dependence of the recovery signal for *Rb. sphaeroides.* Allowing all three pigments, BChl 850, BChl 875 and BChl 896, to be initially excited we have,



Scheme II. *Rb. sphaeroides.*

$$\Delta A(t) = \Psi_1 e^{-k_1 t} + \Psi_2 e^{-k_2 t} + \Psi_3 e^{-k_3 t} \tag{4}$$

where the amplitudes, Ψ_i, have the following form:

$$\Psi_1 = \left[\frac{(\varepsilon_D - \varepsilon_B^*) k_2 k_3}{(k_3 - k_1)(k_2 - k_1)} - \frac{C_0 (\varepsilon_C - \varepsilon_B^*) k_2}{D_0 (k_1 - k_2)} + \frac{B_0}{D_0} (\varepsilon_B - \varepsilon_B^*) \right] D_0 \tag{5}$$

$$\Psi_2 = \left[\frac{-(\varepsilon_D - \varepsilon_C^*) k_3}{(k_2 - k_3)} + \frac{(\varepsilon_D - \varepsilon_B^*) k_2 k_3}{(k_3 - k_2)(k_1 - k_2)} + \frac{C_0}{D_0} (\varepsilon_C - \varepsilon_C^*) + \frac{C_0 (\varepsilon_C - \varepsilon_B^*) k_2}{D_0 (k_1 - k_2)} \right] D_0 \tag{6}$$

$$\Psi_3 = \left[(\varepsilon_D - \varepsilon_D^*) + \frac{(\varepsilon_D - \varepsilon_C^*) k_3}{(k_2 - k_3)} + \frac{(\varepsilon_D + \varepsilon_B^*) k_2 k_3}{(k_2 - k_3)(k_1 - k_3)} \right] D_0 \tag{7}$$

B_0, C_0, ε_B and ε_C have the same meaning as in Scheme I and D_0 represents the initial concentration of excited D, and ε_D, ε_D^* are the ground-state and excited-state absorption coefficients of D at that particular wavelength. For the situation where not all pigments are directly excited by the light-pulse the corresponding parameter(s) of initial concentration is (are) set equal to zero.

The lifetimes and relative amplitudes obtained through a fit of these expressions to the measured curves at several wavelengths are summarized in Tables I and II. We also note that the energy transfer dynamics in the two species are quite similar, except for the added complexity in the *Rb. sphaeroides* kinetics caused by the existence of the B800-850 antenna.

The measured kinetics for *Rb. sphaeroides* and *R. rubrum* are directly related to the ΔA spectra of the various kinetic components. To illustrate this for the more complex system, *Rb. sphaeroides,* we have composed Fig. 3. Here we have approximated the ΔA spectrum of each bacteriochlorophyll-protein complex by a spectrum approximately equal to that measured for *R. rubrum* at room temperature [15]. However, the ΔA spectrum used for B875 is an exception. As mentioned

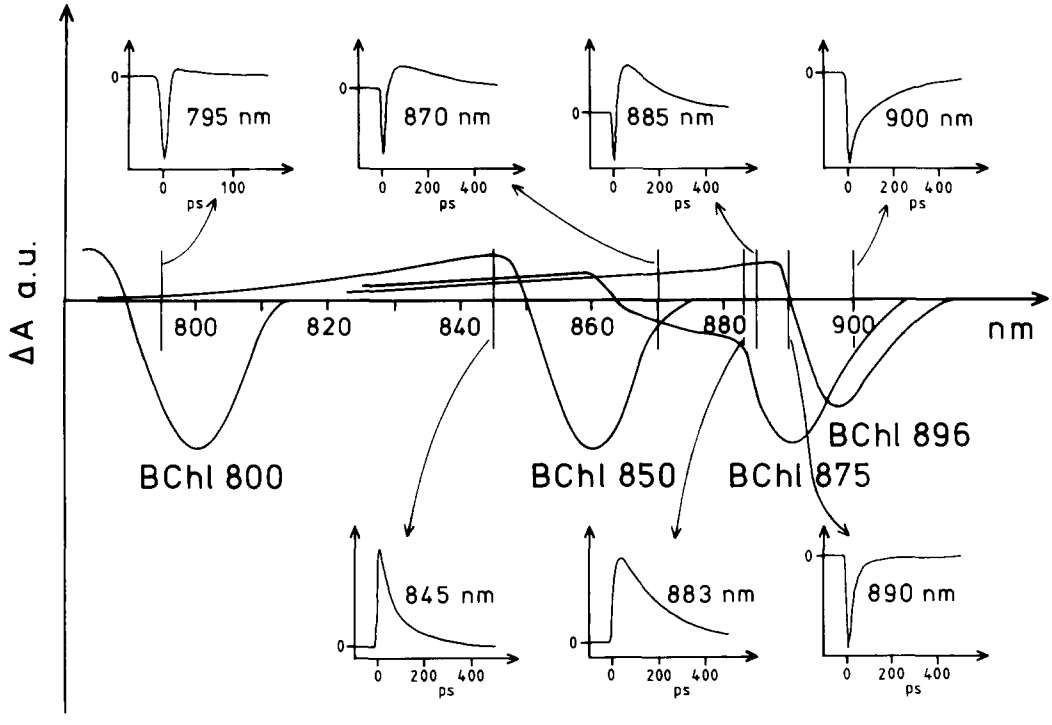


Fig. 3. Relation between calculated recovery kinetics and ΔA spectra of BChl pigments. The calculated kinetics (insets) are obtained by using the lifetimes and amplitude ratios of Table I, convoluted with a Gaussian pulse having 10 ps width.

above, the complex kinetics in the wavelength region around 870 nm suggest the presence of at least two short-lived decay components (20 and 40 ps) with negative amplitude. The approximate ΔA spectrum in combination with the measured isosbestic point of B875 is not compatible with this observation; the $\tau_2 = 20$ ps component should have a positive amplitude at this point in the spectrum. If, on the other hand, the ΔA spectrum of B875 has a negative shoulder in the interval 860–880 nm, as suggested in Fig. 3, all ΔA spectra in this figure would be in at least qualitative agreement with the amplitudes obtained from the absorption recovery experiments (see Table I). Picosecond absorption measurements of the isolated B875 complex at 77 K also suggest a similar shape of the ΔA spectrum in this wavelength region (Bergström, H., et al., unpublished data). It is quite possible that this feature can be related to the long-wavelength shoulder in the BChl 850 absorption band mentioned above. However, it is premature to speculate further about possible explanations for this observation, and additional experiments are necessary.

The position on the wavelength scale of each spectrum in Fig. 3 is determined by the measured isosbestic wavelength of the corresponding pigment. Calculated absorption recovery kinetics, using the ΔA spectra and the lifetimes of Table I, are shown as insets, and in principle can be compared directly with those shown in Fig. 1. Generally, there is seen to be a good agreement between the measured kinetics and that expected from this model. As an example, consider the kinetics measured at 800 nm. Judging by the ΔA spectrum and the kinetic scheme (Scheme II) we expect to observe an initial fast transient bleaching, due to excitation of BChl 800, which decays into excited state absorption of BChl 850*. This absorption recovers to zero signal, since the BChl 875* (or BChl 896*) into which BChl 850* decays has very little excited state absorption at this wavelength. The measured kinetics show that this is exactly what is experimentally observed (Fig. 1H).

A straightforward comparison between the calculated and measured kinetics can also be given for the red part of the spectrum, i.e. $\lambda > 880$ nm, where according to the spectra only B875 and

B896 are directly excited. Consider for instance $\lambda = 900$ nm. According to Fig. 3 and the kinetic Scheme II, we expect to see a fast initial decay of an intense bleaching as BChl 875* transfers its excitation energy to BChl 896, followed by a slower decay of the remaining bleaching when B896* excitations are quenched by closed reaction centers. Again, this is exactly the kind of decay curve that was measured (Fig. 1B).

As a third and final example we consider the more complex kinetics at $\lambda = 870$ nm, where both BChl 850 and BChl 875 are initially excited. Upon excitation and probing at 870 nm, we expect to see an initial bleaching decaying with the two time constants $\tau_2 = 20$ ps and $\tau_3 = 40$ ps into an absorption signal, representing BChl 896*. Despite the small difference in the τ_2 and τ_3 lifetimes, these features are clearly distinguished in the measured decay curve (see Fig. 1F and Table I).

The remaining traces shown in Fig. 3 are based on the kinetic and spectral parameters given in Table I and reproduce the general properties of the experimental decay curves. In conclusion, the assumed spectral data, in combination with Scheme II, give a good description of the excitation transfer dynamics in *Rb. sphaeroides*. Using Scheme I and similar ΔA spectra, the kinetics of *R. rubrum* can be reproduced (however, no shoulder in the B880 spectrum around 870 nm is observed) (not shown).

Time-resolved absorption anisotropy

In this section we shall describe the observed decays of the induced absorption anisotropy in *R. rubrum* and *Rb. sphaeroides*. A few examples are displayed in Fig. 4, and a summary of the anisotropy results at several wavelengths is collected in Tables III and IV. Generally, the observed anisotropies are higher at 77 K than at room temperature [13,23]. This is especially true for anisotropy measured in the red wing of the absorption spectrum. Except for the measurements on *Rb. sphaeroides* at wavelengths above 900 nm (Fig. 4B) there is seen to be a small initial decay of anisotropy with a time-constant of about 20–30 ps (see Fig. 4C–D). This decay time is very similar to the fast isotropic component observed at the same wavelengths, and the time constant of anisotropy decay observed at room temperature [13]. Both

TABLE III

ANISOTROPY OF *RB. SPHAEROIDES* CHROMATOPHORES AT 77 K, WITH CLOSED RC

λ (nm)	$r(0)$	$r(\infty)$
800	0.24	–
802	0.23	–
859	0.12	0.12
866	0.25	–
870	0.26	–
890	0.19	0.11
895	0.2	0.12
898	0.2	0.15
900	0.2	0.15
905	0.26	0.25

Rb. sphaeroides and *R. rubrum* have $r(0) \geq 0.2$ for $\lambda = 900$ nm (Fig. 4B–D), and the $r(\infty)$ values are also considerably higher than the corresponding values observed at room temperature, i.e., $r(\infty) \approx 0.15$. At wavelengths shorter than 890 nm the anisotropy is seen to be significantly lower than for wavelengths above 900 nm; it attains values more like the ones observed at room temperature. This finding is illustrated by the decay of *R. rubrum* at 870 nm, Fig. 4E.

As seen from Tables III and IV the measurements of anisotropy decays have been concentrated to the red wing of the spectrum, and to the region around 800 nm for *Rb. sphaeroides*. The reason for this is that at other wavelengths strong excited-state absorption exists that may distort the anisotropy decay. This is particularly so if the directions of the transition dipole moments of ground and excited states are different. However, from our experimental data there is no indication of such effects; at wavelengths above 890 nm the

TABLE IV

ANISOTROPY OF *R. RUBRUM* CHROMATOPHORES AT 77 K, WITH CLOSED RC

λ (nm)	$r(0)$	$r(\infty)$
870	0.05	≈ 0
880	0.08	0.05
895	0.2	0.09
900	0.2	0.1
905	0.15–0.2	–

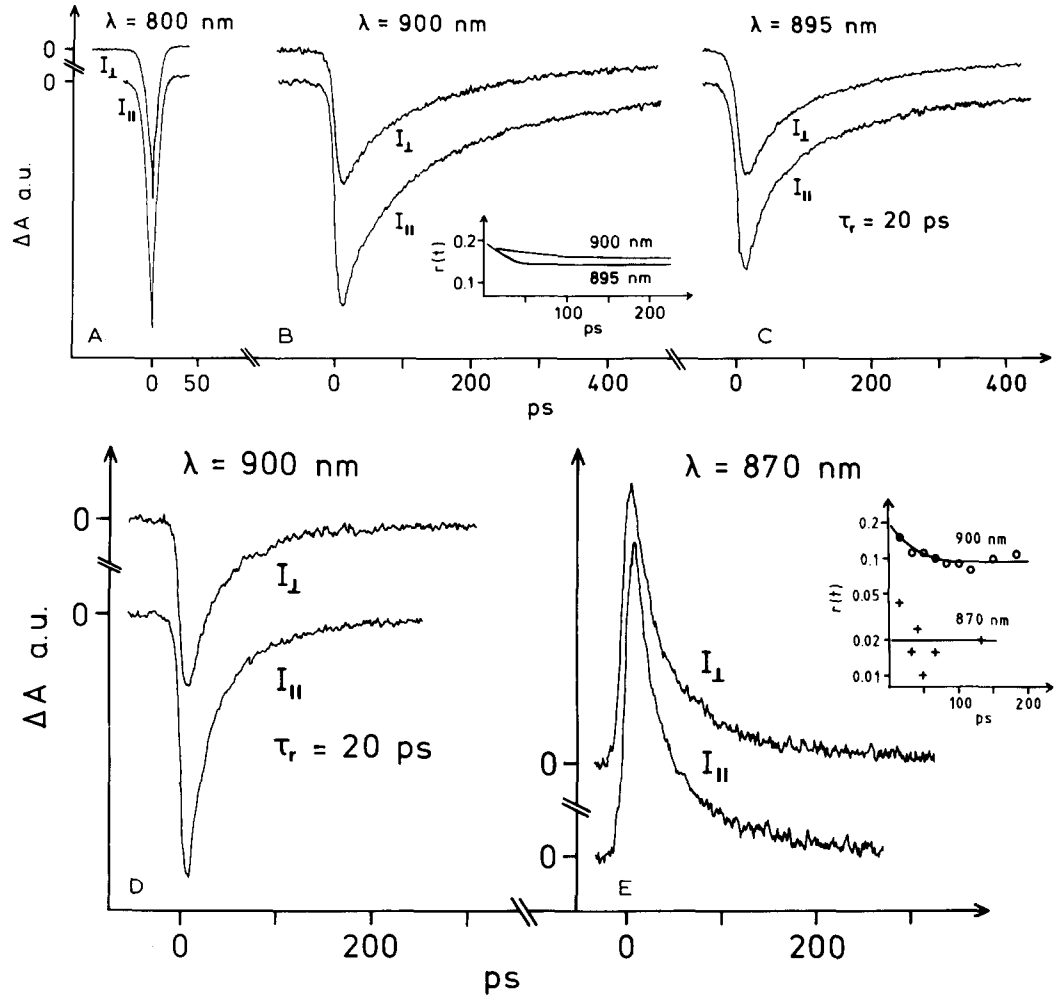


Fig. 4. Decays of induced anisotropy, with closed traps. 77 K. (A–C) *R. sphaeroides* at 800, 900 and 895 nm. (D, E) *R. rubrum* at 900 and 870 nm.

measured anisotropy is high and independent of wavelength, and for wavelengths below 890 nm (840–890 nm) the anisotropy is very low ($r \leqslant 0.05$) and also here independent of wavelength.

We have also measured the anisotropy around 800 nm for *Rb. sphaeroides* chromatophores (Fig. 4A), and found a relatively high value, $r(0) \approx 0.25$, which does not seem to change with time. However, due to the very short lifetime of the decay, it is difficult to resolve the time evolution of the anisotropy. The anisotropy measured at room temperature was also observed to be high [13,23], but due to the limited time-resolution and a complex decay signal, a definite value could not be obtained. The fact that $r(0)$ at 77 K is lower than the maximum value of 0.4 suggests that some fast energy transfer among the BChl 800 molecules precedes the BChl 800 → BChl 850 transfer, in agreement with earlier steady-state fluorescence data [5].

Fast absorption decay kinetics around 892 nm in R. rubrum

Finally, we turn the attention to some interesting results of isotropic kinetics obtained for *R. rubrum* in the vicinity of the apparent isosbestic wavelength for absorbance changes due to B880. The three kinetic curves shown in Fig. 5 displays a

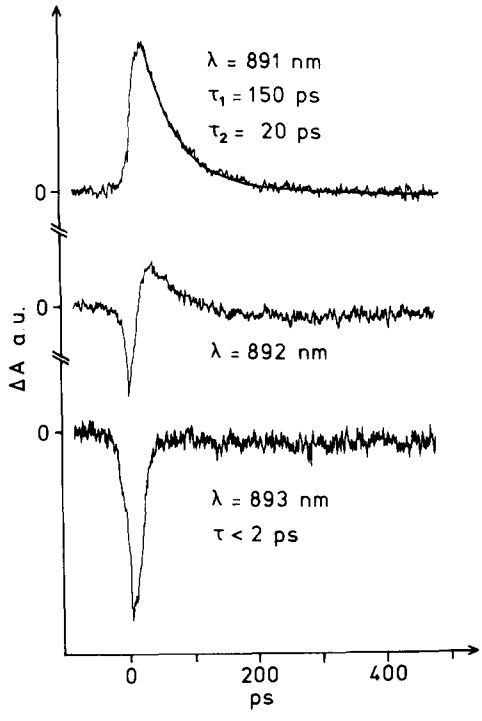


Fig. 5. Absorption recovery kinetics of *R. rubrum* at 77 K around the isosbestic wavelength of B880.

remarkable variation over the narrow wavelength interval 891–893 nm. The characteristics of the kinetics are seen to change completely through this interval. At the shortest wavelength 891 nm the decay is characterized by a fast and slow decay ($\tau_2 \approx 20$ ps and $\tau_1 \approx 150$ ps) due to B880 → B896 transfer and B896 quenching, respectively (see Scheme I). Both components are seen as absorptions. At $\lambda = 892$ nm a very short-lived bleaching is superimposed upon this signal and the τ_2 component is hardly distinguishable. Another nanometer to the red the slow component, τ_1, has disappeared and now only the very rapid, close to pulse-limited decay remains. A few nanometers further to the red, i.e., $\lambda = 895$ nm (see Fig. 2B), both τ_2 and τ_1 have reappeared with lifetime values typical of these components measured at other wavelengths (see Table II). The disappearance of τ_1 at 893 nm we attribute to the passage through the isosbestic wavelength of B896. The low intensity of the B880 signal (component τ_2) in this interval is similarly ascribed to the proximity of its isosbestic wavelength, combined with the distortion of the kinetics caused by the very fast superimposed component.

These phenomena are strongly reminiscent of observations made in our previous room-temperature picosecond study [13]. In *Rb. sphaeroides* at a wavelength close to the apparent isosbestic wavelength of the BChl 850* absorption changes, similar fast decays were detected.

Discussion

The isotropic decay kinetics

The excited-state decays in chromatophores of *R. rubrum* and *Rb. sphaeroides* can very well be described by assuming the presence of a long-wavelength antenna pigment, B896. The simplest excitation transfer schemes places B896 between the major long-wavelength pigment, B875/880, and the reaction center. A satisfactory fit to the observed absorption decay kinetics can be obtained both for *Rb. sphaeroides* and *R. rubrum* using Scheme II and Scheme I, respectively, which are based on this hypothesis.

Thus, in *Rb. sphaeroides* quenching of the B896 excited state by the closed reaction centers (rate constant k_1 in Scheme II) proceeds with a typical time constant of $\tau_1 = 190 \pm 10$ ps, quite similar to the value at room temperature [13]. This process seems somewhat faster in *R. rubrum*, $\tau_1 = 150 \pm 20$ ps (see Table II).

The B875/B880 → B896 excitation transfer rate (rate constant k_2 in Schemes I and II), is, within experimental error, identical in the two organisms and corresponds to $\tau_2 = 20 \pm 5$ ps. This points to a rather similar organization and relative orientation of B875/B880 and B896 pigments in *Rb. sphaeroides* and *R. rubrum*.

The rate of B850 → B875 transfer (time constant $\tau_3 = k_3^{-1} = 40$ ps in Scheme II) is somewhat lower than the B875 → B896 rate, and quite similar to the corresponding room temperature rate obtained from the equilibration time and equilibrium constant [2]. The small increase in rate constant at low temperature could be due to a more favorable spectral overlap for the BChl 850 → BChl 875 transfer at low temperature.

Our previous picosecond measurements at room temperature [13] suggested a value of the BChl

800 → BChl 850 transfer time of less than 1 ps. The measured decays were all pulse-limited. When the same experiments are repeated at 77 K the time constant seems to have become longer, long enough to broaden the autocorrelation function of the laser pulse slightly (10%). A deconvolution of the measured decay curve with a Gaussian pulse of the same width as the autocorrelation function yields a value of $\tau_{800-850} = 2.2 \pm 1.0$ ps (see Fig. 1H), in reasonable agreement with the value calculated from the BChl 800 emission yield at 4 K [24]. This is clearly larger than the corresponding value at room temperature, which indicates that the BChl 800 → BChl 850 transfer rate is temperature dependent. In our previous room-temperature work [13,23] we suggested such a temperature dependence to explain the observed difference in BChl 800* absorption anisotropy at room temperature and fluorescence anisotropy at 4 K [5]. Thus, the room temperature absorption anisotropy of the BChl 800 excited state was high, whereas steady-state fluorescence anisotropy at 4 K pointed towards a lower anisotropy. The presently observed temperature dependence of the BChl 800 → BChl 850 transfer rate could very likely give rise to the observed difference. At room temperature the BChl 800 → BChl 850 transfer is dominating over BChl 800 ↔ BChl 800 transfer, resulting in a high BChl 800* polarization. At low temperature the rates of energy transfer in the two channels have become comparable, due to the decrease of the BChl 800 → BChl 850 rate. Consequently, BChl 800* depolarization occurs during the lifetime of the BChl 800 excited state.

Time-resolved absorption anisotropy decay

The high degree of fluorescence anisotropy observed at 4 K upon selective excitation of B896 [11] should also be manifested in the absorption anisotropy measurements. The results summarized in Fig. 4 and Tables III and IV appear to confirm the fluorescence anisotropy data. Thus, we see that the absorption anisotropy at 77 K measured at $\lambda \geq 900$ nm is higher than both the corresponding room temperature value and the anisotropy at shorter wavelengths (i.e., in the BChl 850 and BChl 875 bands) at 77 K. However, we notice that our values of the absorption anisotropy are lower than the limiting value $r \approx 0.3$ observed in the fluorescence depolarization measurements [11], and only for *Rb. sphaeroides* do we approach this value with $r(0) = r(\infty) = 0.26$ at 905 nm. By comparison with the fluorescence anisotropy of *Rb. sphaeroides* and *R. rubrum* measured by Kramer et al. [11] (their Figs. 6A and 9), we conclude that the anisotropy does not reach its maximum value until $\lambda > 910$ nm. This is particularly true for *R. rubrum*, in which the B880 band is red-shifted as compared to the B875 band of *Rb. sphaeroides*. This means that longer wavelengths are required to selectively excite B896 in *R. rubrum* than in *Rb. sphaeroides*. Thus, at $\lambda = 900$ nm, where most of our anisotropy measurements were performed also some B875 will have been excited, which reduces the observed initial polarization due to rapid energy transfer among BChl 875/880 molecules. The initial decay of anisotropy with a time constant of about 20 ps (Fig. 4C) is a further indication of a partial excitation of B875/880, since it suggests depolarizing B875/880 → B896 transfer. Conversely, at $\lambda = 900$–905 nm in *Rb. sphaeroides*, where a high value of the initial anisotropy ($r(0) = 0.26$) is observed, very little of the 20 ps decay of $r(t)$ is seen (Fig. 4B).

Another reason why our values of the anisotropy are lower than those of Kramer et al. [11] is that their measurements were performed at 4 K, whereas ours were at 77 K. At the higher temperature the absorption bands are wider and more extensive spectral overlap occurs. This will reduce the possibilities of selective excitation of B896 and thereby decrease the anisotropy.

The strongly increased anisotropy upon selective excitation of B896 implies either that little or no energy transfer between different BChl 896 molecules occurs, or that the BChl Q_y transition moments are highly oriented. This may be taken as an indication that the BChl 896 molecules are organized differently as compared to B880/B875 and B800–850 (see below).

At 800 nm, a relatively high value of the absorption anisotropy was observed, $r(0) \approx 0.25$. Although the value at room temperature could not be accurately measured, due to limited time-resolution, the 77 K value apears to be lower than the room temperature value. In any case, the currently observed magnitude of the anisotropy suggests that some energy transfer among BChl 800 mole-

cules occurs during the lifetime of the BChl excited state. At 77 K transfer from BChl 800 to BChl 850 and transfer among BChl 800 molecules appears to proceed on a similar time-scale. The observed anisotropy, $r = 0.25$, suggests a more parallel orientation of the BChl 800 Q_y transition moments than that given in the model of the B800–850 complex by Kramer et al. [5].

B896

In the past, the presence of a special pigment protein complex connecting the major long-wavelength antenna B875/B880 with the reaction center was often hypothesized [8,19,25–28]. The arguments were derived from the anomalously low fluorescence yield of the light harvesting antenna, even in the absence of reaction cennters [25,26]. Fluorescence lifetime measurements at 77 K on the isolated B875 complex and on RC-less mutants of *Rb. sphaeroides* [8,19] as well as room-temperature absorption decay measurements in chromatophores of *R. rubrum* [27,28] supported this suggestion.

This model for the pigment organization was put on a firm basis by fluorescence depolarization measurements at 4 K by Kramer et al. [11], who explained the observed increase in fluorescence polarization upon excitation in the red wing of the B875 or B880 absorption band by the presence of the long-wavelength antenna component, B896. The presented interpretation of the BChl excited state absorption decay kinetics at 77 K, together with our earlier room-temperature results [13] further substantiate these ideas. The observation that B896 is coupled to B875/B880 both in the native membrane and in the isolated protein complex [10,11], combined with the fact that B875/B880 $\rightarrow$ B896 energy transfer is very efficient ($\tau \approx 20$ ps), shows that these two pigment-groups probably are very tightly coupled.

At this moment we have several alternative explanations for B896.

(a) B896 is an intrinsic property of the B880/B875 complex, for instance for each five BChl 880/875 pigments bound in a B880/B875 minimum unit, one BChl 896 occurs, representing a BChl molecule in a different environment.

(2) B896 arises from specific interactions between different B880/B875 complexes. For instance, two BChl 880/875 molecules on different complexes could interact to produce the red-shifted absorption and high polarization.

(3) B896 arises from the specific interactions between B880/B875 complexes and the reaction center. Although the experiments with the isolated B875 complex of *Rb. sphaeroides* seems to argue against this possibility, we shall not discard it at this moment.

(4) B896 is a separate pigment-protein complex, tightly associated to B880/B875, with highly oriented pigment molecules.

One final remark about B896. In the past, Razjivin et al. [28] have invoked charge transfer states, located on a minor, long-wavelength antenna pigment, which they called B905, to be involved in energy transfer to the reaction center. It is quite possible that this pigment is identical to the presently observed B896. However, our measurements clearly show that B896 can be considered as an antenna pigment taking an active part in the overall energy transfer sequence, and there is no reason to propose a transfer mechanism of a different nature than that of the other transfer steps, i.e., of the Förster type.

A further heterogeneity of the long-wavelength B880 antenna?

The presence of the very fast (1–5 ps) transient around 892 nm in *R. rubrum* as shown in Fig. 5 may indicate a further heterogeneity of the B880 antenna. In that case, these kinetics may represent either the fast energy transfer between slightly different B880 molecules, or possibly, between closely associated BChl 880 and BChl 896 molecules.

Another possible origin for this fast kinetic component could be a rapid spectral shift induced by the excitation of the BChl 880 molecules. In fact, the B880 ΔA spectrum has been suggested to arise from a bleaching of one BChl molecule and a blue-shift of the remaining BChl molecules in the B880 complex [4,15]. Thus, our observation in the wavelength interval around the isosbestic wavelength of B880 might be the kinetic manifestation of this process. Similar spectral shifts have previously been observed in reaction centers [29,30], and assigned to electric-field-induced shifts of absorption bands. In the present case the excitation

could be viewed as a breaking of the exciton interaction between coupled BChl 880 molecules and a concomitant spectral shift. A similar observation was made in our previous room-temperature picosecond study [13] for *Rb. sphaeroides* antenna at a wavelength close to the apparent isosbestic wavelength of BChl 850. Thus, from this limited experience, very rapid kinetics around the isosbestic wavelengths, tentatively signaling spectral shifts, might be viewed as a general property of BChl antenna pigment-protein complexes of purple bacteria.

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