Time-Resolved and Steady-State Spectroscopic Analysis of Membrane-Bound Reaction Centers from *Rhodobacter sphaeroides*: Comparisons with Detergent-Solubilized Complexes†

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ABSTRACT: The spectroscopic analysis of the antenna-deficient *Rhodobacter sphaeroides* strain RCO1 has been extended to an investigation of the kinetics and spectroscopy of primary charge separation. Global analysis of time-resolved difference spectra demonstrated that the rate of charge separation in membrane-bound reaction centers is slightly slower than in detergent-solubilized reaction centers from the same strain. A kinetic analysis of the decay of the primary donor excited state at single wavelengths was carried out using a high repetition rate laser system, with the reaction centers being maintained in the open state using a combination of phenazine methosulfate and horse heart cytochrome *c*. The kinetics of primary charge separation in both membrane-bound and solubilized reaction centers were found to be non-monoexponential, with two exponential decay components required for a satisfactory description of the decay of the primary donor excited state. The overall rate of charge separation in membrane-bound reaction centers was slowed if the primary acceptor quinone was reduced using sodium ascorbate. This slowing was caused, in part, by an increase in the relative amplitude of the slower of the two exponential components. The acceleration in the rate of charge separation observed on removal of the reaction center from the membrane did not appear to be caused by a significant change in the electrochemical properties of the primary donor. The influence of the environment of the reaction center on primary charge separation is discussed together with the origins of the non-monoexponential decay of the primary donor excited state.

Photochemical reaction centers (RCs) transduce the energy of light into electrical energy. In the RCs of purple bacteria this is achieved by a vectorial light-driven electron transfer reaction involving a series of bacteriochlorophyll (Bchl), bacteriopheophytin (Bphe), and quinone cofactors arranged across the membrane dielectric (Feher et al., 1989). Research on the mechanism of electron transfer within RCs has been greatly stimulated by the availability of high-resolution X-ray structures for the RCs from *Rhodopseudomonas viridis* (Deisenhofer et al., 1985) and *Rhodobacter sphaeroides* (Allen et al., 1987; Chang et al., 1991) and by the advent of genetic techniques for the alteration of the RC through site-directed mutagenesis (Coleman & Youvan, 1990).

The primary donor of electrons in the purple bacterial RC is a pair of Bchl molecules (P) located near the periplasmic side of the membrane. Electron transfer is triggered by the formation of an excited electronic state of the primary donor (P*), either by the direct absorption of a photon or by transfer of excitation energy from the Bchls of the antenna complexes which surround the RC (van Grondelle et al., 1994). An electron is then transferred to the acceptor Bphe on the "active" pigment branch of the RC (H_L) in approximately 3 ps at room temperature and then on to the primary acceptor quinone (Q_A) in approximately 200 ps (Feher et al., 1989; Fleming & van Grondelle, 1994). Despite intensive study by a number of groups, a consensus view of the mechanism of primary electron transfer from P* to H_L is yet to emerge (Fleming & van Grondelle, 1994). A major point of controversy is the role of the accessory Bchl on the active branch (B_L); one proposal, experimental evidence for which has been presented in Holzapfel et al. (1990) and Arlt et al. (1993), has electron transfer occurring via B_L, with the $P^+B_L^-H_L$ state being formed as a distinct intermediate. Other groups have challenged this interpretation, claiming that a discrete $P^+B_L^-H_L$ state is not required to account for time-resolved data (Kirmaier et al., 1985a; Martin et al., 1986; Breton et al., 1986; Kirmaier & Holten, 1991; Woodbury et al., 1994). In the alternative view, the Bchl is seen as acting to enhance the electronic coupling between P* and H_L and facilitating a mechanism for electron transfer based upon superexchange (Bixon et al., 1989; Parson et al., 1990). A

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consensus view has also been proposed in which both mechanisms operate in parallel, with their relative importance varying with temperature (Chan et al., 1991; Nagarajan et al., 1993).

Recently, the picture of primary electron transfer was further complicated by two phenomena. The first is the observation of coherent oscillations in the kinetic data used to measure the decay of the P* state and which have been proposed to reflect the motion of a vibrational wave packet on the potential surface of the P* excited state (Vos et al., 1993). Such oscillations have been observed at cryogenic temperatures in data acquired using isolated RCs from *R. sphaeroides* strain R26 (Vos et al., 1991) and at both cryogenic temperatures and room temperature in membranes and isolated RCs of the RCO1 strain of *R. sphaeroides* used in the present study (Vos et al., 1994a–c). Oscillations have also been observed in measurements of spontaneous emission from *R. sphaeroides* RCs (Stanley & Boxer, 1995). The fact that these oscillations are observed suggests that, under the conditions of the experiment, vibrational relaxation is taking place on a time scale similar to that of primary electron transfer. Although it is yet to be proven that such coherent nuclear motion forms an intrinsic part of the mechanism of charge separation, these data, together with other recent observations (Woodbury et al., 1994), have raised the question of whether the primary charge separation reaction is better described as an adiabatic process (Vos et al., 1994c; Woodbury et al., 1994).

The second observation is that with recent improvements in the signal to noise characteristics of picosecond time scale kinetic data, and particularly in experiments measuring spontaneous emission, more than one exponential term has been required to fit data used to measure the decay of P* (Arlt et al., 1993; Du et al., 1992; Hamm et al., 1993; Jia et al., 1993; Vos et al., 1991). The origin of this bi- or multiexponentiality is unclear but seems to indicate that the energetics of charge separation are more complex than had first been thought (Gudowska-Nowak, 1994; Lyle et al., 1993; Small et al., 1992).

In order to address some of the questions raised by recent experimental findings it has become necessary to explore possible sources of RC heterogeneity. The first consideration is that the RC may have an inherent heterogeneity, in that an individual complex may be capable of residing in two or more (conformational) states that are stable on the time scale of primary electron transfer. This could give rise to a population of RCs which exhibit a range of electron transfer rates. Such heterogeneity was invoked by Kirmaier and Holten (1990) in order to account for a detection wavelength dependence of the rate of primary electron transfer and for the observation that the reaction speeds up at cryogenic temperatures. A second possible source of heterogeneity may stem from the fact that in a culture of bacteria RCs are synthesized and degraded as the cells grow, divide, and die. Hence it is possible that a minor population of the RCs isolated from bacterial cells may be structurally perturbed as a result of the normal biosynthetic activities of the cell. Finally, it is possible that, even if the inherent heterogeneity of a population of RCs is insufficient to give rise to a range of electron transfer rates, the procedures used to purify the RCs may induce sufficient destabilization of the RC structure as to give rise to non-monoexponential kinetics. This question of the integrity of RCs in the presence of detergent may be a particular problem for complexes that have been altered by site-directed mutagenesis; the instability of a number of mutant complexes is well documented (Bylina & Youvan, 1988; Coleman & Youvan, 1990; Gray et al., 1990; Mattioli et al., 1991a).

In seeking to minimize sources of artefactual heterogeneity in RC samples, one possibility is to examine the biophysical properties of RCs while they are still in the natural membrane. However, in wild type strains of *R. sphaeroides*, kinetic studies of the primary reactions within the RC are precluded by the presence of the Bchls of the antenna complexes, the absorbance bands of which overlap those of the RC primary donor. In a recent publication, Schmidt et al. (1993) presented a time-resolved study of membrane-bound RCs from *Rhodobacter capsulatus* strain U43 (pTXA6-10). This strain is deficient in both types of antenna complex (LH1 and LH2) by virtue of point mutations in the *puc* (LH2) and *puf* (RC/LH1) operons (Dörge et al., 1990). The rates of the reactions $P^*H_L \rightarrow P^+H_L^-$ and $P^+H_L^-Q_A \rightarrow P^+H_LQ_A^-$ in membrane-bound RCs were observed to be in broad agreement with rates determined using detergent-solubilized RCs from *R. sphaeroides* and *R. capsulatus* by the authors (Hamm et al., 1993; Schmidt et al., 1993) and from *R. capsulatus* by other groups (Du et al., 1992). In the absence of any major differences between membrane-bound and solubilized RCs, three interesting points were noted. The first was that the time constant for decay of the P* state was somewhat slower in the membrane-bound RCs (4.5 ps) than the range of values normally quoted for solubilized RCs (between 2.3 and 3.5 ps). Secondly, in achieving a satisfactory kinetic fit to data on P* decay, a slow (90 ps), minor component was required in membrane-bound RCs. In accounting for this it was suggested that a trace amount of the LH1 antenna complex may have been present in membranes of strain U43 (pTXA6-10) (Stiehle et al., 1990). Finally, only one exponential in the sub-15 ps range was required for a satisfactory fit to the data on P* decay, in contrast to the two required in previous studies on purified RCs (2.3–2.7 and 7–12.1 ps) (Du et al., 1992; Hamm et al., 1993). It was, however, noted that the 90 ps component might mask a weak component in the 10 ps region which would be predicted from studies of solubilized complexes.

In an earlier publication we described the construction of a strain of *R. sphaeroides* that is antenna-deficient by virtue of deletion of the genes encoding the structural subunits of both the LH1 and LH2 complex (Jones et al., 1992a). The strain, named RCO1, grows photoheterotrophically but at a reduced rate in comparison with antenna-containing strains. The absorbance, linear dichroism, and circular dichroism properties of membrane-bound RCs from this strain were very similar to those determined for purified *R. sphaeroides* RCs (Jones et al., 1992a). In the present report we have further characterized the spectroscopic properties of membrane-bound RCs, including a time-resolved study of the primary charge separation step. To facilitate a direct comparison, we have also characterized RCs purified from membranes of the RCO1 strain.

MATERIALS AND METHODS

Biological Material. Construction of the *R. sphaeroides* strain RCO1 was described in Jones et al. (1992a). Semi-

aerobic/dark growth of cells, preparation of membranes, and purification of RCs were as described in Jones et al. (1994).

Steady-State Spectroscopy. Fourier transform resonance Raman spectroscopy was carried out as described in Jones et al. (1994). Room and low-temperature (77 K) absorbance spectra were measured on the laboratory-built spectrophotometer described in Kwa et al. (1994).

Time-Resolved Spectroscopy. The transient absorbance difference spectra were measured on an instrument described elsewhere (Visser et al., 1995). It consists of a mode-locked Nd:YAG laser (Antares, Coherent) which synchronously pumps a hybrid dye laser (Satori, Coherent) with intracavity group velocity dispersion (GVD) compensation. Using rhodamine and DODCI dyes, this system generated pulses centered at 590−600 nm with an average width of 200 fs. The pulses were amplified to 0.5 mJ in a three-stage dye amplifier pumped by a 30 Hz regenerative amplifier. The beam was split into an excitation beam (5%), which was put though a variable optical delay and a detection beam (95%) which was used to create a white light continuum by focusing the beam into a flowing water cell. The white light continuum was put through a prism pair to compensate for GVD and was split in two equal portions, one for probing the excited spot of sample and the other for use as a reference to compensate for instabilities in the white light continuum. Both were projected on a double-diode array detector, giving 130 nm wide spectra. In order to cover the full 720−945 nm range, two experiments were performed on each sample, detecting from 720 to 850 nm and from 815 to 945 nm. The GVD compensation was tuned to be optimal by changing the amount of prism in the pathway and measuring the birefringence signal of CS2 between two crossed polarizers (Visser et al., 1995).

To prevent any buildup of long-lived states in the RCs the measurements were performed in a 1 mm thick rotating cell with a diameter of 13 cm. The optical density of the samples was between 0.3 and 0.25 mm^{-1} at 860 nm, and the excitation density was tuned to be 30%, giving a maximum absorbance difference at 860 nm of <0.1 mm^{-1}. Due to the relatively low optical density of the sample, many shots had to be averaged (typically 1000−2000) in order to obtain good-quality spectra. Under these conditions absorption differences down to 0.005 mm^{-1} could be accurately measured. Experiments were performed either on undiluted RC-only membranes or on purified RCs diluted 1:100 in 50 mM Tris/0.03% LDAO/10 mM EDTA (pH 7.8).

We also performed stimulated emission studies of the mutants measured with a high repetition rate instrument (25−250 kHz) consisting of an amplified Ti:sapphire laser (Mira-RegA, Coherent). The amplified pulse (830 nm) was split into two unequal parts (70/30), the larger of which was used to create a stable white light continuum. The detection wavelength was selected using a 20 nm bandpass filter. The excitation beam was modulated using a chopper with a frequency of 1 kHz and put through a variable optical delay. Both the excitation and probe beam were then focused and overlapped in a rotating cell. The probe beam was passed through a monochromator to avoid scatter from the excitation beam, detected using a photodiode, and measured using a lock-in amplifier.

To keep the RCs in an active state and to avoid the generation of a large fraction of RCs in the $P^+Q_A^-$ state during interrogation with the high-repetition rate laser, two methods were used. In the first method, the quinone molecules were chemically prereduced with sodium ascorbate, blocking forward electron transfer at the state $P^+H_L^-$. Since the time constant for recombination of $P^+H_L^-$ is on the order of 20 ns, all of the RCs will have returned to the $PH_LQ_A^-$ state before the next pulse arrives. In the experiments with the ascorbate-treated RCs a repetition rate of 250 kHz was used together with a detection wavelength of 920 nm and the sample was contained in a static cuvette stirred by a platinum wire attached to an electric toothbrush. Identical results were achieved by reduction of Q_A using sodium dithionite (data not shown). In the second method a combination of 500 μM phenazine methosulfate (PMS) and 100 μM reduced horse heart cytochrome *c* (cyt *c*) was used to keep the RCs open, using the rotating cell described above as sample holder and a detection wavelength of 915 nm. In this case the recombination of $P^+Q_A^-$ is accelerated by the donation of electrons to P^+ from cyt *c* and the reoxidation of Q_A^- by PMS. To confirm that the RCs were in the active state we measured the amplitude of the decay of the H_L^- state at 675 nm, a measurement which also yielded the rate of the secondary electron transfer step from H_L^- to Q_A. These experiments were performed at a repetition rate of 25 kHz. Samples of RC-only membranes and purified RCs were diluted 1:1 and 1:100, respectively, in a buffer consisting of 50 mM Tris/10 mM EDTA (pH 7.8).

Global Analysis of Time-Resolved Difference Spectra. The time-gated difference spectra (40 total for each sample) resulting from the experiments performed over the two wavelength ranges described above were subjected to a global analysis using a single-model function to describe the data. The analysis permits the information contained in the noisy experimental data to be condensed into a small number of parameters which may be estimated more precisely. The system response was described by a Gaussian with a pulse width of about 400 fs (fwhm). A kinetic model consisting of three sequentially-decaying components, $1 \rightarrow 2 \rightarrow 3$, was used (van Stokkum et al., 1993), which contains as parameters the decay rates (reciprocals of lifetimes) of the components and their species-associated spectra (SAS).

Equilibrium Redox Titrations of P. Chemical redox titrations were carried out in a home-built redox cuvette that was maintained at 293 K. Purified RCs or RC-only membranes were suspended in 100 mM Tris buffer (pH 8.0) containing the redox mediators at the following final concentrations: 3 μg of *N,N,N',N'*-tetramethylphenylenediamine (TMPD)/mL, 12 μg of diaminodurene (DAD)/mL, 7 μg of PMS/mL, 7 μg of phenazine ethosulfate (PES)/mL. Redox potentials were measured using a platinum electrode and a calomel reference electrode. The electrode pair was calibrated using several different ratios of ferri/ferrocyanide and corrected to values relative to the standard hydrogen electrode. Corrections for ionic strength were made as described in Kolthoff and Tomsicek (1935) and O'Reilly (1973). Redox titrations were carried out by the addition of ferricyanide and subsequent additions of small aliquots of ascorbate. The amount of reduced P was calculated from the initial amplitude of the bleaching at 814 nm observed after a saturating xenon flash in a home-built single-beam spectrophotometer.

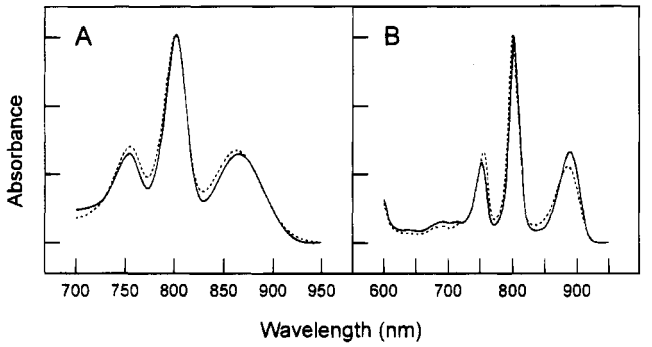

FIGURE 1: (A) Room temperature and (B) 77 K absorption spectra of the Q_y region for detergent-solubilized (dashed line) and membrane-bound (solid line) RCs of *R. sphaeroides* strain RCO1. For the purposes of comparison, spectra were normalized to the height of the absorbance band at approximately 800 nm.

RESULTS

Room Temperature and 77 K Spectra of Membrane-Bound and Solubilized Reaction Centers. Before embarking on a comparison of time-resolved optical properties of the two forms of the *R. sphaeroides* RC, we first carried out a more detailed comparison of their room temperature absorbance spectra, focusing on the near-infrared spectral region encompassing those bands attributed to the Q_y transitions of the RC Bchls and Bphes. As can be seen in Figure 1A, on removal of the RC from the membrane the band attributed principally to the monomeric Bchls underwent a slight blue shift (from 802 to 801 nm) and that attributed to the Bphes underwent a small red shift (from 754 to 755 nm). The band attributed to the lower exciton component of the Q_y transition of P shifted from 866 to 862 nm on solubilization of the RC. This 4 nm spectral shift was also manifested in the maximum of the bleach of this band observed in time-resolved difference spectra (see next section), which was located at 872 nm in membrane-bound RCs and at 868 nm in solubilized complexes. As can be seen from the spectra in Figure 1, which for the purposes of comparison have been normalized to the height of the absorbance band at 800 nm, there were also some minor differences in the relative amplitudes of the Q_y absorbance bands. These differences in relative amplitude were also present in spectra recorded at 77 K (Figure 1B). Attempts to normalize the two sets of spectra by extraction of Bchl and Bphe from the samples of purified RCs and RC-only membranes using acetone/methanol were hampered by the large amount of non-RC carotenoid present in the RC-only membranes and the low levels of other pigments which led to a consistent overestimate of the amount of Bphe present in the membrane samples.

Time-Resolved Difference Spectroscopy of Membrane-Bound and Solubilized Reaction Centers. Time-resolved difference spectra were recorded for both types of sample over the range 720−945 nm. The general features of the two sets of spectra obtained were very similar to one another and were in general agreement with the features seen in data published by other groups on the detergent-solubilized form of the wild type *R. sphaeroides* RC (Woodbury et al., 1985; Williams et al., 1992). The spectra of the membrane-bound RCs (Figure 2A) showed an initial rapid bleaching of the P absorbance band accompanied by stimulated emission on the red side of this bleach. At later times this emission showed the expected decay, displaying more clearly the bleach of

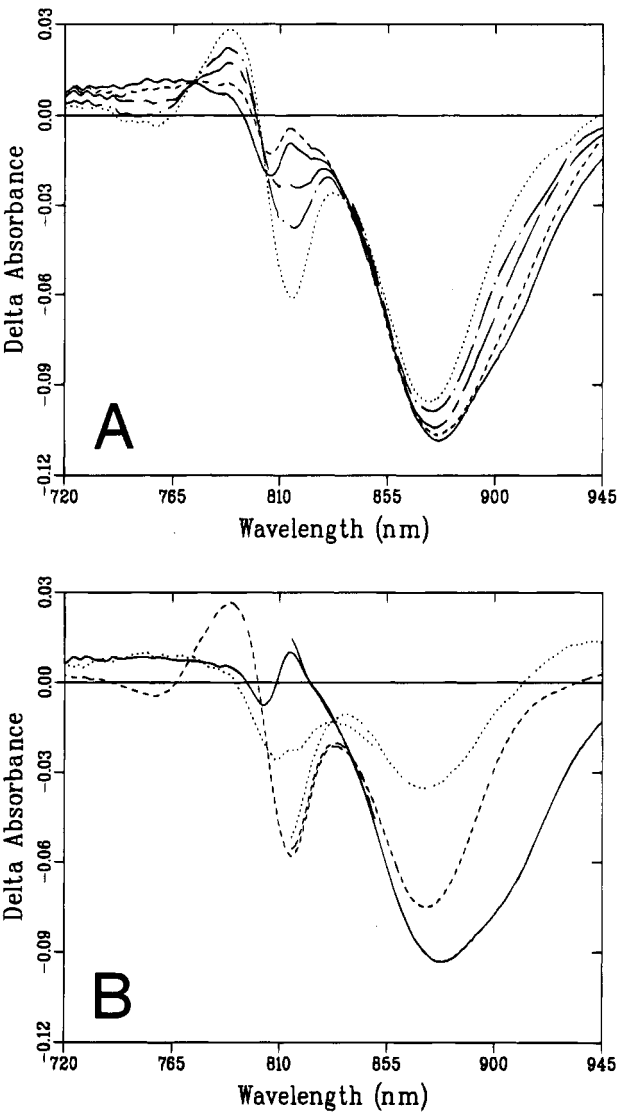

FIGURE 2: (A) Time-resolved difference spectra for membrane-bound RCs of *R. sphaeroides* strain RCO1; the spectra were acquired at intervals of 0.8 ps (solid line), 1.7 ps (dashed line), 3.3 ps (chain dash line), 5.0 ps (chain dot line), and 10 ps (dotted line) after the pump pulse (B) species-associated spectra derived from global analysis of the data in A. Spectra are of species with a lifetime of 300 fs (dotted line), a lifetime of 4.5 ps (solid line), and an infinite lifetime (dashed line).

the P ground-state absorbance band, which was centered at a wavelength corresponding to the maximum of the absorbance band (shown in Figure 1A). In order to obtain information on the rate of decay of the stimulated emission, and hence on the decay of the P* state, global analysis was carried out on the full spectral region, using a fitting window of 13 ps and a set of parameters that took into account the excitation wavelength of ~600 nm in these experiments and hence ultrafast (~100 fs) relaxation between the Bchl Q_x and Q_y transitions as well as energy transfer from the accessory Bchls to P (~300 fs) (Jia et al., 1995).

The global analysis of the spectra acquired for membrane-bound RCs using a kinetic model consisting of three sequentially-decaying components (1 → 2 → 3) resulted in the species-associated spectra shown in Figure 2B. The first state (dotted line) decayed with a time constant of approximately 300 fs and was probably associated with ultrafast energy transfer from the accessory Bchls to P (see Discus-

sion). The second state (solid line), which decayed with a time constant of 4.5 ps, had the spectral characteristics of the P* state, with a large negative feature between 830 and 945 nm consisting of bleaching of the P ground-state absorbance and stimulated emission from P* together with a second small negative feature at approximately 803 nm, the origin of which was not entirely clear. This feature was also seen in experiments performed with solubilized RCs and in experiments with membrane-bound mutant RCs. This feature may have arisen from one of a number of possible processes that would be expected to contribute to absorbance changes in this region (or a combination thereof) and will be discussed in detail elesewhere (L. Beekman, R. Monshouwer, A. Reijnders, I. H. M. van Stokkum, F. van Mourik, P. McGlynn, R. W. Visschers, M. R. Jones, and R. van Grondelle, unpublished results, 1995). The final state (dashed line), the lifetime of which was infinite on the time scale of the measurement, had the well-known features of the charge separated state ($P^+H_L^-$) with the bleaching of the P ground-state absorbance, a band shift of the accessory Bchl band to the blue and bleaching of the Bphe absorbance at approximately 760 nm. As expected, the species-associated spectra derived from data acquired using detergent-solubilized RCs showed the same general features (data not shown); the time constant for the decay of the P* state derived from these spectra was 3.3 ps, somewhat faster than the value of 4.5 ps determined for membrane-bound RCs.

Kinetics of P Decay in Solubilized Reaction Centers.* In order to examine the kinetics of decay of stimulated emission from the P* state more closely, experiments were also conducted at a single wavelength using a high repetition rate laser system, which yields kinetic data with higher time resolution and excellent signal to noise characteristics. Experiments were carried out at room temperature with a repetition rate of 25 kHz. In order to prevent accumulation of inactive RCs (i.e., complexes in the $P^+Q_A^-$ state), RC samples were housed in a rotating cell in the presence of PMS and cyt *c* (see Materials and Methods).

We first examined the kinetics of decay of the P* state at 915 nm in isolated RCs, using a fitting window of approximately 60 ps. The kinetic traces were fitted with either a single exponential term and an offset (Figure 3A) or two exponential terms and an offset (Figure 3B). The offset was used to compensate for a bleach at this wavelength, which has a lifetime that is infinite on the time scale of these measurements and which arose from the formation of P^+. Although the data were reasonably well approximated by the fit using a single exponential term with a time constant of 4.05 ps (Table 1, row 3), the residuals from this fit showed some structure, indicating that the decay was not purely monoexponential (Figure 3A). When a fit with two exponential terms was calculated (Figure 3B), the structure in the residuals disappeared, with a significant improvement in χ^2. The time constants and relative amplitudes of the parameters used for the two-component fit were 3.16 ps (85%) and 13.2 ps (15%) (Table 1, row 4), in reasonable agreement with published values for the *R. sphaeroides* RC (Arlt et al., 1993; Du et al., 1992; Hamm et al; 1993; Vos et al., 1991) and with the time constant for P* decay in isolated RCs of 3.3 ps estimated from the global analysis described above.

Kinetics of P Decay in Membrane-Bound Reaction Centers.* Measurements of P* decay were also carried out

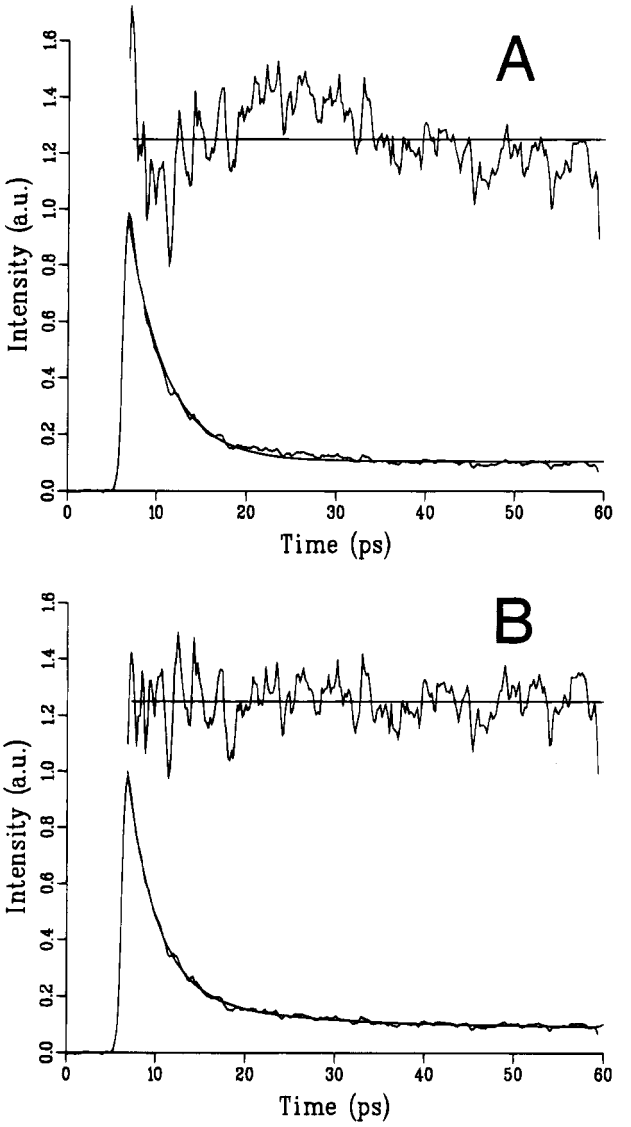

FIGURE 3: Kinetic data at 915 nm for detergent-solubilized RCs from strain RCO1 incubated in the presence of PMS and cytochrome *c*, showing fits and residuals obtained with an offset plus a single exponential term (A) and an offset plus two exponential terms (B). The residuals are shown on an absorbance scale that has been expanded 10-fold. The parameters used to fit the data are shown in Table 1.

at 915 nm using membrane-bound RCs that had been incubated with PMS and cyt *c* (Figure 4). We once again found that a biexponential fit with parameters $\tau_1 = 3.67$ ps (84%) and $\tau_2 = 11.9$ ps (16%) (Figure 4A and Table 1, row 6) was more satisfactory than a fit made with a single exponential term with $\tau = 4.75$ ps (Table 1, row 5). The requirement for two exponential terms to obtain a satisfactory fit to the decay was dependent upon the signal to noise ratio of the data. Individual traces obtained using the same sample (i.e., with no averaging) could be fitted with a single exponential term (data not shown) with no discernible improvement in the fit on adding a second component; in such cases the time constant for P* decay was approximately 4.5 ps, in good accord with the monoexponential fit to averaged data and with the data in Figure 2 obtained with the lower repetition rate system. We also examined the effect of varying the length of the fitting window on the parameters of the fit. Using a shorter window produced some minor alterations in the decay times and relative amplitudes for

Table 1: Fits to Kinetic Data Obtained at 915 nm for Membrane-Bound and Detergent-Solubilized RCs from Strain RCO1[a]

sample	τ (ps)	τ_1 (ps)	a_1 (%)	τ_2 (ps)	a_2 (%)	exptl conditions
DS	3.3					global analysis of time-resolved spectra
MB	4.5					global analysis of time-resolved spectra
DS	4.05					PMS/cyt *c*, 60 ps fitting window
DS		3.16 ± 0.09	85	13.2 ± 1.8	15	PMS/cyt *c*, 60 ps fitting window
MB	4.75					PMS/cyt *c*, 120 ps fitting window
MB		3.67 ± 0.12	84	11.9 ± 1.2	16	PMS/cyt *c*, 120 ps fitting window
DS		3.29 ± 0.04	90	15.4 ± 0.8	10	ascorbate, 100 ps fitting window
MB		4.04 ± 0.17	75	13.3 ± 1.0	25	ascorbate, 100 ps fitting window

[a] DS, detergent-solubilized; MB, membrane-bound. The results of a fit with a single exponential term are shown in column 2. For biexponential fits (columns 3–6), the relative amplitudes of the two components are shown as percentages and the standard errors are also shown.

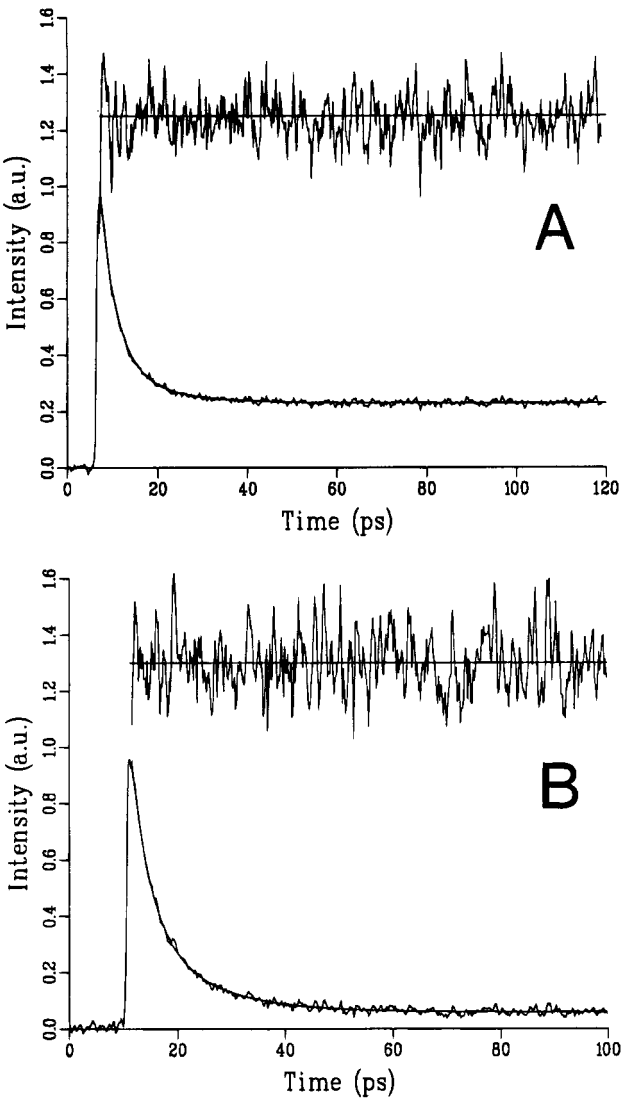

FIGURE 4: Kinetic data for membrane-bound RCs from strain RCO1, showing biexponential fits and residuals. (A) Data obtained at 915 nm for membranes incubated in the presence of PMS and cytochrome *c* and (B) data recorded at 920 nm for membranes incubated in the presence of sodium ascorbate. The residuals are shown on an absorbance scale that has been expanded 10-fold. The parameters used to fit the data are shown in Table 1.

biexponential fits, but there was only a minor effect (<0.2 ps) on the average rate of decay.

The amplitude of the offset required in fitting the data obtained at 915 nm with solubilized RCs (Figure 3) was clearly smaller than that required for a fit to the data obtained with membrane-bound RCs (Figure 4A). This difference in amplitude was consistent with the blue shift of the P Q_y ground-state absorbance band of solubilized complexes relative to that of membrane-bound RCs, as observed in both the absorbance spectra in Figure 1 and the time-resolved difference spectra obtained with the 30 Hz system.

Effect of Ascorbate on the Rate of P Decay.* As an alternative to the use of PMS and cyt *c* to keep the RC samples in the active state, sodium ascorbate may be used to reduce the acceptor quinone (Q_A) and hence block forward electron transfer from H_L^-. In Q_A-reduced RCs, the radical pair $P^+H_L^-$ state recombines with a time constant of approximately 20 ns, and hence even excitation rates of the order of 250 kHz will not lead to the photoaccumulation of P^+. In measurements of P* decay at 920 nm in ascorbate-reduced RCs and membranes, the higher pulse rate (250 versus 25 kHz) and the use of a static, stirred cell led to an improvement of the noise characteristics of the data such that kinetic fits could be made to individual traces, such as that shown in Figure 4B, and averaging of traces was not required. Once again a fit made with an offset and a single exponential term (τ = 5.2 ps) was insufficient to described the decay of the P* state in membrane-bound RCs (not shown) and two exponential terms of 4.04 ps (75%) and 13.3 ps (25%) were required for a satisfactory fit to the data (Figure 4B and Table 1, row 8). The slowing of the time constant used for the monoexponential fit to the ascorbate-treated membranes (5.2 ps) relative to the comparable fit made to PMS/cyt *c*-treated membranes (4.75 ps) was manifested in the biexponential fits by a slowing of both components in the ascorbate-treated membranes (3.67 ps/11.9 ps slowing to 4.04 ps/13.3 ps), and an increase in the relative amplitude of the slow component, which rose from 16% in the PMS/cyt *c* membranes to 25% in the ascorbate-treated membranes. Thus the overall slowing of P* decay in the ascorbate-treated membranes was due in part to a more pronounced multiexponential character.

Comparison of the Rate of Secondary Electron Transfer from H_L^- to Q_A. The rate of electron transfer from H_L^- to Q_A was estimated for both types of RC from data collected at 675 nm (not shown), where the absorbance change is indicative of the formation and loss of Bphe anions (Davis et al., 1979; Holzapfel et al., 1989). The window used to fit the decay of the signal at this wavelength extended from 30 ps after the excitation pulse to 600 ps after the pulse. With solubilized RCs the best fit to the decay of the signal at 675 nm was given with a single exponential term with a time constant of 180(±20) ps, whereas with membrane-bound complexes an exponential decay of 210(±20) ps was satisfactory. Both values are consistent with the value of 200–220 ps estimated for this reaction by others (Kaufmann et al., 1975; Kirmaier et al., 1985b; Rockley et al., 1975).

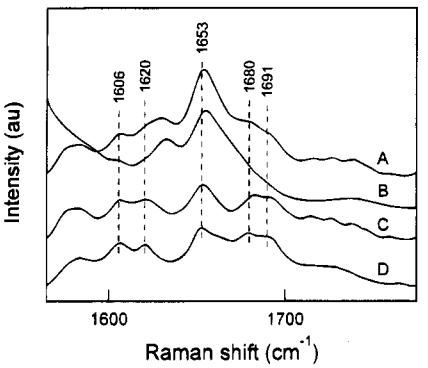

FIGURE 5: (A) FT Raman spectrum of RCO1 membranes; (B) FT Raman spectrum of membranes from the deletion strain DD13/G1; (C) difference spectrum obtained by subtraction of B from A; (D) FT Raman spectrum of detergent-solubilized RCs.

Determination of the Electrochemical Midpoint Potential for P/P$^+$ in Membrane-Bound Reaction Centers. In chromatophore membranes prepared from wild type strains of *R. sphaeroides*, the midpoint potential (E_m) for the P/P$^+$ redox couple lies in the region of +445 mV (Dutton & Jackson, 1972; Jackson et al., 1973). In contrast, several more recent publications have reported an E_m for P/P$^+$ of between +485 and +520 mV in wild type, detergent-solubilized *R. sphaeroides* RCs (Moss et al., 1991; Murchison et al., 1993; Nagarajan et al., 1993; Peloquin et al., 1994; Williams et al., 1992). One interpretation of this data is that the E_m of P increases on removal of the RC from the membrane. In order to investigate this possibility, equilibrium redox titrations were carried out on RC-only membranes and purified RCs (see Materials and Methods). No significant difference was found between the E_m for P in membrane-bound RCs (+488 ± 10 mV) and that determined for solubilized complexes (+493 ± 10 mV), with both values lying significantly higher than the +445 mV established for membrane-bound reaction centers from a wild type (antenna-containing) *R. sphaeroides* (Dutton & Jackson, 1972; Jackson et al., 1973).

FT Raman Spectroscopy of Membrane-Bound Reaction Centers. Near-infrared FT Raman spectroscopy involves the excitation of RCs with 1064 nm light and, in the presence of ascorbate, is selective for features of the vibrational spectrum of P (Mattioli et al., 1993). The signals arising from these features may be used to assess the number and strength of the hydrogen bonds to the 2-acetyl and 9-keto carbonyl groups of P_L and P_M. The assignment of individual bands in the spectrum of wild type *R. sphaeroides* RCs to particular carbonyl groups (Robert, 1990; Mattioli et al., 1991b) was confirmed by site-directed mutagenesis in a recent publication (Mattioli et al., 1994). To date, all measurements of the FT Raman spectrum of P have been conducted on purified complexes.

The FT Raman spectrum of RCO1 membranes is shown in Figure 5A. The spectrum was dominated by a band at 1655 cm^{-1} which appeared to be due to a membrane component other than the RC. This band was also observed in FT Raman spectra of membranes from LH-only strains of *R. sphaeroides* bearing site-directed mutations which led to a low level of LH complex expression (B. Robert, G. J. S. Fowler, J. D. Olsen, and C. N. Hunter, unpublished observations, 1995).

In an attempt to compensate for this "membrane feature", an FT Raman spectrum was recorded using membranes prepared from the appropriate background deletion mutant, DD13/G1 (Jones et al., 1992b) (Figure 5B). This strain lacks both the RC and LH complexes. After normalization of the spectra to the feature at 1655 cm^{-1}, the spectrum in Figure 5B was subtracted from that in Figure 5A, resulting in the spectrum shown in Figure 5C; the spectrum of detergent-solubilized RCs is shown for comparison in Figure 5D. As can be seen, all the major features observed in the spectrum of purified complexes were also seen in the corrected spectrum for membrane-bound RCs. In particular the positions (and relative intensities) of the bands corresponding to the 2-acetyl and 9-keto carbonyl groups of P_L and P_M (at 1620, 1653, 1680, and 1691 cm^{-1}) were conserved within the resolution of the measurement (±2 cm^{-1}).

DISCUSSION

In this report we have extended our analysis of *R. sphaeroides* strain RCO1 to a more detailed examination of the properties of membrane-bound RCs. In general, we find that the optical properties of the RC are not changed by any great extent on removal of the complex from the membrane. However, some small but consistent differences were observed between membrane-bound and solubilized complexes in terms of both absorption maximum and band intensity, indicating that the environment of the RC has the capacity to modulate the properties of the complex. Although this modulation seems to represent only a fine-tuning of charge separation in the wild type complex, we believe that the fact that the environment of the complex can be shown to exert an effect should be borne in mind when considering the properties of mutated complexes. A number of mutant RCs have been found to be unstable on removal from the membrane (Bylina & Youvan, 1988; Coleman & Youvan, 1990; Gray et al., 1990; Mattioli et al., 1991a), and the majority of these have been characterized in the detergent-solubilized form.

The most pronounced alteration in the near-infrared absorption spectrum of the RC on removal of the complex from the membrane was a 4 nm blue shift in the position of the Q_y absorbance band of P, which was also manifested in time-resolved difference spectra. Similar but larger shifts in the position of this band (10−15 nm) have been reported as arising from detergent-solubilization of the wild type reaction center from *R. capsulatus* (Prince & Youvan, 1987; Rautter et al., 1994; Wang et al., 1994), and from solubilization of wild type and mutated reaction centers from *R. sphaeroides* (Farchaus et al., 1993; Wachtveitl et al., 1993). In some cases, the magnitude of this shift has been reported to be dependent upon the type of detergent used or upon the detergent concentration (Farchaus et al., 1993; Wang et al., 1994). We also observed from absorption spectra taken at both room temperature and 77 K that the relative intensity of the bands in the Q_y region was altered on removal of the RC from the membrane. At present we are not able to say what the precise change in intensity is for the three main bands in the near-infrared and can only note that their relative intensities change. We are currently examining ways of obtaining an independent, accurate estimate of the concentration of RCs in the two types of sample in order to be able to throw further light upon this.

The data presented in Figure 5 provide the first example of an FT Raman spectrum of the primary donor 2-acetyl and

9-keto carbonyl groups for a RC that is still embedded in the natural membrane. This spectrum cannot be measured directly in wild type strains of *R. spheroides* because of interference from antenna bacteriochlorophylls and contributions from other components of the membrane. In the case of membranes from the RCO1 strain the antenna complexes are absent, and any contributions from other components can be corrected for by recording the FT Raman spectrum of the appropriate background double-deletion strain, in this case DD13/G1 (Jones et al., 1992a,b). The excellent conservation of the position and of the relative strength of the primary donor carbonyl bands in spectra obtained with membrane-bound and solubilized RCs argues against any significant purification-induced alterations in the conformation of the primary donor Bchls and the surrounding protein, suggesting that the modest shift in the position of the Q_y absorbance band of P observed on removal of the RC from the membrane does not arise as a consequence of gross changes in the structure of the complex in the vicinity of the primary donor. In additional support of this observation there did not appear to be any significant shift in the E_m for the P/P^+ redox couple on removal of the RC from the membrane, with values in the region of +490 mV being found for both types of complex. This finding rules out the possibility that the discrepancy between reported values for the E_m of the P/P^+ couple in purified RCs and in chromatophore membranes described in detail in the Results arises as a consequence of detergent-solubilization and purification of the RC. Rather it seems to arise from the biochemical or genetic separation of the RC from the attendant light-harvesting complexes.

The time-resolved difference spectra recorded for membrane-bound RCs (Figure 2A) showed absorbance changes that were consistent with those observed in experiments with detergent-solubilized complexes performed by ourselves and reported previously by others (Nagarajan et al., 1990; Williams et al., 1992; Woodbury et al., 1985). Global analysis of the data revealed three spectrally-distinct states (Figure 2B), the shortest lived of which ($\tau \approx 300$ fs) showed negative features centered at approximately 810 and 870 nm and reflected energy transfer from the excited state of the monomeric Bchls to P (Jia et al., 1995), all four of the Bchls of the RC having been excited in their Q_x transitions by the ~600 nm pump pulse. The second intermediate state showed the characteristic negative feature in the 830−945 nm region that has been associated with the bleaching of the ground state absorbance band of P together with stimulated emission from P*.

Global analysis of the time-resolved difference spectra presented in Figure 2 demonstrates that the rate of primary charge separation is slightly slower (τ = 4.5 ps) in membrane-bound RCs than in solubilized complexes (τ = 3.3 ps), a finding that is in good accord with the results of Schmidt et al. (1993) who used a similar strain of *R. capsulatus*. The difference between the two rates probably reflects a small change in one or more of the parameters that control the rate of the primary reaction; for example, modest changes (<10 mV) in the E_m for P/P^+ have been shown to bring about comparable changes in the rate of P* decay in mutant *R. capsulatus* RCs (Jia et al., 1993). The difference in E_m for P/P^+ observed in our experiments when membrane-bound RCs were compared with solubilized complexes (5 mV) fell within the experimental error, and hence the difference in rates of P* decay cannot be simply explained by changes in this parameter alone. It is intriguing that the removal of the RC from the membrane accelerates the rate of charge separation, as it suggests that in the membrane the rate of this reaction is not optimal. In this context it is relevant to note that at least one mutant complex has been reported (Phe L181 → Tyr) in which the rate of charge separation is faster than in the wild type complex (measured in solubilized complexes) (Jia et al., 1993) and that we have also observed an acceleration in this rate in the mutant Tyr M210 → His, in experiments conducted on membrane-bound RCs (L. Beekman, R. Monshouwer, A. Reijnders, I. H. M. van Stokkum, F. van Mourik, P. McGlynn, R. W. Visschers, M. R. Jones, and R. van Grondelle, unpublished results, 1995). It is also not known what effect removal of the antenna complexes by genetic or biochemical means has on the rate of charge separation in RCs. The nonoptimal rate of P* decay that we observe in membrane-bound RCs may also arise as a consequence of a need to optimize the efficiency of trapping of antenna excitations by the RC by minimizing the back-transfer of excitations to the antenna rather than as a requirement for a maximal rate of primary electron transfer. Studies of the rate of decay of the LH1 antenna excited state have demonstrated that the rate of excitation trapping by the reaction center is largely insensitive to variations in the rate of charge separation over a range between 2.5 and 10 ps (Beekman et al., 1994).

As stated in the introduction, in recent years it has become apparent that the kinetics of P* decay cannot be adequately described by a single exponential function, but rather it requires the use of at least two exponential components or a distribution of lifetimes (Du et al., 1992; Hamm et al., 1993; Jia et al., 1993; Nagarajan et al., 1993; Vos et al., 1991). A possible source of the non-monoexponentiality of P* decay in isolated RCs is heterogeneity in the sample induced by detergent-solubilization and purification of the complex. In order to examine whether the decay of the P* state in membrane-bound *R. sphaeroides* RCs is mono- or biexponential, we have examined RCO1 membranes using a Ti:sapphire laser system that yields data at a single wavelength with excellent signal to noise characteristics.

The kinetics of P* decay measured in solubilized RCs with the high repetition rate system were similar to those observed by others using related techniques based either on absorbance or fluorescence spectroscopy at single wavelengths to monitor stimulated and spontaneous emission from P*, respectively (Du et al., 1992; Hamm et al., 1993; Jia et al., 1993; Vos et al., 1991), or on global analysis of time-resolved spectra (Nagarajan et al., 1993). Monoexponential fits to the kinetics of P* decay measured at 915 nm gave time constants which were in good agreement with the results obtained with the low repetition rate laser system. Despite the high repetition rate of the Ti:sapphire laser, RCs could be kept in the open state by using a combination of PMS and cyt *c*. RCs could also be kept open by reduction of the Q_A quinone using sodium ascorbate, as described in more detail below.

Accurate fitting of the kinetics of P* decay in solubilized RCs incubated with PMS and cyt *c* required two exponential terms in addition to an offset (Figure 3). The time constants and relative amplitudes of the two exponential terms which gave the best fit to the data [τ_1 = 3.16 ps (85%); τ_2 = 13.2

ps (15%)] were of the same order and ratio as those determined by others (Arlt et al., 1993; Du et al., 1992; Hamm et al; 1993; Vos et al., 1991), although the time constant of the faster component was somewhat slower than the 2.3−2.7 ps determined by spontaneous emission measurements (Arlt et al., 1993; Du et al., 1992; Hamm et al., 1993). The significance of this difference is unclear as, in addition to differences in the source of the wild type RCs and the protocols used to isolate them, further differences in the method used to measure the rate of P* decay may influence the time constant(s) obtained. For example, extending the length of the fitting window in our experiments accelerated both τ_1 and τ_2 in membrane-bound RCs (data not shown), and the requirement for a second exponential component was dependent upon the noise in the decay data. Finally, the parameters required to fit the kinetics of P* decay may show a small wavelength dependence over the stimulated emission band (Vos et al., 1994c; L. Beekman, M. R. Jones, and R. van Grondelle, unpublished results, 1995). Such factors should be borne in mind when comparing data obtained at different wavelengths in different laboratories and with different instruments.

In accord with the results of the global analysis (Table 1), the time constants for P* decay in membrane-bound RCs were somewhat slower than those measured for solubilized RCs under identical conditions (Figure 4A). Once again, accurate fitting of the kinetics required at least two exponential terms in addition to an offset (Table 1). At present, it is not clear whether the two time constants required to fit the kinetics of P* decay in both membrane-bound and solubilized RCs reflect the fact that the kinetics are truly biexponential, suggesting two populations of RC that are discrete on the time scale of the measurement, or whether they reflect the fact that the kinetics are actually multiexponential, arising from a distribution in one or more of the parameters that govern the rate of charge separation. We are currently examining the possibility that distributions in the driving force for charge separation (the change in free energy, ΔG) or the electronic coupling between the donor and acceptor pigments (V) may give rise to a distribution in charge separation rates, using a set of mutant RCs in which the rate of P* decay is attenuated.

If sodium ascorbate was used to keep the RCs open rather than PMS/cyt *c* (Figure 4B) then the rate of charge separation in membrane-bound RCs was slowed. When a single exponential term was used to fit the kinetics of P* decay the time constant obtained was 5.2 ps, some 10% slower than the equivalent value obtained with PMS/cyt *c*-treated membranes (4.75 ps). The effect of ascorbate can be rationalized on the basis of the negative charge on Q_A making the adjacent chromophore H_L more difficult to reduce. This would be manifested in a decrease in the E_m for the H_L/H_L^- redox pair and hence a decrease in ΔG.

In the present study we have not detected any evidence for a 90 ps component (10% amplitude) in the kinetics of P* decay as observed by Schmidt et al. (1993), either in the global analysis of the time-resolved difference spectra or in the higher resolution kinetic data obtained with the high repetition rate laser system. The source of this discrepancy may be the different approaches taken to the construction of the antenna-deficient *R. sphaeroides* and *R. capsulatus* strains. In the present case the RCO1 strain is antenna-deficient by virtue of deletion of the genetic material encoding the subunits of the LH1 and LH2 complexes (Jones et al., 1992a,b). In contrast, the *R. capsulatus* strain examined by Schmidt and co-workers (1993) was LH1-deficient by virtue of a point mutation in the plasmid-borne copies of the *pufB* (LH1 β subunit) gene (Dörge et al., 1990) and was LH2-deficient by virtue of a point mutation that has been mapped to the *pucC* gene, an open reading frame that lies immediately downstream of the *pucBA* genes and which codes for a protein of uncertain function that is required for the correct synthesis and/or assembly of the LH2 complex (Tichy et al., 1989). As suggested by Schmidt et al. (1993), it is likely that the 90 ps component observed in *R. capsulatus* but not in *R. sphaeroides* is due to a contaminating level of antenna complex.

As stated in the introduction, the non-monoexponentiality of the decay of the P* state has been the subject of some discussion, but as yet the origins of this phenomenon are unclear. The data presented in this report demonstrates that a heterogeneity in the RC population induced by purification of the complex from the bacterial membrane cannot be the source of this phenomenon, as more than one exponential term was required to provide a satisfactory fit to the decay of P* in membrane-bound RCs. The non-monoexponential behavior of P* decay in the wild type RC did not appear to be exacerbated on removal of the RC from the membrane.

Finally, we turn to the question of whether the light-harvesting antenna complexes modulate the kinetics of fast electron transfer within the RC. The documented difference in the E_m for P/P^+ measured for RCs in the presence or absence of the antenna complexes (see above) demonstrates that at least one of the parameters than governs the rate of charge separation in the RC (through its influence on ΔG) is affected by the removal of the antenna complexes by either genetic or biochemical means. We are currently examining the influence of the antenna complexes on the E_m for P using strains with RC^+LH1^+, RC^+LH2^+, and $RC^+LH1^+LH2^+$ phenotypes (Jones et al., 1992b). It is also known that the LH complexes can influence the stability of mutant RCs (Bylina et al., 1990), with some mutated RC complexes only being stable in the presence of the LH1 complex (Robles & Youvan, 1993). It is not known whether the presence of the antenna complexes would influence the relative populations of different conformational states of the RC or the characteristics of a distribution in a parameter such as ΔG that could give rise to multiexponential kinetics of P* decay. At present, problems of selective excitation of the RC pigments and the detrapping of excitations from P* into the antenna mean that it is not possible to obtain unambiguous data on the kinetics of charge separation in the presence of the antenna complexes. We are currently investigating the use of genetically-modified RC/LH systems in an attempt to minimize these problems.

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