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Comparative study of reaction centers from purple photosynthetic bacteria: Isolation and optical spectroscopy

S. Wang[1], S. Lin, X. Lin, N. W. Woodbury & J. P. Allen*
*Department of Chemistry and Biochemistry and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287-1604, USA; [1]Current address: Duke University Medical School, Division of Anesthesiology, Durham, NC 27710, USA; *Author for correspondence*

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Abstract

Reaction centers from two species of purple bacteria, *Rhodospirillum rubrum* and *Rhodospirillum centenum*, have been characterized and compared to reaction centers from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. The reaction centers purified from these four species can be divided into two classes according to the spectral characteristics of the primary donor. Reaction centers from one class have a donor optical band at a longer wavelength, 865 nm compared to 850 nm, and an optical absorption band associated with the oxidized donor at 1250 nm that has a larger oscillator strength than reaction centers from the second class. Under normal buffering conditions, reaction centers isolated from *Rb. sphaeroides* and *Rs. rubrum* exhibit characteristics of the first class while those from *Rb. capsulatus* and *Rs. centenum* exhibit characteristics of the second class. However, the reaction centers can be converted between the two groups by the addition of charged detergents. Thus, the observed spectral differences are not due to intrinsic differences between reaction centers but represent changes in the electronic structure of the donor due to interactions with the detergents as has been confirmed by recent ENDOR measurements (Rautter J, Lendzian F, Lubitz W, Wang S and Allen JP (1994) Biochemistry 33: 12077–12084). The oxidation midpoint potential for the donor has values of 445 mV, 475 mV, 480 mV and 495 mV for *Rs. rubrum*, *Rs. centenum*, *Rb. capsulatus*, and *Rb. sphaeroides*, respectively. Despite this range of values for the midpoint potential, the decay rates of the stimulated emission are all fast with values of 4.1 ps, 4.5 ps, 5.5 ps and 6.1 ps for quinone-reduced RCs from *Rs. rubrum*, *Rb. capsulatus*, *Rs. centenum*, and *Rb. sphaeroides*, respectively. The general spectral features of the initial charge separated state are essentially the same for the four species, except for differences in the wavelengths of the absorption changes due to the different donor band positions. The pH dependence of the charge recombination rates from the primary and secondary quinones differ for reaction centers from the four species indicating different interactions between the quinones and ionizable residues. A different mechanism for charge recombination from the secondary quinone, that probably is direct recombination, is proposed for RCs from *Rs. centenum*.

Abbreviations: RC – reaction center; P – bacteriochlorophyll dimer; H – bacteriopheophytin; Q – quinone; Rb – Rhodobacter; Rs – Rhodospirillum; Rps – Rhodopseudomonas; EDTA – (ethylenediamine)tetraaceticacid; LDAO – N,N-dimethyl-dodecylamine-N-oxide; CTAB – cetyltrimethylammonium bromide; DOC – deoxycholate; Tris – Tris-(hydroxymethyl)aminomethane; ns – nanosecond; ps – picosecond; fs – femtosecond

Introduction

The primary photochemical event of photosynthesis, the conversion of light into a charge separated state, occurs in an integral membrane protein-pigment complex called the reaction center (RC) (for reviews see Feher et al. 1989; Parson 1991). RCs isolated from purple non-sulfur bacteria are generally composed of three protein subunits (L, M and H), four bacteriochlorophylls, two bacteriopheophytins (H), two ubiquinones (Q), and one non-heme iron. The amino acid sequences of reaction centers for three species that contain bacteriochlorophyll-*a*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum*, are homologous and the binding sites for the cofactors are generally conserved (Williams et al. 1983, 1984; Youvan et al. 1984; Belanger et al. 1988).

The similarity in primary structure among the reaction centers of purple non-sulfur bacteria is consistent with the widespread assumption that the functional properties of these reaction centers are very similar. Electron transfer events in RCs from *Rb. sphaeroides* and *Rb. capsulatus* have been well characterized (for reviews see Kirmaier and Holten 1987; Woodbury and Allen 1994) and the spectral features of the initial charge separated state in RCs from *Rs. rubrum* have been described (Kirmaier et al. 1983). These results show that light excites the primary electron donor, a bacteriochlorophyll dimer, P, to its first excited singlet state. After excitation, an electron is transferred from the donor to a bacteriopheophytin H_A within 3.5 ps. Despite a considerable amount of study, the mechanism for this reaction is strongly debated (for reviews see Deisenhofer and Norris 1993). Electron transfer continues to the primary quinone Q_A in 200 ps and then to the secondary quinone Q_B in 200 μs. The quantum yield of this charge separation process is essentially unity as the forward rates at each step are orders of magnitude faster than the competing back reaction rates. An exogenous cytochrome transfers an electron to the oxidized donor allowing the donor to be re-excited by light. After the quinone has been doubly reduced and has accepted two protons from nearby amino acid residues (Paddock et al. 1989; Takahashi and Wraight 1990; Hanson et al. 1992), it is released into the membrane. The proton uptake is associated with a proton gradient across the membrane that creates the driving force for the formation of ATP (Dutton 1986).

In order to investigate the assumption of functional similarity, we present a comparative analysis of RCs from four bacteriochlorophyll-*a* containing purple bacteria: *Rb. sphaeroides*, *Rb. capsulatus*, *Rs. rubrum*, and a recently discovered bacterium, *Rhodospirillum centenum* (Favinger et al. 1989). In addition to the three subunits present in RCs from the other three species, the RCs from *Rs. centenum* have a bound tetraheme cytochrome that is thought to be similar to the tetraheme subunit present in RCs from *Rhodopseudomonas viridis* (Yildiz et al. 1992). In this study we address the question of whether key functional properties of the RCs from *Rs. rubrum* and *Rs. centenum*, that have not been well characterized, are similar to those of *Rb. sphaeroides* and *Rb. capsulatus*. The spectral features, the P/P^+ midpoint potential, and the kinetics of electron transfer, as measured by femtosecond optical absorption spectroscopy is discussed. To probe the environment of the Q_A and Q_B region of *Rs. rubrum* and *Rs. centenum*, the pH dependence of the charge recombination rates from the quinones has been measured using microsecond optical absorption spectroscopy and compared to that of *Rb. sphaeroides* and *Rb. capsulatus*.

Materials and methods

Materials. DEAE (diethylaminoethyl) Sephacel was obtained from Supelco. The detergents N,N-dimethyldodecylamine-N-oxide (LDAO), Triton X-100, and cholate were from Fluka. Sodium dodecyl sulfate was from Biorad. Deoxycholate, chenodeoxycholate, ursodeoxycholate, taurocholate, glycocholate, cetyltrimethylammonium bromide (CTAB), β-octyl glucopyranoside, Triton X-114, dodecylmaltoside, and zwittergent 3–14 were from Calbiochem. The phospholipids phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine were obtained from Sigma.

Bacterial strains. The strains used for these experiments include the carotenoidless mutant strain R-26 from *Rb. sphaeroides*, *Rs. rubrum* wild type S1, and *Rs. centenum* wild type strain (ATCC 43720). For *Rb. capsulatus* an antennaless strain was used that is derived from U43, in which the *puf* operon including the RC and light-harvesting I (B875) genes were deleted and the light-harvesting complex II (B880–850) was lost by a chromosomal point mutation (Youvan et al. 1985). The wild type reaction center genes were carried on a

derivative of the plasmid pU2922 (Bylina et al. 1986) and conjugated into the deletion strain. On this plasmid a putative bacteriochlorophyll binding ligand, residue histidine 32 of the a subunit of the light-harvesting complex I was changed to arginine by site-specific mutagenesis (Bylina et al. 1988). This mutant does not assemble light-harvesting complex I in the chromatophore membranes. The RCs are expressed from the plasmid when the strain is grown semiaerobically.

Growth media. Rb. capsulatus was grown in medium RCV-PY, which combines the defined medium RCV (Weaver et al. 1975) with peptone and yeast extract. Per liter the medium RCV-PY contains 10 ml 10% $(NH_4)_2SO_4$, 40 ml 10% DL malic acid (pH 6.8), 50 ml super salts solution,15 ml 0.64 M KH_2PO_4 (pH 6.8), 2 g yeast extract and 2 g peptone. Per liter the super salts contains 0.4 g 1% disodium EDTA, 4 g $MgSO_4 \cdot 7H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 20 ml trace elements, 0.24 g $FeSO_4 \cdot 7H_2O$, and 20 mg thiamine. Per liter the trace elements solution contains 1.59 g $MnSO_4 \cdot H_2O$, 2.8 g H_3BO_3, 0.04 g $Cu(NO_3)_2 \cdot 3H_2O$, 0.24 g $ZnSO_4 \cdot 7H_2O$, and 0.75 g $NaMoO_4 \cdot 2H_2O$. The reaction center yields in this medium were comparable or higher than those grown in other media such as MPYE (Yen et al. 1977) and RCV^+ (RCV with dimethyl sulfoxide, see Yen et al. 1977). *Rs. rubrum* and *Rb. sphaeroides* were grown in a medium consisting of 3 g peptone, 4 g yeast extract and 1.33 ml of 15% succinate per liter. The medium was adjusted to a pH of 7.2 with NaOH. *Rs. centenum* was grown in modified CENMED media (Favinger et al. 1989) that contains per liter 20 ml 1 M phosphate buffer, 1 ml 1% disodium EDTA, 2 ml 0.002% vitamin B_{12}, 5 g yeast extract, 6 g casamino acids, 5 g soytone, and 7.5 ml vitamin solution. The medium was adjusted to a pH of 6.0. Per liter the vitamin solution contains 100 g nicotinic acid, 15 mg thiamine, and 2 ml 1 mg/ml biotin in 50% ethanol.

Growth conditions. The *Rb. capsulatus* strain was streaked on RCV-PY agar and incubated for 2–3 days at 31 °C under dark conditions. One colony was picked and used to inoculate two test tubes, each containing 6 ml of medium. The cultures were grown for 2 days in test tubes in an incubator shaker (New Brunswick Scientific Co.) at 31 °C and 200 rpm under dark conditions. These cultures were used to inoculate a 2.8 L Fernbach flask containing 2 L of RCV-PY medium with 12.5 μg/ml kanamycin and cells were grown for

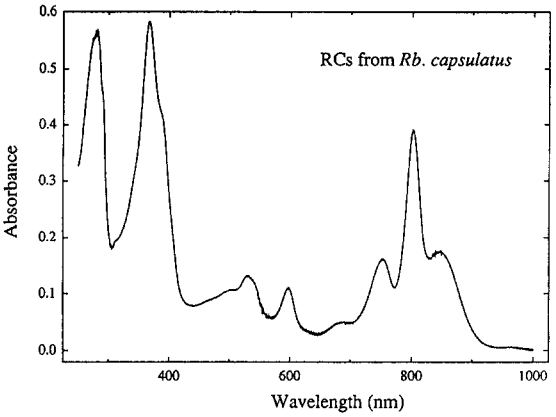

Fig. 1. Steady state optical absorption spectrum of purified reaction centers from *Rb. capsulatus*. The purity of the sample is indicated by an A_{280}/A_{800} ratio of 1.4. RCs were in 10 mM phosphate buffer pH 7.4 and 0.05% LDAO. Note the dimer absorbance peak position is at 850 nm compared to its initial value of 865 nm in chromatophores.

3–4 days in the incubator shaker under dark conditions. The other three species were grown under photosynthetic conditions. A single colony from an agar plate, which had been streaked and incubated 2 days in the dark at 31 °C, was used to inoculate 1 L of medium in a screw cap bottle. The culture was grown in the dark overnight to allow the depletion of oxygen and then put into a light box, which was maintained at 31 °C for *Rb. sphaeroides* and *Rs. rubrum*, and at 45 °C for *Rs. centenum*. After 24 h the 1 L of culture was used to inoculate 12 L of medium and grown for 2–3 days at 31 °C under the light.

Isolation procedures. RCs were isolated from *Rb. capsulatus* using the following modifcations of a previously reported protocol (Bylina and Youvan 1988). After preparation of chromatophores, RCs were solubilized by the addition of 0.45% LDAO. The crude RCs were washed several times by adding 1/3 volume of DEAE Sephacel equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 0.05% LDAO, centrifuging in an SS-34 rotor at 5000 rpm for 5 min, and resuspending in 10 mM potassium phosphate buffer pH 7.4 and 0.05% LDAO. Then crude RCs bound to DEAE were poured onto a column, washed first with 10 mM potassium phosphate buffer pH 7.4 and 0.05% LDAO then the same solution plus 100 mM KCl. Finally, RCs were eluted with a 100 to 300 mM KCl gradient in 10 mM potassium phosphate buffer pH 7.4 and 0.05% LDAO and fractions were collected. The fractions containing reaction centers, that typically had a A_{280}/A_{800} ratio of 1.8 to 2.5 as measured with a Cary 5 spec-

trophotometer (Varian), were dialyzed against 10 mM potassium phosphate buffer pH 7.4 and 0.05% LDAO to remove the salt. Reaction centers of higher purity with a A_{280}/A_{800} ratio of 1.4 and a yield of over 20 mg (from 12 L of culture) were obtained by a second DEAE chromatography step (Fig. 1). After isolation the RCs were concentrated in a pressure cell (Amicon), and exchanged by dialysis into 10 mM potassium phosphate pH 7.4 and 0.05% LDAO.

RCs were isolated from *Rb. sphaeroides* according to published procedures (Feher and Okamura 1978), concentrated, and dialyzed against 15 mM Tris-Cl pH 8.0, 0.025% LDAO, and 1 mM EDTA. RCs were isolated from *Rs. centenum* following the procedures Yildiz et al. (1992) with the modification that the ammonium sulfate precipitation step that removes the tetraheme cytochrome was omitted. After isolation the RCs were concentrated in an pressure cell (Amicon), and dialyzed against 15 mM Tris-C1 pH 8.0, 0.025% LDAO and 5 mM EDTA. RCs were isolated from *Rs. rubrum* following the protocol of Snozzi and Bachofen (1979) with modification. Crude *Rs. rubrum* RCs were solubilized in 0.3% LDAO and 100 mM NaCl. Crude RCs were bound to a DEAE column equilibrated with 15 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 0.025% LDAO. The RCs were washed with 15 mM Tris-C1, pH 8.0, 1 mM EDTA and 0.025% LDAO with 60 mM NaCl. The RCs were eluted with a 60 to 120 mM NaCl gradient in 15 mM Tris-Cl, pH 8.0, 1 mM EDTA and 0.025% LDAO. The RCs were then concentrated and exchanged into 15 mM Tris-CI pH 8, 0.025% LDAO and 1 mM EDTA.

The purity of RCs was estimated by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue. Three major bands were found in all four species and were at similar positions. Only very minor additional bands were observed indicating that there was little contaminating protein. The steady state optical spectra were measured using a Cary 5 spectrophotometer (Varian). For the light bleaching study, the sample compartment was modified to allow illumination with a 1000 W tungsten light (Oriel) that was filtered with a broad band green filter.

For most of the spectroscopic measurements the RCs from *Rb. capsulatus* were in 10 ml mM potassium phosphate pH 7.4, 0.025% LDAO, and 1 mM EDTA and the RCs from *Rb. sphaeroides*, *Rs. centenum*, and *Rs. rubrum* were in 15 mM Tris-Cl pH 8, 0.025% LDAO and 1 mM EDTA. For the femtosecond absorption measurements and the redox titrations the LDAO was exchanged with 0.05% Triton X-100. For the femtosecond absorption measurements sodium dithionite was added to a concentration of 4 mM to reduce the primary quinone. For $P^+Q_A^-$ charge recombination rate measurements, terbutryn was added to the sample to 0.5 mM final concentration (1.5 mM for RCs from *Rs. centenum*). For $P^+Q_B^-$ charge recombination rate measurements with RCs from *Rb. sphaeroides* and *Rb. capsulatus*, ubiquinone-10 was added to the sample to a concentration of 40 mM. For RCs from *Rs. rubrum* and *Rs. centenum*, no exogenous quinone was added.

Detergent and phospholipid effects. To test the effect of charged detergents and phospholipids on the optical spectrum, RCs ($A_{802}^{1cm}\sim0.5$) were titrated with either detergent or phospholipid. For each of the tests, RCs were titrated with 0.03% to 0.1% of the following detergents: (1) the negatively charged detergents deoxycholate, sodium dodecyl sulfate, chenodeoxycholate, cholate, ursodeoxycholate, taurocholate and glycocholate, (2) the positively charged detergent CTAB, (3) the nonionic detergents β-octyl glucopyranoside, Triton X-114 and dodecylmaltoside, and (4) zwitterionic detergents LDAO, CHAPS, and zwittergent 3–14. Four phospholipids were also tested: phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine. Ten percent phosphatidyl choline dissolved in chloroform was added to the RCs to a 0.05% final concentration. Phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine were suspended in chloroform at a concentration of 2.5% and were added to the RCs to a final concentration of 0.02%.

Redox titration. RCs ($A_{802}^{1cm}\sim0.5$) were placed into a flow cell with an external mixing chamber as has been described previously (Williams et al. 1992). The potential was adjusted by the addition of potassium ferricyanide or sodium ascorbate and measured with a platinum electrode and a calomel reference electrode. The oxidation state of the donor was determined by monitoring the absorption of the Q_Y band of the dimer at each potential. Additional details concerning the measurements and analysis of the data have been previously described (Williams et al. 1992).

Transient absorption spectroscopy. Subpicosecond-resolution transient absorption measurements of the primary electron transfer in the RCs ($A_{802}^{1cm}\sim5$) was measured using 150 fs excitation flashes at 860 nm. The time evolution of the absorbance was measured in the ranges 700 nm to 840 nm and 870 nm to 1000

nm. Lifetimes were obtained from exponential data fits of the absorbance decays. Further experimental details have been described elsewhere (Taguchi et al. 1992; Woodbury et al. 1994).

For optical measurements of the rates involving the quinones, the RCs (A_{802}^{1cm}~0.7) were excited at 532 nm using 5 ns excitation flashes from a Surelite Nd YAG laser (Continuum) while monitoring the absorption changes at 860 nm (or 850 nm) using a system of local design. For measurements of the pH dependence of the rates a mixture of buffers was added to the sample, consisting of 2 mM final concentration of each of sodium citrate, cyclohexylamino-ethanesulfonic acid (CHES), 1,4-piperazine-diethanesulfonic acid (PIPES), 3-(cyclohexylamino)-propanesulfonic acid (CAPS) and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). The pH of the solution was adjusted by adding HCl or NaOH. A series of titrations with HCl and NaOH were performed for each sample, the same value for the rate at a given pH was always measured within the error of the data.

Results

Steady-state optical absorption spectroscopy. For all four species, absorption peaks are present in the optical spectrum of isolated RCs at 540 nm, 600 nm, 760 nm, ~800 nm and ~850–865 nm as is characteristic of RCs containing bacteriochlorophyll-*a* and bacteriopheophytin-*a* (Fig. 1). All four exhibit peaks that are typical for a Bchl dimer with the position of the Q_Y absorption band at ~850 nm for *Rb. capsulatus* and *Rs. centenum*, or at 865 nm for *Rb. sphaeroides* and *Rs. rubrum* (Fig. 2A). The peak position of the band at ~800 nm also varied by approximately 5 nm. This variation may be due to the change in contribution of the edge from the dimer Q_Y band or an altered contribution of an exciton band from the dimer. In the presence of saturating light the dimer Q_Y band bleached, the ~800 nm band shifted approximately 5 nm to the blue, and a new absorption band at 1250 nm was observed (Fig. 2). Unlike the dimer Q_Y band the oscillator strength of the 1250 nm band varies significantly among the four species when the spectra are normalized at ~760 nm. For *Rb. sphaeroides* and *Rs. rubrum* the peak amplitude is 10 to 12% that of the 760 nm band while for *Rs. centenum* and *Rb. capsulatus* the peak amplitude is 2 to 5% that of the 760 nm band. Thus, the spectral characteristics of the RCs from *Rb. sphaeroides*

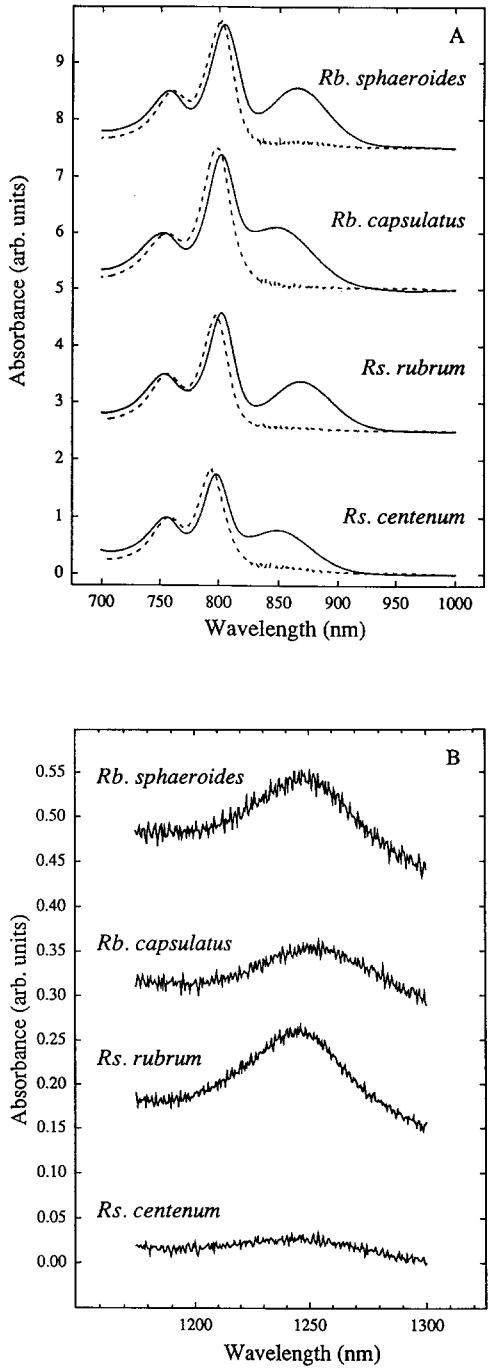

Fig. 2. Steady state optical absorption spectrum of purified reaction centers. (A) Spectra in the range of 700 nm to 1000 nm in the absence (solid line) and presence (dashed line) of a saturating light. (B) Spectra in the range of 1150 nm to 1300 nm in a saturating light. The spectra shown in Fig. 2B were measured at the same time as those in Fig 2A. All spectra have been normalized so that A_{760} is 1.0.

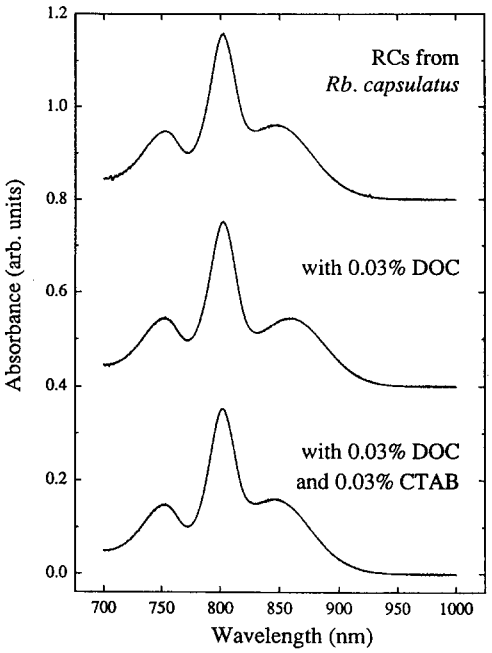

Fig. 3. Optical absorption spectra of *Rb. capsulatus* reaction centers from 700 nm to 1000 nm in the presence of different detergents. RCs were in 10 mM phosphate buffer pH 7.4 and 0.05% LDAO. With no other detergents present the absorbance peak position of the electron donor was 850 nm. When 0.03% deoxycholate, DOC, was added the absorbance peak position of the donor shifted to 865 nm and the other absorbance peaks were unchanged. Upon subsequent addition of 0.03% cetyltrimethylammonium bromide, CTAB, the absorbance peak position shifted back to 850 nm. These shifts were reversible with the removal of detergent by dialysis.

and *Rs. rubrum* are clearly different from those of *Rs. centenum* and *Rb. capsulatus*.

Effect of charged detergents on the optical spectra. During the purification process the dimer absorption peak of RCs isolated from *Rb. capsulatus* shifted from 865 nm to 850 nm during dialysis after the first DEAE chromatography step (data not shown). Titration of the sample with deoxycholate shifted the dimer absorption peak to longer wavelengths with no changes to any of the other absorption peaks (Fig. 3). With 0.03% deoxycholate present, the dimer absorption peak reached 865 nm. Upon subsequent addition of 0.03% CTAB, the absorbance peak shifted back to 850 nm (Fig. 3). When the reaction centers were dialyzed to remove the deoxycholate, the dimer peak returned to 850 nm. Thus, deoxycholate caused the dimer peak to shift reversibly. This effect was also investigated using other detergents. Negatively charged detergents caused the Q_Y band of the dimer to shift to 865 nm. Addition of a positively charged detergent, CTAB, resulted in a shift to 850 nm. Non-ionic and zwitterionic detergents did not change the absorption peak position. The Q_Y band for RCs from *Rs. centenum* also shifted in a similar manner, although the extent of the shift was smaller. For RCs from *Rb. sphaeroides* R-26, the addition of 0.03% CTAB shifted the dimer Q_Y band from 865 nm to 850 nm. After subsequent addition of 0.03% deoxycholate, the dimer peak returned to 865 nm. Similar shifts of the Q_Y band for RCs from *Rs. rubrum* and *Rs. centenum* are observed except that the magnitude of the shift was smaller ($\sim$ 10 nm).

One explanation for the shift of the Q_Y band of the dimer during the isolation process is that charged phospholipids, that interact electrostatically with the dimer, are lost during the isolation process. This possibility is supported by the observation that the ENDOR spectra of the RCs in chromatophores for all four species are essentially identical (Rautter et al. 1994). To test this idea we measured the optical spectra of RCs from *Rb. capsulatus* in the presence of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl glycerol, which are the major phospholipids in chromatophore membranes of *Rb. sphaeroides* (Marinetti et al. 1981). The position of the Q_Y band did not change from 850 nm upon the addition of any of these phospholipids or phosphatidyl serine. Thus, the feature of the membrane that apparently is responsible for the positioning of the band at 865 nm was not duplicated by the addition of these phosopholipids. This indicates that either one of these lipids is tightly bound to the RC in the membrane but cannot be replaced once removed or that another component, such as a minor phosolipid, is bound to the RC. A $\sim$10 nm blue shift of this band has been reported for RCs with the quinones depleted (Debus et al. 1985); both quinones were nearly fully occupied for all results reported in this study.

P/P^+ midpoint potential. Redox titrations were performed on RCs from all four species (data not shown). Fitting the data to the Nernst equation ($n = 1$) yielded midpoint potentials of 445 mV, 475 mV, 480 mV and 495 mV for RCs from *Rs. rubrum*, *Rs. centenum*, *Rb. capsulatus* and *Rb. sphaeroides*, respectively. The titrations were found to be reversible and the estimated error of each midpoint potential is $\pm$ 10 mV.

Femtosecond transient absorption spectroscopy. The kinetics of the initial charge separation in *Rb. sphaeroides*, *Rb. capsulatus*, *Rs. rubrum* and *Rs. centenum* were studied in quinone-reduced RCs at room

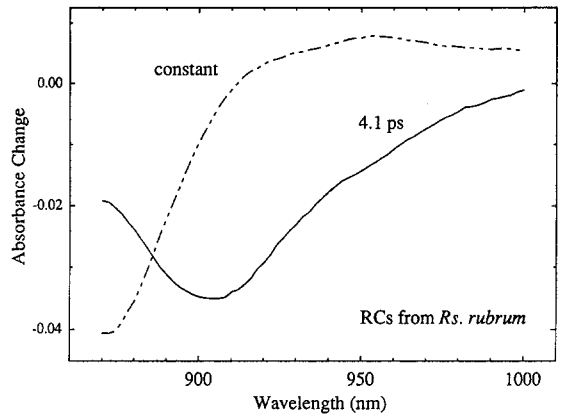

Fig. 4. The spectrum of the preexponential amplitude and constant term that resulted from fitting the ΔA vs. time and wavelength surface of *Rs. rubrum* to $A_1(\lambda)\, e^{-t/\tau_1} + A_2(\lambda)$ where $A_1(\lambda)$ and $A_2(\lambda)$ are the amplitude of spectrum shown and τ_1 is the decay time listed (4.1 ps). The RCs, $A_{802}^{1cm} \sim 5$, were in 50 mM Tris-Cl buffer pH 8.0, 0.05% Triton X-100, and 1mM EDTA. To reduce the primary quinone 4mM sodium dithionite was present.

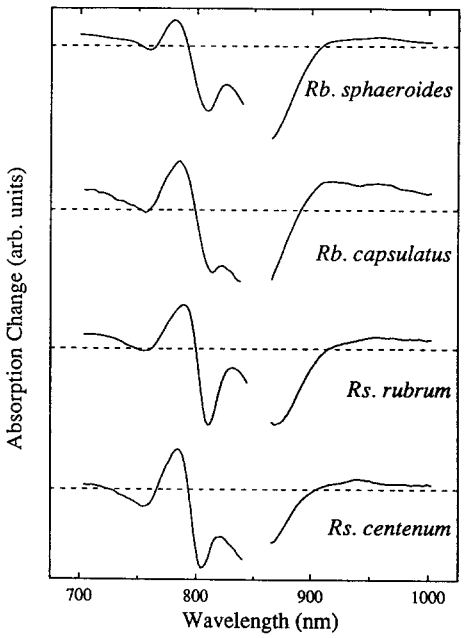

Fig. 5. Time-resolved spectral changes of reaction centers isolated from *Rb. sphaeroides*, *Rb. capsulatus*, *Rs. rubrum* and *Rs. centenum* 15 ps after excitation with a 150 fs pulse at 860 nm. At this time the RCs are primarily in the charge separated state $P^+H_A^-$. The RCs, $A_{802}^{1cm} \sim 5$, were in 0.05% Triton, 50 mM buffer (phosphate pH 7.4 for *Rb. capsulatus* and 50 mM Tris-Cl buffer pH 8.0 for the other three species), and 1 mM EDTA. To reduce the primary quinone 4 mM sodium dithionite was present.

temperature. Excited singlet state lifetimes of the primary donor (P*) were measured by monitoring the decay of the stimulated emission at 70 wavelengths between 870 and 1000 nm at 100 time points covering a 25 ps timespan. The absorption change vs. time and wavelength surfaces that resulted were fit using a global exponential decay analysis (Woodbury et al. 1994) assuming one exponential decay time and a constant term.

The decay lifetimes for *Rs. centenum* and *Rs. rubrum* were 4.1 ps and 5.5 ps. These lifetimes are very similar to the measured values of 4.5 ps and 6.1 ps for *Rb. capsulatus* and *Rb. sphaeroides* respectively. A representative plot of the amplitude spectra associated with the exponential decay term and the constant term for *Rs. rubrum* reaction centers is shown in Fig. 4. The exponentially decaying absorption changes in this spectral region have a broad spectral signature with a band near 905 nm that has been identified as stimulated emission from P* in *Rb. capsulatus* (Woodbury et al. 1985; Martin et al. 1986). Similar results were obtained with the other three species, though the exact position of the peak of this feature and the spectral shape within 20 nm of the excitation wavelength (860 nm) varied somewhat between species.

Individual time resolved absorption change spectra of reaction centers from each of the four species were measured between 700 and 1000 nm at 15 ps after excitation in order to determine the difference spectrum of the early charge separated state in each species (Fig. 5). Similar spectra are observed in each of the reaction center samples, though the exact peak positions and amplitudes vary depending upon the spectral features of the steady state absorption spectrum of the RCs. For *Rb. capsulatus*, the optical spectrum of the $P^+H_A^-$ state was measured from 700 nm to 950 nm and showed that the bleaching of the dimer Q_Y transition also shifted with deoxycholate (data not shown). The spectrum of $P^+H_A^-$ in RCs from *Rs. rubrum* is in general agreement with the spectra previously reported at a longer time of 50 ps (Kirmaier et al. 1983) and the data for *Rb. sphaeroides* and *Rb. capsulatus* are similar to those previously published (Kimaier and Holten 1987, 1988).

pH Dependence of recombinution rates. After excitation of the RCs by a saturating light pulse, the decay of the state P^+ was measured by monitoring the optical absorption changes at 865 nm or 850 nm. The representative kinetics of charge recombination from the primary and secondary quinone are shown in Fig. 6 for RCs from *Rb. capsulatus*. The $P^+Q_A^-$ recombination

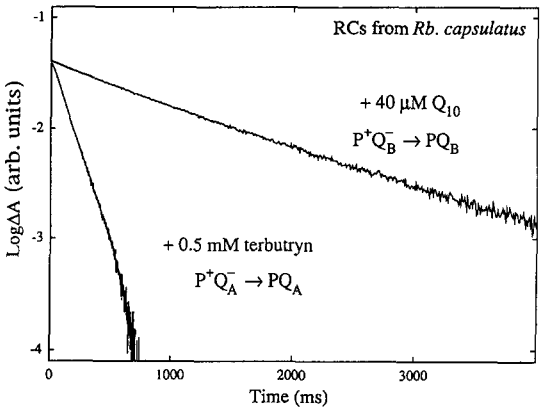

Fig. 6. Kinetics of charge recombination from the quinones at pH 7.4 in RCs isolated from *Rb. capsulatus*. The left lower curve is the decay of the P^+ bleaching in the presence of 0.5 mM terbutryn and represents $P^+Q_A^-$ recombination with a rate of 7.7 s^{-1}. The right upper curve is the decay of the P^+ bleaching in the presence of 40 μM exogenous quinone and represents the $P^+Q_B^-$ recombination with a rate of 0.8 s^{-1}. The RCs, A_{802}^{1cm}~0.7, were in 10 mM phosphate buffer pH 7.4 and 0.05% LDAO.

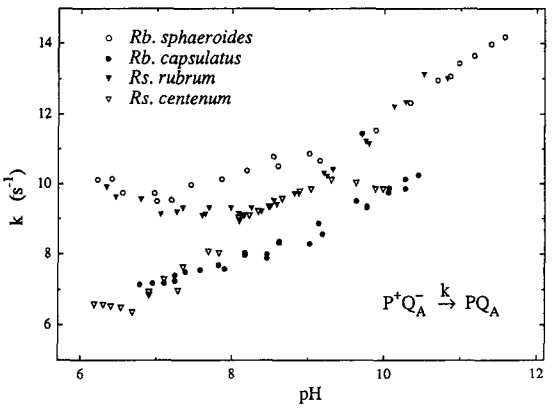

Fig. 7. The pH dependence of the charge recombination rate from $P^+Q_A^-$ to PQ_A. The RCs from *Rb. capsulatus* were in 10 mM phosphate buffer and 0.05% LDAO and the RCs from the other three species were in 15 mM Tris-Cl buffer, 0.025% LDAO and 1mM EDTA. The RC concentration was adjusted to A_{802}^{1cm}~0.7, the herbicide terbutryn was added at a concentration of 0.5 mM to block electron transfer from Q_A^- to Q_B, and a mixture of buffers, consisting of 2 mM CHES, PIPES, CAPS and HEPES, was added.

rate was found to be 7.7 s^{-1} for RCs in the presence of terbutryn at pH 7.4 in *Rb. capsulatus* in agreement with previous results (Prince et al. 1987). With no herbicide added, the rate was biphasic with the dominant component (70–80% of the total amplitude) having a decay rate of 0.8 s^{-1} and representing the recombination rate from $P^+Q_B^-$. In the presence of exogenous quinone, this slow component contributed 95% to the total amplitude indicating that almost all RCs had two quinones (Fig. 6). This rate is in agreement with earlier studies of RCs with exogenous quinone added (Prince et al. 1987). For RCs from the other three species a slow and fast component were also observed and the slow component was eliminated by the addition of herbicide. With no herbicide present, approximately 70% of the total amplitude was due to the slow component in these species.

The pH dependence of the rate of charge recombination from the primary quinone to the donor was measured (Fig. 7). For *Rs. rubrum*, the rate decreased from 10 s^{-1} to 9.3 s^{-1} as the pH was increased from 6 to 8 and increased steadily to 13 s^{-1} as the pH was increased to 10.5. For *Rs. centenum*, the rate steadily increased from 6.2 s^{-1} at pH 6.2, 7.8 s^{-1} at pH 8.0, and 9.9 s^{-1} at pH 9.0 and was pH independent above pH 9. The rates were 9.9 s^{-1} and 7.5 s^{-1} for *Rb. sphaeroides* and *Rb. capsulatus*, respectively, at pH 7.4. Above pH 8 the rates steadily increased from 10.2 s^{-1} and 7.8 s^{-1} at pH 8 to values of 12.2 s^{-1} and 10 s^{-1} at pH 10.5

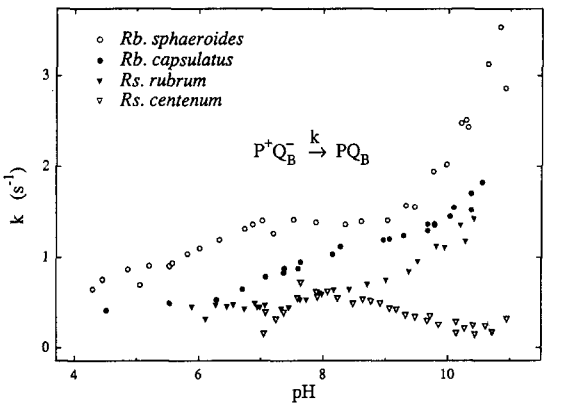

Fig. 8. The pH dependence of the charge recombination rate from $P^+Q_B^-$ to PQ_B. The RCs from *Rb. capsulatus* were in 10 mM phosphate buffer and 0.05% LDAO and the RCs from the other three species were in 15 mM Tris-Cl buffer, 0.025% LDAO, and 1mM EDTA. The RC concentration was adjusted to A_{802}^{1cm}~0.7 and a mixture of buffers, consisting of 2 mM CHES, PIPES, CAPS and HEPES, was added.

for *Rb. sphaeroides* and *Rb. capsulatus*, respectively. These data for *Rb. sphaeroides* and *Rb. capsulatus* are in agreement with previous studies (Feher et al. 1988; Takahashi et al. 1990; Hanson et al. 1992).

For recombination from the secondary quinone, the pH dependence for *Rs. centenum* was clearly different compared to that of the other three species. In contrast to a general increase in rate with increasing pH for the other three species, for *Rs. centenum*, the rate was 0.5

s^{-1} and approximately independent of pH from 7 to 8 and steadily decreased to 0.25 s^{-1} at pH 11 (Fig. 8). The rate is decreased by only 20% as the temperature was lowered from 24 °C to 6 °C. For *Rs. rubrum*, the rate was approximately 0.5 s^{-1} and pH independent from pH 6 to 7.5 and increased steadily to 1.3 s^{-1} as the pH was increased to 10.5. The measured pH dependences for *Rb. sphaeroides* and *Rb. capsulatus* are in agreement with previous studies (Feher et al. 1988; Takahashi et al. 1990; Hanson et al. 1992), with values of 1.3 s^{-1} and 0.8 s^{-1} measured for *Rb. sphaeroides* and *Rb. capsulatus*, respectively, at pH 7.

Discussion

Isolation protocol. A critical aspect for the isolation of the *Rb. capsulatus* RCs in this report was the use of a detergent concentration of 0.45% LDAO for solubilization. Previous protocols used 2% and 1.5% LDAO for the solubilization of the RCs (Prince et al. 1973, 1987). The use of 0.3% and 1% LDAO has also been reported for the solubilization of RCs from genetically modified strains (Bylina and Youvan 1988; Hanson et al. 1992). In the previous reports of modified preparations neither the purity nor the yield of the preparation were discussed. We have tested the effectiveness of different concentrations of LDAO for the isolation of RCs from the antennaless strain. At low concentrations of LDAO, 0.15% to 0.35%, it was possible to solubilize RCs but the yield was low (for example, 0.15% LDAO resulted in yields four-fold less than 0.45% LDAO). At a concentration of 0.45% LDAO the yield was essentially maximum. The purest RC's have been found to have a A_{280}/A_{800} ratio of 1.4, as was reported by Prince et al. (1987). Based upon the kinetic studies discussed below, the occupancy of the Q_B site is about 70–80%. In contrast, RCs isolated using the earlier procedures contained essentially no Q_B (Prince et al. 1987) due to the use of high concentrations of LDAO.

Observation of two spectral classes of RCs. The spectral features of the isolated RCs can be divided into two classes. One class of RCs has a P/P* absorption band at ~865 nm and a P^+ absorption band at ~1250 nm whose amplitude is approximately 10% that of the 760 nm band. The second class of RCs have a P/P* absorption band at ~850 nm and a ~1250 nm absorption band whose amplitude is approximately 3% that of the 760 nm band. These spectral differences of isolated RCs are due to large differences in the electronic structure of the donor as shown by ENDOR spectra that reveal signifcant differences in the spin distribution asymmetry (Rautter et al. 1994). Under normal buffering conditions, RCs from *Rb. sphaeroides* and *Rs. rubrum* belong to the first class and RCs from *Rb. capsulatus* and *Rs. centenum* belong to the second class. The RCs can be converted from one class to the other by the addition of charged detergents as measured by the changes in the position of the spectral features of the dimer, such as the position of the Q_Y absorption band (Fig. 3) and the ENDOR spectra (Rautter et al. 1994). The difference in spin density appears to influence the rate of charge recombination as discussed in Rautter et al. (1994). For *Rb. capsulatus*, the recombination rate increases by ~20% at all pH values upon the addition of 0.03% DOC to the solution. The ability of the RCs to convert between the classes indicates that the shructural differences between the two classes are not due to intrinsic differences but due to different interactions with detergents. Based upon the ENDOR results (Rautter et al. 1994), in chromatophores all RCs have features characteristic of the first class only.

Initial electron transfer. Based upon conventional models of electron transfer (Marcus and Sutin 1985), the initial electron transfer process should be sensitively dependent upon the energy levels of P* and $P^+H_A^-$. The $P^*/P^+H_A^-$ energy difference is different for the four RCs studied in this paper for two reasons. First, the measured values of the P/P^+ midpoint potentials range from 445 mV to 495 mV. Second, the 15 nm differences in the dimer Q_Y band correspond to a difference of ~25 meV for the P*/P transition. Together these two factors should result in the $P^*/P^+H_A^-$ energy gap being different for the four species by an amount comparable to the estimated value of 120 meV for the energy difference for RCs from *Rb. sphaeroides* that is based upon the time resolved measurements of fluorescence from P* (Williams et al. 1992). The rate of P* decay was different for the four species with measured rates ranging from 4.1 to 6.1 ps, but this variation was only roughly correlated with the P/P^+ midpoint potential. Similar changes in rates were found for mutants of RCs from *Rb. capsulatus* and *Rb. sphaeroides* that have the $P^*/P^+H_A^-$ energy difference altered by similar amounts (Kirmaier et al. 1991; Williams et al. 1992; Taguchi et al. 1992; Murchison et al. 1993; Jia et al. 1993; Nagarajan et al. 1993). Furthermore, no change in rate was observed in *Rb. capsulatus* RCs for the decay of the stimulated emission from P* nor the

electron transfer rate from H_A to Q_A upon addition of deoxycholate (data not shown). Thus, the $P^*/P^+H_A^-$ energy gap does not appear to be a critical parameter in determining the initial electron transfer rate in RCs from purple bacteria.

The decay lifetimes of P^*, as determined by the global exponential decay analysis, were fit by single exponentials for all four species. The use of a larger number of exponential terms to fit the decay did not statistically improve the fit in any of the samples except for *Rb. sphaeroides*. In this case adding one additional exponential decay term significantly improved the fit and resulted in 2.7 and 15.7 ps lifetimes. This result agrees well with high time resolution measurements of spontaneous emission decay (Muller et al. 1992; Du et al. 1992; Hamm et al. 1993) as well as previous measurements of the stimulated emission (Vos et al. 1991; Woodbury et al. 1994). Although the data from the other three species fit best with only one exponential term, it cannot be excluded that there is a second component that was not resolved at the signal to noise levels of these data. Previous measurements of the spontaneous emission have indicated that, at least in *Rb. capsulatus* reaction centers, there is a small (20%) 10–20 ps kinetic component to the P^* decay (Du et al. 1992).

The reported initial charge separation rate for RCs from *Rb. sphaeroides* and *Rb. capsulatus* varies from 2.6 to 4.1 ps (Woodbury et al. 1985; Chan et al. 1991; Lauterwasser et al. 1991). In our experiments, with the quinones reduced with sodium dithionite to block electron transfer from H_A to Q_A, a value of 6.1 ps was obtained for the single exponential lifetime of RCs from *Rb. sphaeroides*. The presence of the negative charge on Q_A results in a longer decay time for this rate than is measured for RCs with quinones that are unreduced (Woodbury et al. 1985; Martin et al. 1986).

pH Dependence of charge recombination rates from Q_A^-. In *Rb. sphaeroides*, recombination from the primary quinone proceeds directly with the native ubiquinone present (Kleinfeld et al. 1988) and the pH dependence for this rate does not arise from direct protonation of Q_A^- but from electrostatic interactions of Q_A^- with a number of nearby residues (Maroti et al. 1988; McPherson et al. 1988). The pH dependence for RCs from *Rb. capsulatus* is similar to that of *Rb. sphaeroides* indicating similar interactions involving Q_A^-. The slower rate for *Rb. capsulatus* by a factor of 0.8 compared to that of *Rb. sphaeroides* may be due to the a preferential stabilization of the charge separated state in *Rb. capsulatus* due to the difference in spin density of the primary donor as discussed in Rautter et al. (1994). Comparing *Rs. rubrum* and *Rb. sphaeroides*, a different pH dependence is evident between pH 7 and 10. This suggests that there are similar interactions with residues whose pKs are ~6 and ~9.5 but there is one (or more) amino acid residues with a pK of ~8 interacting with Q_A^- in *Rb. sphaeroides* but not in *Rs. rubrum*. In contrast to RCs from the other species, the rate for *Rs. centenum* has a steady increase from pH 6.5 to 8, suggesting a much different interaction with residues whose pKs are ~6 and an apparent loss of interaction with one (or more) residues with an apparent pK of ~9.5. Thus, differences among the four species are observed for interactions involving Q_A. A sequence comparison of *Rb. sphaeroides*, *Rb. capsulatus* and *Rs. rubrum* shows no differences in residues forming the immediate Q_A binding site. This suggests that either Q_A interacts differently with the conserved residues forming the site or that longer range interactions with non-conserved residues lying outside the immediate Q_A binding site are important.

pH Dependence of charge recombination rates from Q_B^-. The pH dependence of the charge recombination rate in *Rb. sphaeroides* and *Rb. capsulatus* has been shown to arise from an interaction between Q_B and nearby amino acid residues. In *Rb. sphaeroides*, the mutation of Glu L212 to Gln resulted in loss of the pH dependence from pH 9.5 to 11 that is observed in wild type (Paddock et al. 1989). Mutation of Asp L213 to Asn in *Rb. sphaeroides* resulted in the loss of the pH dependence below pH 8 (Takahashi et al.1990). In *Rb. capsulatus*, an insensitivity of this rate to pH was observed for the double mutant Glu to Ala at L212 and Asp to Ala at L213 (Hanson et. al. 1992). Together with other experiments, a model has been developed that residues Glu L212 and Asp L213 are critical proton donors for Q_B (Paddock et al. 1989; Takahashi et al. 1990; Hanson et. al. 1992; Feher et al. 1992). The pH dependence then serves as a key test for the presence of similarly positioned proton donors in other RCs.

For *Rs. centenum* the rate is nearly pH independent above pH 8 in contrast to the strong dependence observed for the other three species. This could arise if the equivalent residue to L212 in *Rb. sphaeroides* was a non-ionizable residue such as Gln. However, this is unlikely as L212 has been proposed to play a critical role in the protonation of Q_B that is necessary for cyclic electron turnover and has been found to be conserved in all sequences of purple bacteria that have been sequenced. This suggests that recombination occurs

by a different mechanism, direct recombination, in *Rs. centenum* rather than indirect recombination through Q_A. Direct recombination is expected to be much less sensitive to the energy level of Q_B^- and thus electrostatic interactions with nearby carboxylates, as has been observed for direct recombination from Q_A (for a review see Feher et al. 1988). In futher agreement, the rate in *Rs. centenum* is relatively independent of temperature and the observed value of 0.25 s^{-1} at pH 9.5 is equal to the direct recombination rate that is dominant in RCs from *Rb. sphaeroides* that have the mutation Asp to Asn at L213 (Labahn et al. 1994).

For *Rs. rubrum*, the rate was approximately pH independent from 5.8 to 8. In contrast the rate for *Rb. sphaeroides* and *Rb. capsulatus* increases with pH from 4.5 to 7. Residue Asp L213, that has been found to be critical for the pH dependence below 8 in *Rb. sphaeroides* and *Rb. capsulatus*, is not conserved in *Rs. rubrum*, as L213 is Asn instead of Asp (Belanger et al. 1988). It has been suggested that the role of L213 in *Rs. rubrum* may be fulfilled by alternate residues, such as Asp M44 (Takahashi et al. 1990; Hanson et al. 1992; Feher et al. 1992). If Asp M44 is indeed interacting with Q_B in such a manner then the approximate pH independence of the rate suggests that in *Rs. rubrum* its pK would be below the apparent pK of 4.5 for Asp L213 in *Rb. sphaeroides*.

In summary, the basic mechanism and pathway of early electron transfer are apparently common to all of these species. A striking similarity evident in the fast transient kinetic analysis of the four reaction centers is the similarity in the P* decay times as determined from the decay of the stimulated emission between 870 and 1000 nm and the general spectral features observed in the 700–850 nm region. Despite different $P^*/P^+H_A^-$ energy gaps the RCs in all four species have a fast forward rate and a much slower competing back reaction rate. The electron transfer process is also independent of the spin distribution of P^+ and thus appears to be determined largely due to other features of the RC, such as the distance between the donor and acceptor, the reorganization energy, or the coupling among cofactors (discussed in Woodbury et al. 1994). Although the primary electron transfer process appears to be similar, the RCs have different pH dependencies of the charge recombination rates that are probably due to different interactions between the quinones and nearby protonatable residues. A detailed understanding of these electron and proton transfer properties will require the development of models using the primary structure of the RC from *Rs. centenum* that is now being determined, and ideally, the three dimensional structure of all four RCs.

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