Structure, Spectroscopic, and Redox Properties of *Rhodobacter sphaeroides* Reaction Centers Bearing Point Mutations near the Primary Electron Donor

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ABSTRACT: Single mutations of three amino acid residues in the vicinity of the primary electron donor, P, in the reaction center (RC) from *Rhodobacter* (*Rb.*) *sphaeroides* were constructed and characterized in order to study the effects of hydrogen-bonding on the physical properties of P. The mutations, Phe M197 → Tyr, Met L248 → Thr, and Ser L244 → Gly, represent single amino acid changes near P designed to introduce residues found in *Rhodopseudomonas* (*Rps.*) *viridis* and to, thus, probe the effects of nonconserved residues. The mutations were designed to change the nonconserved H-bonding interactions of P in *Rb. sphaeroides*, at the level of a C_2 acetyl, a C_9 keto, and a C_{10} ester carbonyl of P, respectively, to those present in *Rps. viridis*. The Fourier transform (pre)resonance Raman (FTRR) spectra of P, in its reduced and oxidized states, from reaction centers of these mutants were studied to determine modifications of H-bond interactions of the π-conjugated C_2 acetyl and C_9 keto carbonyl groups and the C_{10} carbomethoxy ester carbonyl groups of P. The vibrational spectra of reduced P in the Met L248 → Thr and Ser L244 → Gly mutants reveal no evidence for changes in the H-bonding pattern of P; this suggests that for *Rb. sphaeroides* wild type, Ser L244 is not H-bonded to the C_{10} ester carbonyl of P_L. The vibrational spectrum of reduced P from the Phe M197 → Tyr mutant compared to that of wild type can unambiguously be interpreted in terms of the formation of a new H-bond with an acetyl carbonyl of P, specifically P_M. Correlating with the new H-bond, the Phe M197 → Tyr mutant exhibits an electronic absorption spectrum where the P absorption band is significantly perturbed. Intact cell and chromatophore photobleaching spectra of the same mutant indicate that the P absorption band has red-shifted by ca. 10 nm; no such behavior is observed for the other mutants. As well, the P → $BPhe_L$ electron transfer rate does not seem to strongly depend on the H-bonding of the C_2 acetyl carbonyl of P_M to a tyrosine residue. The EPR zero-field splitting parameters, E and D, of the primary donor triplet are only slightly modified in the mutant reaction centers, on the order of 1%. The FT Raman spectrum of the oxidized primary donor, P^+, of the Phe M197 → Tyr mutant shows a 3 cm^{-1} upshift of the C_9 keto carbonyl band, as compared to wild type, which may indicate that the + charge is slightly more localized on the P_L component in $P^{\bullet+}$. Optical redox titrations of the Met L248 → Thr mutant indicate that the redox midpoint potential is unchanged (within ±10 mV) compared to that of wild type; the Phe M197 → Tyr and Ser L244 → Gly mutants showed a small but significant increase of +20–30 and +30–40 mV, respectively, indicating that the tyrosine-donated H-bond to the C_2 acetyl carbonyl of P_M in the Phe M197 → Tyr mutant plays no dominant role in modulating the redox properties of P. This study suggests that the introduction of a new H-bond on the C_2 acetyl carbonyl of P in *Rb. sphaeroides*, by a tyrosine residue at position M197, only modestly modifies its spectral properties and is not a dominant determinant in its redox and triplet EPR properties.

The photosynthetic reaction center (RC) of purple bacteria is a membrane protein consisting of three polypeptide subunits (named L, M, and H) and represents the minimum protein unit wherein the primary light reactions and subsequent stable charge separation takes place. In addition to these three subunits, the isolated RC from *Rhodopseudomonas* (*Rps.*)[1] *viridis* possesses a tightly-bound tetraheme cytochrome subunit. The protein cofactors of the isolated RC from *Rhodobacter* (*Rb.*) *sphaeroides* consist of six bacteriochlorin pigments (four bacteriochlorophyll and two bacteriopheophytin molecules), one carotenoid, two quinones, and one non-heme iron. The bacteriochlorins and quinones are arranged in pairs in the L and M subunits, along a pseudo-C_2-symmetry axis which runs from the primary donor to the non-heme iron atom, forming two symmetric possible electron transfer branches. Once the primary electron donor (P), a dimer of bacteriochlorophyll (BChl) molecules, is excited by a photon or singlet exciton, an electron is unidirectionally transferred to the acceptor bacteriopheophytin molecule on the so-called L-branch ($BPhe_L$) in about 3 ps (Woodbury *et al.*, 1985; Martin *et al.*, 1986) via a monomeric accessory BChl molecule on the same branch, $BChl_L$, whose role is still a matter of debate (Holzapfel *et al.*, 1990). This asymmetric, unidirec-

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[1] Abbreviations: FTRR, Fourier transform resonance Raman; *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; *Rsp.*, *Rhodosprillum*; RC, reaction center; P, primary electron donor; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; WT, wild type; Phe, phenylalanine; Tyr, tyrosine; Met, methionine; Thr, threonine; Ser, serine; Gly, glycine; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; ZFS, zero-field splitting.

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tional electron transfer in the bacterial RC is still not fully understood.

With the solved crystal structure for the two bacterial RCs, *Rps. viridis* (Deisenhofer & Michel, 1989) and *Rb. sphaeroides* (Allen *et al.*, 1987a,b; El-Kabbani *et al.*, 1991), site-directed mutagenesis has been used intensively in probing the role of the protein in the electron-transfer properties of the RC (reviewed in Coleman and Youvan, 1990). Much attention has been paid to the spectroscopic properties and to the asymmetric functioning of the bacterial RC. Several amino acid targets have been those which have inequivalent C_2 symmetry-related partner residues. For instance, in the RC from *Rps. viridis* a glutamic acid residue (L104) H-bonds to the C_9 keto carbonyl of $BPhe_L$ on the photoactive L branch whereas no H-bond candidate is found on the similar carbonyl at the C_2 symmetry-related $BPhe_M$ molecule (Deisenhofer and Michel, 1989). Mutation of this Glu residue (which is conserved in *Rb. sphaeroides*, *Rb. capsulatus*, and *Rhodosprillum* (*Rsp.*) *rubrum*) in *Rb. capsulatus* to a residue incapable of H-bonding resulted only in a minor decrease in kinetics and no change in the electron transfer pathway (Bylina *et al.*, 1988). Another example is the mutation of the tyrosine M210 residue in *Rb. sphaeroides*, which is located between the primary donor and the primary electron acceptor $BPhe_L$ and which is in van der Waals contact with P, $BChl_L$, and $BPhe_L$. The C_2 symmetry-related residue is phenylalanine L181. The mutation of Tyr M210 in *Rb. sphaeroides* (Gray *et al.*, 1990) did not result in the breaking or formation of any H-bonds on any conjugated carbonyl of the primary donor (Mattioli *et al.*, 1991a) yet the initial electron-transfer kinetics were significantly slower in *Rb. sphaeroides* (Finkele *et al.*, 1990; Nagarajan *et al.*, 1990) as well as in *Rb. capsulatus* (Chan *et al.*, 1991). Furthermore, the electron-transfer pathway remained the same.

These intriguing results have prompted us to further explore the asymmetric structure of the primary donor, the starting-point of the asymmetric electron transfer in the RC.

An important problem to address is to what extent the asymmetric functioning of the RC is related to the unequal charge distribution in P. For instance, theoretical work suggests that there is an imbalance in the electronic charge distribution in P* in wild-type RCs toward half of the dimer (Plato *et al.*, 1988a; Scherer & Fischer, 1989; Parson *et al.*, 1990). As well, an asymmetric distribution of the spin density of the unpaired electron in P^+ of *Rb. sphaeroides* R26 has been concluded on the basis of ENDOR measurements (Lendzian *et al.*, 1990; Rautter *et al.*, 1992; reviewed in Lubitz, 1991). We have recently shown for wild-type RCs from *Rb. sphaeroides* R26 that, in the P^+ state, the + charge is primary localized on P_L on the time scale of the resonance Raman effect (Mattioli *et al.*, 1991b). Recent work studying the photochemistry of both heterodimer mutants of *Rb. sphaeroides* (McDowell *et al.*, 1991) indicates that the complete localization of the + charge on either BChl component of P is not the sole reason governing the asymmetric electron transfer to $BPhe_L$. However, the different electron transfer rates exhibited by the two heterodimer mutants suggest that the balance of the + charge repartition between P_L and P_M could influence this property. In this respect, it is important to determine which factors influence the localization of the + charge in $P^{\bullet+}$. One question which remains open at present is how the protein affects this asymmetric charge distribution and which residues around P significantly contribute to this effect. Toward this end, it is of interest to study the influences of direct protein interactions, such as H-bonds, on charge distribution in the primary donor and as well as on other physicochemical properties.

Vibrational spectroscopy is a powerful tool in elucidating structural changes resulting from the point mutation. In particular, resonance Raman spectroscopy is sensitive and selective in monitoring changes in the protein–pigment interactions of the resonant chromophore (Lutz & Robert, 1988). Recently we have reported the use of near-infrared (near-IR) excited Fourier transform resonance Raman spectroscopy (FTRR) in studying the structure of the primary electron donor in bacterial reaction centers (Mattioli *et al.*, 1991b, 1993). This method, which excites the Raman spectrum of the RC with 1064-nm radiation, is highly sensitive and selective for the primary donor in its reduced and oxidized states. Structural information such as protein–pigment interactions in the binding pocket of the primary donor, via the central Mg atoms and the π-conjugated carbonyls of P or $P^{\bullet+}$, is directly obtained without resorting to difference techniques. Hydrogen-bonding states of the conjugated carbonyls of the BChl *a* molecules are, in particular, directly ascertainable from the FTRR spectra of P (Mattioli *et al.*, 1991b). As well, the FTRR spectra of P^+ yield information concerning the localization of the + charge in the oxidized primary donor (Mattioli *et al.*, 1991b, 1993). Thus, the possible influence of certain amino acid residue changes in mutants on this charge localization can also be explored.

We report here the effects of specific mutations near the primary donor in three site-directed mutant reaction centers from *Rb. sphaeroides* and describe the related structural changes and their influence on P/P^+ oxidation potentials and on several spectroscopic parameters. These mutations in the P binding pocket serve as probes to investigate the effects of nonconserved residues between *Rps. viridis* and *Rb. sphaeroides*. The residues genetically altered in this work are designed to change the H-bond pattern in the primary donor of *Rb. sphaeroides* in a way which makes its environment more "viridis-like" (see Figure 1). The mutations in *Rb. sphaeroides* studied here are Phe M197 → Tyr, Met L248 → Thr, and Ser L244 → Gly (we gratefully acknowledge Dr. Marianne Schiffer for suggesting these mutations). Their expected changes in protein–pigment interactions, based on the published X-ray crystal structures of *Rb. sphaeroides* and *Rps. viridis*, are given in Table I. It is interesting to compare the spectroscopic properties of the RCs from *Rps. viridis* to those of these mutant *Rb. sphaeroides* RCs. Significant differences in the spectroscopic properties of these two bacterial RCs include the relative red-shift of the primary donor Q_y absorption band with respect to the Q_y absorption band of the monomeric BChls *in vitro*, the EPR spectra of triplet P (Norris *et al.*, 1989; reviewed in Angerhofer, 1991), the temperature dependence of electron transfer rates from P to $BPhe_L$ (Fleming *et al.*, 1988), and the asymmetric electron spin density distribution in the oxidized primary donors (reviewed in Lubitz, 1991; Plato *et al.*, 1991).

The effects of H-bonding with conjugated carbonyls of BChl macrocycles on physicochemical properties of chlorophylls are widely discussed, but the lack of precise experimental data renders unsatisfying conclusions as to their purported roles. Recently, several reaction center mutations have been reported where significant changes in the redox midpoint potential (greater than 60 mV) of P have occurred; these redox changes changes have been postulated to have resulted from the formation of new H-bonds between histidine residues and the C_9 keto carbonyls (Williams *et al.*, 1992) or the C_2 acetyl carbonyl (Stocker *et al.*, 1992) of P, as well as the

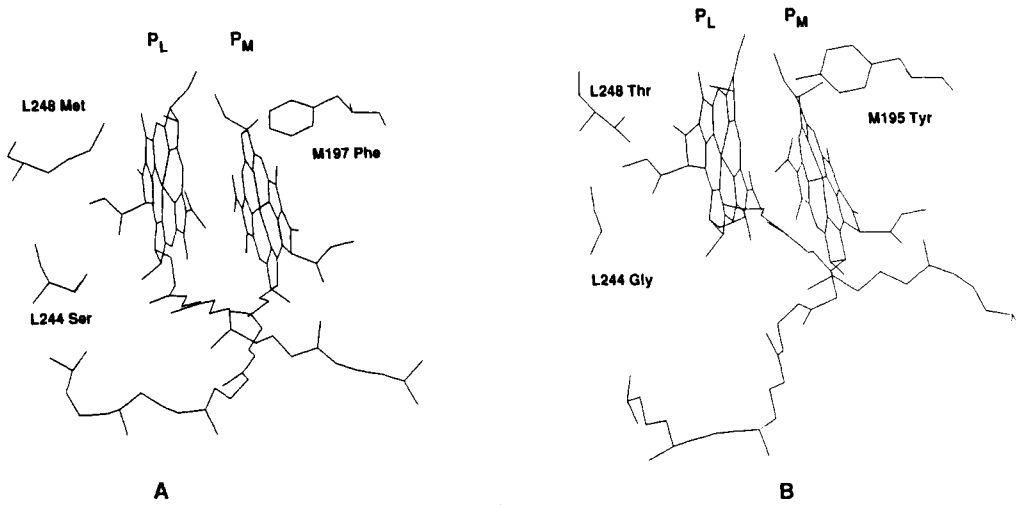


FIGURE 1: Residues around the primary donor of *Rps. viridis* (B) which have been genetically substituted into the reaction center of *Rb. sphaeroides* (A) as described in this work. Illustrated is the geometry of tyrosine M195 (phenylalanine M197 in *Rb. sphaeroides*), methionine L248 (threonine L248), and serine L244 (glycine L244).

Table I: Point Mutations in *Rb. sphaeroides* Studied in This Work Compared with Residues in *Rps. viridis*

residue	*Rb. sphaeroides*[a]	*Rb. viridis*[b]	mutation in *Rb. sphaeroides*	expected possible change in *Rb. sphaeroides*[c]
M197	Phe	Tyr	Phe → Tyr	H-bond formed on P_M C_2=O
L248	Met	Thr	Met → Thr	H-bond formed on P_L C_9=O
L244	Ser	Gly	Ser → Gly	H-bond broken on P_L C_{10}=O

[a] Allen *et al.*, 1987a,b; El-Kabbani, *et al.*, 1991. [b] Michel & Deisenhofer, 1989. [c] These expected changes are based upon the H-bond pattern in the crystal structure of *Rps. viridis* (Michel & Deisenhofer, 1989).

removal of a H-bond donated by a histidine residue to the C_2 carbonyl (Murchison *et al.*, 1993) of P. With the Phe M197 → Tyr mutant studied in this work, we can now compare properties of the *Rb. sphaeroides* and *Rps. viridis* primary donors in terms of their electronic absorption spectra, EPR spectra, redox midpoint potentials, and their electron-transfer kinetics to gauge the effect and role of the protein environment, in terms of H-bond formation donated by a tyrosine residue, in tuning the physicochemical properties of P.

EXPERIMENTAL PROCEDURES

Materials. Cell growth conditions for *Rb. sphaeroides* strains and the construction of the site-directed mutants are described in Gray *et al.* (1990). The plasmids carrying the mutated reaction center genes were introduced individually into the neurosporene-accumulating *puf LMX* deletion strain PUFΔ LMX21/3 as described in Farchaus and Oesterhelt (1989). Unless otherwise stated, the deletion strain which was complemented *in trans* with the 5.3-kb *puf* operon shuttle fragment, carrying the wild type genes, is referred to as "wild type" (WT). Chromatophore membrane fragments were prepared by incubating the cell suspension with lysozyme and DNase for 45 min at room temperature and then passing the cells through a French press cell twice. Isolated chromatophores were obtained after two centrifugation steps (14 000*g*, 30 min, and 100 000*g*, 90 min). Reaction centers were isolated and purified as described elsewhere (Gray *et al.*, 1990). Final preparations of the wild-type and mutant RCs were in 0.8% octyl glucoside and 20 mM Tris buffer at pH 8.

Methods. Construction of Mutants and Culture Growth. The mutants described here were constructed by using the gapped duplex DNA method (Stanssens *et al.*, 1989). The recombinant DNA techniques are described in detail elsewhere (Farchaus *et al.*, 1993). Double strand sequencing of the entire gapped duplex region confirmed that the desired mutations were present and that no other genetic alteration occurred in this region. For large scale fermentations, the cultures were grown at 30 °C in the dark under chemoheterotrophic conditions. Generation times of cultures growing under chemoheterotrophic or photoheterotrophic conditions were obtained as described in Farchaus *et al.* (1993).

Room-temperature steady-state photobleaching experiments on whole cells, chromatophores, or reaction center preparations were recorded using a photodiode array spectrophotometer as described in Uhl *et al.* (1985). The measuring beam intensity was reduced using a 1% neutral density filter and an appropriate cutoff filter (RG 665, Schott, Mainz). Changes induced by the actinic measuring beam were measured as the difference between the initial spectrum and a spectrum recorded 100 ms later. Samples were excited for 100 ms, and 100 spectra in the spectral region 700–950 nm were recorded every 1 ms to follow the bleaching.

Low-temperature (77 K) absorption spectra of reaction centers were recorded using an Aminco DW2A dual-beam spectrophotometer equipped with a Hamamatsu IR-photomultiplier (Model 473). Samples were held in a liquid nitrogen dewar containing sample and reference compartments each of 2 mm path length. Ascorbate and ferricyanide were used to ensure that the RCs were fully reduced or fully oxidized. Spectral resolution was 2.5 nm at 800 nm.

Optical redox titrations were performed as described in Wachtveitl *et al.* (1993). A modified version of the thin layer cell (optical pathlength, 0.2 mm) described in Hawkridge and Ke (1976) was used. The working electrode was an optically transparent gold mesh electrode (50% transmittance, 500 lines/in., Buckbee-Mears Co.) that was glued onto a mylar foil. A platinum wire was threaded and glued to the top of the gold mesh electrode. The counter electrode was constructed of a platinum wire, and the reference electrode was a Ag/AgCl electrode filled with 1 M KCl as electrolyte. Measurements were performed at 20 °C using a scanning potentiostat (EG&G Princeton Applied Research). The light-

induced absorbance changes were recorded during the oxidative part of the titration and the remaining photobleachable RC was taken as the amount of reduced P. In the following reductive part of the titration, it was confirmed that the signal could be restored to its original amplitude, and therefore no RC activity was lost during the potentiometric cycle. The sample was protected from light by a shutter that blocked the measuring beam between individual absorption measurements, during equilibration and dark adaption. The following redox mediators were used at 50 μM final concentrations: phenazine methosulfate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, ferricyanide, and porphyrexide (kind gift of A. W. Rutherford). The RCs were in 0.8% octylglucoside at a concentration of 12.5 μM, in 20mM Tris at pH 8, at 20 °C.

The EPR measurements were performed as described in Wachtveitl (1992). Reaction center samples for the EPR experiments were prereduced with ascorbate (100 mM, final concentration), bubbled with nitrogen for several minutes, and frozen in liquid nitrogen. The EPR triplet spectra were recorded on a Varian E-3 spectrometer equipped with an Air Products EPR cryostat and a Scientific Instruments (series 5500) temperature controller. Conditions for the EPR experiments were as follows: microwave power, 2 mW; field modulation amplitude, 20 G; field modulation frequency, 100 kHz; light modulation frequency, 700 Hz; temperature, 15 K. The experimental details are described elsewhere (Rustandi, 1991).

The Fourier transform (FT) Raman spectra were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA Raman module as described elsewhere (Mattioli *et al.*, 1991b). Typically, ca. 180 mW of laser power from a continuous wave diode-pumped Nd:YAG laser was used. Samples were contained in a sapphire cell described elsewhere (Schrader *et al.*, 1990). All spectra were recorded at room temperature and were the result of the coaddition of 2000 interferograms unless otherwise stated. Spectral resolution was 4 cm^{-1}. Some spectra were corrected for luminescent background using a polynomial fit.

Room-temperature absorption spectra in the 800–1300-nm region were obtained with the same FT Raman apparatus in absorption mode using a tungsten lamp and a Ge diode detector. For these measurements a 1-mm quartz cell containing the RC samples (ca. 1.6 μM) was used. Ascorbate or ferricyanide were added to the sample to ensure the RCs were completely reduced or oxidized, respectively.

RESULTS

Generation times under both chemoheterotrophic (ca. 6 h) and photoheterotrophic culture conditions (ca. 4.5 h at 70 W m^{-2} light intensity) were found to be identical for the wild type and the three strains carrying the point mutations M197 (Phe → Tyr), L248 (Met → Thr), and L244 (Ser → Gly), respectively.

Absorption Spectra of P and P$^+$. The $P^+ - P$ room-temperature photobleaching electronic absorption difference spectra of the *Rb. sphaeroides* WT and the M197 mutant chromatophores are shown in Figure 2; these photobleaching spectra are essentially the same as those of whole cells (data not shown). Comparison of the $P^+ - P$ difference spectra of the L248 and L244 mutants (data not shown) with that of WT shows no significant differences in the minima and bandwidths of the bleached 860-nm band of P. As well, the electrochromic shift of the 803-nm band is similar for WT and the mutants. The Phe M197 → Tyr mutation, however, significantly alters the near-IR absorption band of P, whose

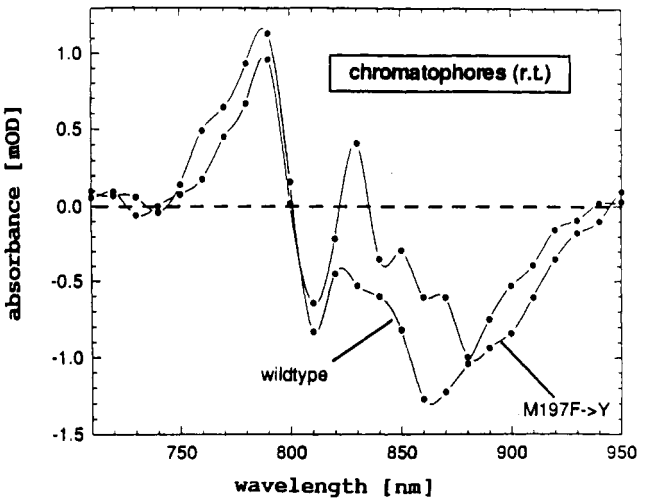


FIGURE 2: Room-temperature photobleaching ($P^+ - P$) difference spectra of *Rb. sphaeroides* chromatophores, wild type (WT), and Phe M197 → Tyr mutant.

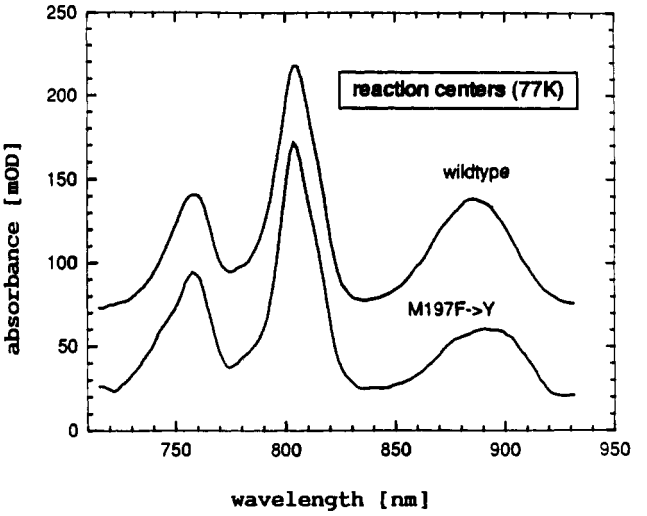


FIGURE 3: Low-temperature (77 K) electronic absorption spectra of *Rb. sphaeroides* reaction centers, wild type, and Phe M197 → Tyr mutant.

maximum is red-shifted by ca. 10 nm with respect to that of WT. As well, this photobleached P band exhibits a smaller extinction coefficient at its maximum (see Figure 2), and appears broader, as compared to that of WT. The photobleaching experiments on chromatophores and whole cells ensure that the red-shift and other spectral changes of the P band seen for the Phe M197 → Tyr mutant do not arise from preparation factors such as RC extraction and purification.

The low-temperature (77 K) absorption spectra of purified reaction centers of the Phe M197 → Tyr mutant and WT are shown in Figure 3, and the results for the other mutants are summarized in Table II. The Phe M197 → Tyr mutant RC preparation exhibits a near-IR absorption band of P at 889 nm. Relative to the 759-nm band, this P band has a smaller apparent extinction coefficient, and is ca. 4 nm broader, as compared to that of WT. These trends in spectral features are similar to those found in the chromatophore photobleaching spectrum of this mutant (Figure 2). The observed red-shift (relative to WT) of the P band for the Phe M197 → Tyr mutant RC preparation is only 1–2 nm (Figure 3 and see Table II) as compared to 10 nm observed in the chromatophore and whole cell photobleaching spectra (Figure 2). However, compared to the room-temperature photobleaching spectra of the chromatophores, the room-temperature absorption

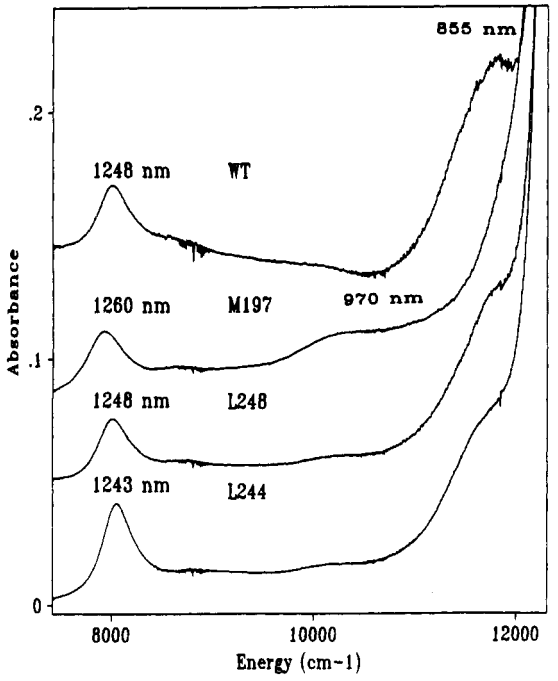


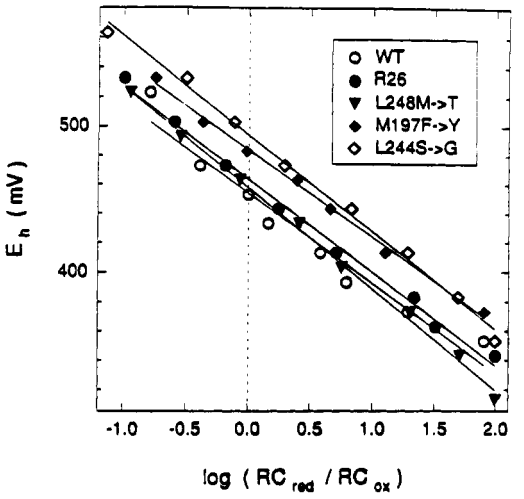
FIGURE 4: Room-temperature near-infrared electronic absorption spectra of oxidized reaction centers of wild type and mutants. The spectra were all normalized to the 800-nm band, which is not shown on this scale. Spectral resolution was 2 cm^{-1}; 120 interferograms were coadded.

Table II: Observed Electronic Absorption Maxima (nm) of *Rb. sphaeroides* Wild-Type and Mutant Reaction Centers and Relative Peak Intensities

	300 K	77 K
wild type	756, 804, 864 (1:2.1:1)	757, 804, 888 (1:2.2:1)
M197F → Y	756, 806, 866 (1:2.4:0.7)	759, 805, 889 (1:2.4:0.7)
L248M → T	757, 803, 859 (1:2.2:1)	756, 803, 873 (1:2.2:1)
L244S → G	757, 804, 857 (1:2.0:1)	757, 803, 878 (1:2.1:1)

spectra of the purified mutant RCs, in general, exhibit P bands that are slightly blue-shifted which seems to arise from RC isolation and purification. These blue-shifts are also seen in the 77 K spectra. Thus, the maximum exhibited by the M197 mutant RCs in Figure 3 should also be affected in a similar fashion and may attenuate the red-shift. However, the broadening and lower relative intensity maximum of the P absorption band is observed only for the M197 mutant, both in chromatophores and isolated RCs.

The room-temperature near-infrared electronic absorption spectra of the ferricyanide-oxidized primary donor, P^+ (normalized to the ca. 800-nm band) for WT and the three mutants (Figure 4) also show that only the Phe M197 → Tyr mutation has significantly altered the P^+ absorption band at ca. 1250 nm. Compared to the wild-type spectrum, this band is red-shifted by 10 nm (or 78 cm^{-1}) for the Phe M197 → Tyr mutant and appears to be slightly broader. For this same mutant, there is a distinct broad band at ca. 970 nm which is more intense in the M197 mutant than in wild type and the other mutants. At present, its origin is unknown. As well, the weak shoulder at 855 nm is visibly diminished or not present at all for this mutant. There is also a blue-shift of 5 nm (or 37 cm^{-1}) observed for the P^+ band of the Ser L244 → Gly mutant.



	WT	R26	M197F->Y	L244S->G	L248M->T
E_h (mV)	453	463	484	494	458
n	0.96	0.94	0.98	0.89	0.86

FIGURE 5: Redox titrations of wild-type and mutant reaction centers. The logarithm of the ratio of reduced and oxidized RCs is plotted versus the ambient potential, E_h. The amount of reduced RC at various potentials was determined by quantitative photobleaching. The curve marked R26 refers to *Rb. sphaeroides* carotenoidless R26 strain titrated for comparison.

Table III: Triplet ZFS Parameters, Redox Potentials, and P* Lifetimes in *Rb. sphaeroides* Wild-Type and Mutant Reaction Centers

	D (10^{-4} cm^{-1})	*E* (10^{-4} cm^{-1})	P/P^+ E_h[a] (mV)	P* decay time[b] (ps)	% P_L^+[c]
wild type	200.9	34.8	453	3.5	80
M197F → Y	199.1	35.8	484	6.0	90
L248M → T	200.9	34.3	458	3.9	78
L244S → G	202.5	35.3	494	6.5	66
BChl *a in vitro*	240.0	57.0			
Rps. viridis wild type	165.0	40.0			

[a] Estimated error is ±10 mV. [b] P* stimulated emission measured at 920 nm (Finkele, U., Lautterwasser, C., and Zinth, W., unpublished data). [c] Estimated percentage of + charge on P_L in the P^{*+} state.

Primary Donor Redox Potentials. The results of electrochemical optical redox titrations of the three mutants are shown in Figure 5 and summarized in Table III. Within the estimated error of ±10 mV, the P/P^+ redox midpoint potentials (E_h) of the M197 and the L244 mutants appear to be significantly different from that of WT, determined to be (453 ± 10) mV (SHE). For comparison, Table III also shows the measured E_h for the carotenoidless R26 strain of *Rb. sphaeroides* which, within error, is similar to that of the WT strain (Table III) used in this work. The E_h value for WT (and for the R26 strain) is in good agreement with that of 450 mV reported by Blankenship and Prince (1985) for *Rb. sphaeroides*, but it is somewhat lower than the 485-mV E_h value reported more recently by Moss *et al.* (1991) and the 495-mV value reported by Williams *et al.* (1992). The reason for this discrepancy is not clear but might arise from methodological differences. The reliability of the redox titration methodology used in this work is discussed in Wachtveitl *et al.* (1993). For the fitting of the Nernst curves in Figure 5, we have left n (number of electrons) as a variable parameter. The obtained slopes (ca. −59 mV) and *n* values (ca. 1) of these curves confirm the reliability of the titrations. The relatively lower *n* values of 0.89 and 0.86 corresponding to the titrations of the Ser L244 → Gly and Met L248 → Thr

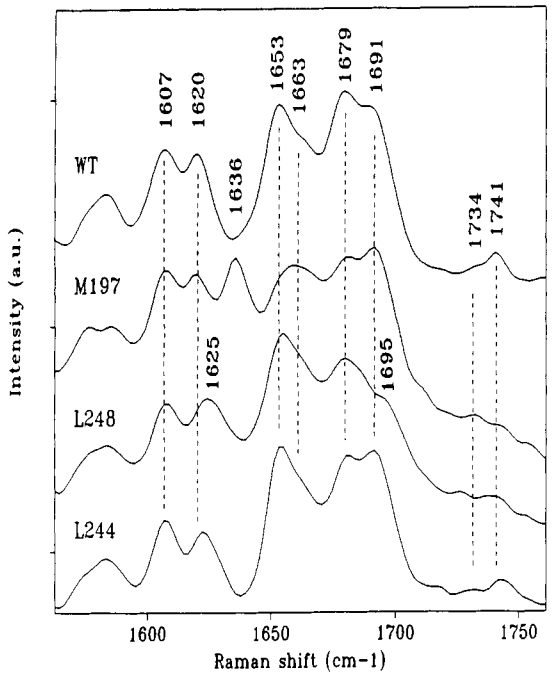


FIGURE 6: Fourier transform preresonance Raman spectra of ascorbate-reduced reaction centers of *Rb. sphaeroides* wild type (WT) and mutants. Spectral resolution was 4 cm^{-1}, and spectra were recorded at room temperature. Spectra represent the coaddition of 4000 interferograms. The top (WT) and bottom (L244) spectra were recorded from samples which were significantly more concentrated than the others, consequently the signal-to-noise ratio is higher. The WT spectrum is that of the R26 strain; this spectrum is virtually identical to that of the wild type strain used in this work.

mutants, respectively, should somewhat increase the uncertainty in their determined P/P^+ E_h values. For the M197 mutant, the introduction of a H-bond to a conjugated carbonyl of P is expected to stabilize the neutral state of P compared to the oxidized state and thus increase the P/P^+ potential (Williams *et al.*, 1992). The increase in the P/P^+ redox potential reported here is significantly smaller than those reported for mutant RCs designed to introduce H-bonds to the C_9 keto carbonyls (Williams *et al.*, 1992) and C_2 acetyl carbonyl (Stocker, *et al.*, 1992) of P.

EPR Measurements of Triplet P. The EPR spectra of the primary donor triplet state in the three mutants reveal that there are no sizable changes in the zero-field splitting triplet parameters as compared to the WT. The results are summarized in Table III. The changes in the zero-field splitting parameters within the WT and mutant reaction center group of data (ca. 1%) are smaller than the changes observed when comparing the reaction center data with chromatophore data (ca. 3%; Wachtveitl (1992)). In other words, RC solubilization and purification alters these triplet parameters more than do the point mutations discussed in this work.

Near-IR FT Raman Spectra of the Reduced Primary Donor, P. Figure 6 shows the room temperature FTRR spectra in the high-frequency region of the WT and mutant RCs in the presence of ascorbate. We have previously discussed that FTRR spectra of reduced RCs excited with 1064 nm radiation involve a preresonance enhancement of the vibrational spectrum of ground-state P via its ca. 865 nm absorption band (Mattioli *et al.*, 1991b). This preresonance condition is sufficiently pronounced so as to enhance the Raman contributions of P over those of the accessory BChl and BPhe molecules. This results in a Raman spectrum where the most intense bands arise from the reduced primary donor.

The assignments of the observed bands in the FTR spectra of P and the corresponding conjugated carbonyls of P_L and P_M have been discussed in detail elsewhere (Mattioli *et al.*, 1991b). The vibrational stretching modes for the C_2 acetyl and C_9 keto carbonyls of BChl *a* are observed at ca. 1660 and ca. 1700 cm^{-1}, respectively, when the carbonyls are not engaged in intermolecular interactions (Lutz, 1984). The frequencies of these modes downshift by as much as 40 cm^{-1} when these carbonyls are engaged in H-bond interactions; the magnitude of the downshift depends upon the strength of the H-bond. *In vitro* studies have shown that, typically, the frequency of the C_2 acetyl carbonyl mode may be observed between 1620 and 1665 cm^{-1} whereas that of the C_9 keto carbonyl may be observed in the range 1650–1710 cm^{-1} (Lutz, 1984). In the FTRR spectrum of reduced RCs of *Rb. sphaeroides* wild type (Figure 6), the 1607-cm^{-1} band arises from a C_aC_m methine bridge stretching mode of P; its narrow bandwidth and its frequency indicate that the central Mg atoms of both BChl components of P possess one axial ligand each (Mattioli *et al.*, 1991b). The 1620- and 1653-cm^{-1} bands arise from the stretching modes of the P_L C_2 acetyl carbonyl H-bonded to His L168 (El-Kabbani *et al.*, 1991) and the free P_M C_2 acetyl carbonyl, respectively. The 1679- and 1691-cm^{-1} bands arise from the stretching modes of the C_9 keto carbonyls of P_M and P_L, respectively; their observed frequencies indicate that they are not engaged in H-bonding interactions. There is a weak shoulder at ca. 1663 cm^{-1}, which probably arises from the accessory BChls or the protein; it persists in the P^+ spectrum and, therefore, cannot be attributable to neutral P.

The FTRR spectrum of reduced P in the Phe M197 → Tyr mutant (Figure 6) shows the appearance of a new band at 1636 cm^{-1} and the disappearance of the 1653-cm^{-1} band. The rest of the spectrum is essentially unchanged. We have measured two different RC preparations of the M197 mutant (data not shown) which differ only in the carotenoid present in the RC (Farchaus & Oesterhelt, 1989). Both preparations exhibit the same spectral features for P indicating that there is no interference from the preresonant scattering of the carotenoid molecule at frequencies greater than 1600 cm^{-1}. These observed changes are totally consistent with the formation of a new H-bond on the C_2 acetyl carbonyl of P_M. Indeed, if the genetic replacement of Phe M197 with a Tyr residue were to form a new H-bond with the C_2 acetyl of P_M (as is the situation in the *Rps. viridis* RC), then according to our assignments, one would expect to observe the disappearance of the band corresponding to the free C_2 acetyl carbonyl of P_M (i.e. the 1653-cm^{-1} band), and a new band appear between 1620 and 1655 cm^{-1} depending on the strength of the H-bond formed. This is precisely what is observed. The downshift of 17 cm^{-1} of the 1653-cm^{-1} band represents an estimated H-bond enthalpy of ca. 2.6 kcal/mol (11 kJ/mol), according to the Badger-type empirical relation given by Zadorozhnyi and Ishchenko (1965) for stretching frequencies of aromatic carbonyls.

For the Met L248 → Thr mutant there is no evidence for the formation of a new H-bond with the keto carbonyl of P_L or any other conjugated carbonyl. Indeed, the formation of such a H-bond to the C_9 keto carbonyl of P_L would be expected to induce the disappearance of the band corresponding to the stretching mode of this carbonyl (i.e. the 1691-cm^{-1} band) and the appearance of a new band between 1695 and 1660 cm^{-1} depending on the strength of the formed H-bond; this is not observed. There is, however, a weakening in intensity of this band and a slight shift to 1695 cm^{-1}. We interpret these observations as a perturbation of this P_L keto carbonyl,

perhaps consisting of a change in its polarizability. Also present in this spectrum is the 3-cm^{-1} upshift of the 1622-cm^{-1} band to 1625 cm^{-1}. This small but significant shift indicates that there is a perturbation on the C_2 acetyl carbonyl of P_L caused by the mutation. Since the point mutation is far from this acetyl carbonyl the perturbation most likely arises from secondary effects in the P binding pocket which alters the H-bond strength of His L168 with the C_2 acetyl carbonyl of P_L. In view of the perturbation on both the C_2 acetyl and C_9 keto carbonyls of P_L, the Met L248 → Thr mutation most likely results in a minor repositioning of P with respect to its binding pocket, especially at the level of P_L. This perturbation could be the result of replacing the methionine residue, possessing a polarizable sulfur atom, with a shorter threonine side-chain possessing a polar OH group which could affect the local binding pocket structure via a change in electrostatic interactions.

For the Ser L244 → Gly mutant, its FT RR spectrum shows no evidence of the breaking of a H-bond with a C_{10} carbomethoxy ester carbonyl of P. In the WT spectrum there are two bands in the spectral region of the C_{10} ester carbonyl stretching region, a weak band at 1741 cm^{-1} and a weaker one at 1734 cm^{-1} (Mattioli *et al.*, 1991b). The 1741-cm^{-1} band is assignable to a free C_{10} ester carbonyl; a similar band at 1740 cm^{-1} is observed for BChl *a* in dry tetrahydrofuran, a solvent not capable of donating a H-bond. The 1734-cm^{-1} band might be interpreted as arising from (i) a (weakly) H-bonded C_{10} carbonyl, as proposed in the crystal structure of El-Kabbani *et al.* (1991), to be that of P_L H-bonded to Ser L244, or from (ii) a similar C_{10} free carbonyl, but in a different dielectric or electrostatic environment. If the genetic replacement of Ser L244 with Gly results in the breaking of a H-bond with the C_{10} ester carbonyl of P_L, then one could expect the disappearance of the 1734-cm^{-1} band and an increase in intensity of the 1741-cm^{-1} band. In the FT Raman spectrum of the Ser L244 → Gly mutant, the double band feature at 1734 and 1743 cm^{-1} is still present thus providing no evidence for the breaking of a H-bond donated by the serine side-chain. It may be argued that the 1734-cm^{-1} band arises from a carbomethoxy carbonyl of P which is H-bonded to the protein backbone at position L244 for both WT and the Ser L244 → Gly mutant, or some other position on the backbone. However, the frequency 1734 cm^{-1}, compared to the infrared data of Ballschmiter and Katz (1972), is most likely too high to signal a H-bond. Rather, a shift of 1741 − 1734 = 7 cm^{-1} for C_{10} carbomethoxy ester carbonyls of chlorophylls can easily be accounted for by a difference in solvent permittivity (Bekarek *et al.*, 1979). Taken as a whole, this body of evidence strongly argues for the case where the serine L244 side-chain in WT *Rb. sphaeroides* does not form a H-bond with P and argues that both C_{10} carbomethoxy ester carbonyl groups of P are free and are probably in different dielectric environments.

FT Resonance Raman Spectra of the Oxidized Primary Donor, $P^{\bullet+}$. As previously discussed, structurally informative new bands appearing in the FTRR spectrum in oxidized WT RCs of R26 that are associated with P^+ formation are the 1600-, 1641-, and 1717-cm^{-1} bands (Mattioli *et al.*, 1991b). They arise from the downshift of the 1607-cm^{-1} C_aC_m band, and the upshift of the 1620- and 1691-cm^{-1} bands of the C_2 acetyl and C_9 keto carbonyls of P, respectively. The trends of these shifts are totally consistent with the one-electron oxidation of a molecule which possesses an A_{1u}-type HOMO orbital (Oertling *et al.*, 1987). The magnitude of the upshift of the C_9 keto carbonyl frequency of P_L upon the oxidation

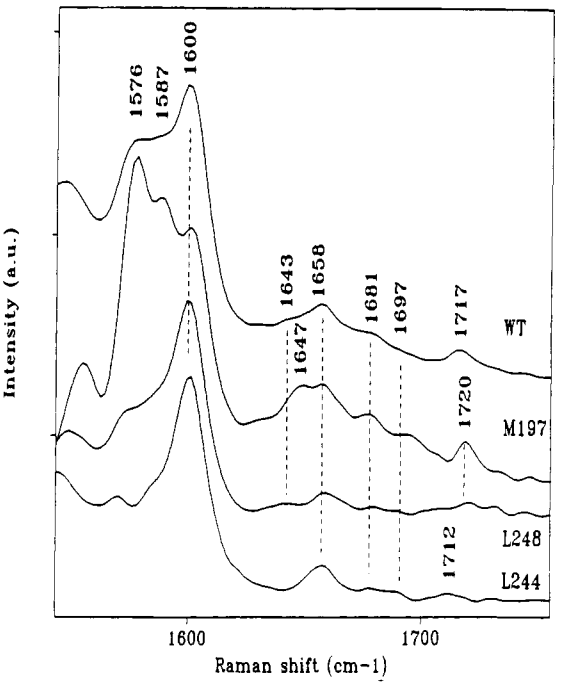


FIGURE 7: Fourier transform resonance Raman spectra of ferricyanide-oxidized reaction centers of *Rb. sphaeroides* wild type (WT) and mutants under the same conditions as in Figure 6.

of P (i.e. +26 cm^{-1} compared to the +32-cm^{-1} shift observed for BChl *a* in a nonprotic solvent by Cotton *et al.* (1980), Mäntele *et al.* (1988), and Heald and Cotton, (1990)) indicates that the + charge of the oxidized P is predominantly localized on one BChl component of the dimer; the 1641-cm^{-1} band, which arises from the upshift of the H-bonded acetyl carbonyl of P_L (vibrating at 1620 cm^{-1} in reduced P) identifies P_L as the component on which the + charge is localized. On the basis of the +26-cm^{-1} upshift of the P_L keto carbonyl upon P oxidation, we have estimated the + charge localization to be 80% favoring P_L, assuming the upshift is linear with localization and that a shift of +32 cm^{-1} would represent 100% localization one of the two BChl molecules in P (Mattioli *et al.*, 1991b, 1993).

For the Phe M197 → Tyr RC mutant, the FTRR spectrum of the RCs in the P^+ state is very similar to that of the wild-type (Figure 7). The 1647- and 1720-cm^{-1} bands are both slightly upshifted as compared to the 1643- and 1717-cm^{-1} bands observed in the WT spectrum. These bands indicate that the + charge is still predominantly localized on P_L. The small 3–4-cm^{-1} upshift of these bands with respect to the corresponding wild type P^+ bands may be indicating that the + charge is slightly more localized on P_L for this mutant. It is interesting to note that, relative to the 1658-cm^{-1} band, which is not attributable to P, the 1647- and 1720-cm^{-1} bands of P^+ are more intense. Similarly, the 1576- and 1587-cm^{-1} bands are also more intense with respect to the 1600-cm^{-1} band yet do not shift in vibrational frequency. These observations indicate that the resonance condition of the P^+ species with 1064-nm excitation has changed in the Phe M197 → Tyr mutant. This is not surprising in light of the shift of the ca. 1250-nm band presented in the near-infrared absorption of P^+ for this mutant as compared to the wild type (Figure 4).

Relative intensity changes in the FT Raman spectra of $P^{\bullet+}$ are also seen in the Met L248 → Thr and Ser L244 → Gly mutants which is also indicative of changes in resonance condition for these mutants. For these mutants, the 1643- and 1717-cm^{-1} marker bands, as well as bands around 1576-

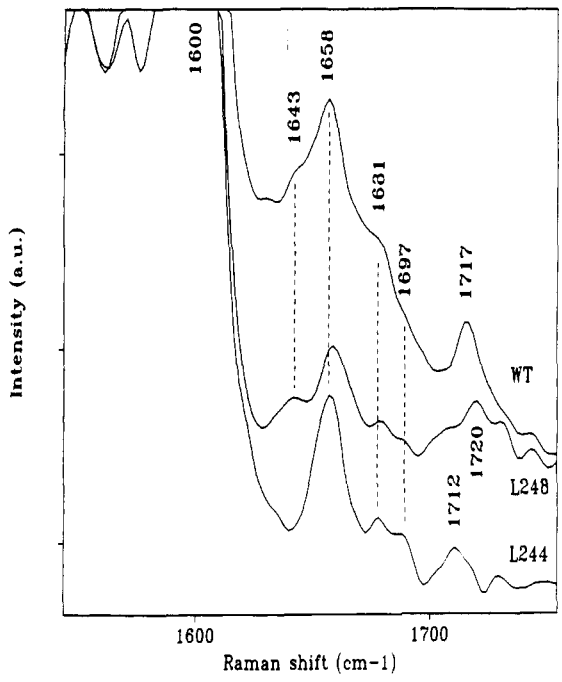


FIGURE 8: Enlargement of Figure 7 showing the weak Raman features of the P^+ Fourier transform resonance Raman spectra of the L248 and L244 mutants compared to that of the wild type.

and 1587-cm^{-1} bands have all lost intensity, with respect to the 1600-cm^{-1} band. This indicates a drastic decrease in the resonance Raman contributions of P_L^+. Figure 8 displays the P^+ spectra of the Met L248 → Thr and Ser L244 → Gly mutant on an enlarged scale compared to that of wild type. One can observe weak, broad bands centered at 1720 and 1712 cm^{-1} for the Met L248 → Thr and Ser L244 → Gly mutants, respectively, which appear above the noise. We may estimate the C_9 keto carbonyl to upshift from 1695 to 1720 cm^{-1}; this would indicate the charge localization to be ca. 78% on P_L for the Met L248 → Thr mutant, similar to that of the wild type (80% on R_L). Similarly for the Ser L244 → Gly mutant, the upshift of the P_L keto carbonyl is estimated to upshift from 1691 to 1712 cm^{-1}, indicating a 66% charge localization on P_L which is significantly lower than in wild type.

DISCUSSION

The near-infrared Fourier transform Raman spectrum of the reduced RCs from the Phe M197 → Tyr mutant of *Rb. sphaeroides* clearly indicates the formation of a new H-bond between the C_2 acetyl carbonyl of P_M and the genetically-introduced tyrosine residue; there is no evidence of any change in the H-bonding pattern of the primary donor as seen in the FTRR spectra of the L248 and L244 mutants. The H-bonded C_2 acetyl carbonyl in the M197 mutant is seen to vibrate at 1636 cm^{-1}. Interestingly, this new H-bond, as gauged by the downshift relative to a free C_2 acetyl carbonyl (ca. 1655 cm^{-1}), appears not as strong as the one observed for the P_L acetyl carbonyl at 1622 cm^{-1} (ca. 4.7 kcal/mol or 19 kJ/mol as compared to ca. 2.6 kcal/mol or 11 kJ/mol, assuming the Badger-type empirical relations as given by Zadorozhnyi and Ishchenko (1965)), which is H-bonded with His L168 (El-Kabbani *et al.*, 1991). Histidine L168 is conserved in *Rps. viridis*, *Rb. sphaeroides*, *Rb. capsulatus*, and *Rhodospirillum rubrum* (Komiya *et al.*, 1988). This genetically-induced H-bond may permit us to correlate and analyse other observed physical properties of the primary donor in terms of the influence of nonconserved H-bonds on the primary electron donor in different species. For instance, *Chloroflexus aurantiacus*, which exhibits different electron transfer kinetics than purple bacteria (Becker *et al.*, 1986; Shuvalov *et al.*, 1986), does not have a histidine residue at the equivalent L168 position in *Rb. sphaeroides* (Shiozawa *et al.*, 1989; Ovchinnikov *et al.*, 1988).

The FTRR data on the Ser L244 → Gly mutant provide no evidence for the breaking of an existing H-bond to the C_{10} ester carbonyl of P_L in WT *Rb. sphaeroides*, as proposed by El-Kabbani *et al.* (1991). This observation would then be consistent with the recent X-ray crystal data of *Rb. sphaeroides*, which proposes that the closest approach of the OH group of the Ser L244 to the C_{10} ester carbonyl of P_L is 4.1 Å (H. Michel, personal communication) which is out of the H-bonding distance. As well, this same serine residue is not within the H-bonding distance of the P_L ester carbonyl in the crystal structure of *Rb. sphaeroides* strain 2.4.1 Y (F. Reiss-Husson, personal communication).

The X-ray structure of the *Rps. viridis* RC indicates that a H-bond exists between Thr L248 and the C_9 keto carbonyl of P_L (Deisenhofer & Michel, 1989). For the *Rb. sphaeroides* Met L248 → Thr mutant reported here, there is no evidence for a similar H-bond. Thus compared to the same residue in the RC from *Rps. viridis*, the Thre L248 residue in the *Rb. sphaeroides* mutant is not in optimum geometric position to make a H-bond with P_L. El-Kabbani *et al.* (1991) have compared the distances of the Met L248 residue to P in *Rb. sphaeroides* and Thr L248 (which is H-bonded to the C_9 keto carbonyl of P_L) to P in *Rps. viridis* (Deisenhofer and Michel, 1989); these two crystal structures indicate that Thr L248 is closer to P in *Rps. viridis* (2.6 Å) than Met L248 in *Rb. sphaeroides* (3.1 Å), even though the methionine sidechain is longer.

FT infrared absorption P^+/P difference spectra have been recently reported for chromatophores of the above-mentioned mutants (Nabedryk *et al.*, 1992). Although protein contributions in the 1620–1665-cm^{-1} IR region masked those of the C_2 acetyl carbonyls of P and precluded any conclusion regarding a new H-bond for the Phe M197 → Tyr mutant, Nabedryk *et al.* (1992) found no evidence for the formation of a new H-bond to a C_9 keto carbonyl of P for the Met L248 → Thr mutant, in agreement with the FT Raman results presented here. However, they did not exclude the possibility that a weak H-bond had been broken for the Ser L244 → Gly mutant; we find no evidence for such a change in the FT Raman spectra reported here.

The 77 K absorption spectra of the reduced, purified mutant RCs indicate that only the Phe M197 → Tyr mutation significantly alters the absorption characteristics of the primary donor P. This is seen by a broadening of the P absorption band at 885 nm and an apparent reduction in its maximum intensity. There are no indications of similar behavior from the Met L248 → Thr or Ser L244 → Gly mutants. Photobleaching experiments performed on the chromatophores or intact whole cells of these three mutants also demonstrate that only the Phe M197 → Tyr mutation results in a change in the P absorption band; this band is seen to broaden and to red-shift by ca. 10 nm while it essentially is unchanged for the other mutants. A similar trend is observed in the absorption spectra of P^+ of the mutant RCs. The P^+ absorption band for the Phe M197 → Tyr mutant is seen to red-shift by 12 nm (or 75 cm^{-1}) compared to WT while for the Met L248 → Thr there is no observable shift. For the Ser L244 → Gly mutant there is an apparent 5-nm upshift.

The significant red-shifts of the P and P^+ absorption bands for the Phe M197 → Tyr mutant seem to correlate with the formation of the new H-bond on a C_2 acetyl carbonyl of P. Based on INDO calculations, Hanson and co-workers have predicted a red-shift of the Q_y absorption band of BChl species forming H-bonds at the C_2 acetyl carbonyl; the opposite trend was predicted with H-bonding to the C_9 keto carbonyl (Hanson *et al.*, 1987). The predicted trend is seen in the P and P^+ absorption spectra of the M197 mutant. The lack of shift observed in the P and P^+ absorption spectra of the Met L248 → Thr mutant would then be consistent with the interpretation of the FTRR data presented here that a new H-bond is not formed in this mutant.

BChl *a* in solution exhibits a Q_y absorption band at ca. 770 nm and the primary donor in RCs of *Rb. sphaeroides* shows a maximum at 865 nm; this represents a difference of ca. 1420 cm^{-1}. The primary donor from RCs of *Rps. viridis* has an absorption band centered at 970 nm while BChl *b* in solution absorbs at 790 nm making this shift ca. 2240 cm^{-1}, significantly greater than the *Rb. sphaeroides* and other primary donors comprised of BChl *a*. The formation of the new H-bond on P in the M197 mutant results in the situation that both acetyl carbonyls of P are H-bonded as they are in the *Rps. viridis* RC and results in the red-shift of the P absorption band. Yet, compared to the data for *Rps. viridis*, the shift of the P absorption band compared to that of the respective BChl molecule *in vitro* is less for the *Rb. sphaeroides* case. This indicates that other factors are at play for the shift in P absorption band; possibly it is (i) an additional H-bond on the C_9 keto carbonyl of P_L in the primary donor of *Rps. viridis*, (ii) the distance between the two BChl components in the dimer (Thompson *et al.*, 1991) which plays a role in the different shifts involved or the oscillator strength of BChl *b*, (iii) the different conjugation pathway in BChl *b*, or (iv) all of the above. As well, the primary donor of *Rps. viridis* is estimated to possess more charge transfer character (in the triplet state) than that of *Rb. sphaeroides* (Norris *et al.*, 1989) which must be considered in theories explaining the red-shift of P as arising from a mixing of charge-transfer transitions and exciton interactions (Parson & Warshel, 1987; Friesner & Won, 1989).

An interesting observation in this work has been that the formation of a new H-bond on the acetyl carbonyl of P_M did not result in sizable changes in the triplet zero-field splitting parameters of the primary donor; thus the degree of localization and charge transfer character of ^3P has not significantly changed. For ^3P in *Rps. viridis*, the triplet excitation is localized on the P_L molecule whereas in *Rb. sphaeroides* the triplet excitation seems to be more distributed on P_L and P_M (Norris *et al.*, 1989). As well, the estimated charge transfer characters of the triplet states are 23% and 13% for *Rps. viridis* and *Rb. sphaeroides* R26, respectively (Norris *et al.*, 1989). With respect to the triplet parameters discussed here, none of the mutants measured showed indications of more "viridis-like" triplet characteristics. Comparing the *D* and *E* values of the chromatophores with those of the RC preparations, the RC triplet parameters are ca. 3% lower for the chromatophores, suggesting that RC solubilization and purification have somewhat altered the π–π interactions in P. The variation of the *D* and *E* values among the mutant RCs is on the order of 1% and, therefore, interpretation of the significance of this small change is not clear. The largest *E* value and the lowest *D* value in Table III are exhibited by the M197 mutant and may be interpreted as a very slight increase in triplet localization or charge-transfer character; this would be a similar trend in agreement with the interpretation of the FTRR spectrum of $P^{\bullet+}$ of this same mutant where the + charge seems to be more localized on P_L. In any event, it is clear from the EPR data that none of the mutations studied significantly alters the triplet parameters of P. The effects of H-bonding upon the triplet properties of P have received little attention, but the results presented in this work would indicate that H-bonding to the C_2 acetyl carbonyl of P_M results in very minor changes in the triplet zero-field splitting parameters.

We have previously reported the FTRR spectrum of $P^{\bullet+}$ from *Rb. sphaeroides* R26 (Mattioli *et al.*, 1991b) excited at 1064 nm. Under these resonance conditions with the ca. 1250-nm absorption band of $P^{\bullet+}$, only the contributions of P_L^+ are clearly observable in the Raman spectrum. The observed upshift of the C_9 keto carbonyl stretching mode upon oxidation of P should reflect the degree of + charge localization in $P^{\bullet+}$ which was estimated to be ca. 80% on P_L for *Rb. sphaeroides* R26 (Mattioli *et al.*, 1991b); the same conclusion is obtained for the wild type RCs in this work. The $P^{\bullet+}$ FTRR spectrum of the Phe M197 → Tyr mutant indicated that the stretching modes of both the C_2 acetyl and C_9 keto carbonyls of $P^{\bullet+}$ were upshifted by 4 and 3 cm^{-1}, respectively, in frequency compared to the same bands in the WT spectrum. The fact that the C_2 acetyl and the C_9 keto carbonyls *both* upshift to similar degrees is a good indication that conformational changes in or around P are most likely not causing these shifts. Indeed, if drastic conformational or H-bonding changes were occuring during/after the oxidation of P then one might expect to observe different degrees of shifting of the carbonyl frequencies, even in opposite directions; this is not observed. The 3-cm^{-1} upshift of the C_9 keto carbonyl of the Phe M197 → Tyr mutant could be interpreted as an increase in the + charge localization of this mutant up to ca. 90% on P_L compared to 80% for WT. This increase in localization seems to correlate with the observed 12-nm red-shift of the NIR $P^{\bullet+}$ absorption band at ca. 1250 nm. For the L248 mutant no change in either the $P^{\bullet+}$ absorption band or + charge localization was observed. Interestingly, the L244 mutant showed a 5-nm upshift of the $P^{\bullet+}$ absorption band and a decrease in + charge localization, estimated to be 66% on P_L.

Femtosecond optical measurements of the stimulated emission from the first excited electronic state of P, P*, measured at 920 nm (Holzapfel *et al.*, 1990), give the following lifetimes for the initial electron transfer step from P to $BPhe_L$: WT, 3.5 ps; M197, 6.0 ps; L248, 3.9 ps; and L244, 6.5 ps (Finkele, U., Lauterwasser, C. and Zinth, W., unpublished data). In general, it is seen that the observed lifetime of the P* state is not drastically altered, the maximum increase being less than a factor of 2. This is to be compared to the change in the primary electron transfer rate constant observed in *Rb. sphaeroides* RC mutants where tyrosine M210 was genetically altered to phenylalanine and leucine (Finkele *et al.*, 1990; Nagarajan *et al.*, 1990) or threonine in *Rb. capsulatus* (Chan *et al.*, 1991). For these mutants, the rate was decreased by a factor significantly greater than 2. Tyrosine M210 is further away from P than are the amino acid residues discussed in this work, and it does not form a H-bond with P (Mattioli *et al.*, 1991a).

At present, the available femtosecond electron transfer kinetic data of the mutants reported in this work seem to correlate only partially with other observables. For instance, the M197 mutant, where a new H-bond has been formed on the C_2 acetyl carbonyl of P_M, shows less than a ca. 2-fold decrease in the electron transfer kinetics to $BPhe_L$; for the

L248 mutant, where no new H-bond is seen to form, the kinetics remain similar to those in WT. For the L244 mutant there is no FT Raman data which supports the breaking of a H-bond on a C_{10} ester carbonyl of P, yet the electron-transfer kinetics also appear to become slower by a factor of 2; this could be due to a slight modification of the conformational structure of the P environment due to secondary effects of the mutation. With this possibility in mind, it is not clear whether the changes in electron transfer rate in the M197 mutant is genuinely due to the new H-bond introduced at the C_2 acetyl carbonyl or to other secondary effects. For example, for the Tyr M210 → Phe, Leu mutants of *Rb. sphaeroides*, where no changes have occurred in the H-bonding interactions of the conjugated carbonyls of P (Mattioli *et al.*, 1991a), the initial electron transfer rates were observed to be 16 and 22 ps, respectively (Finkele *et al.*, 1990). Interestingly, when comparing the femtosecond data with the + charge localization on P_L, there appears to be a weak relationship. The Met L248 → Thr mutant displays the same P* kinetics as WT as well as P_L charge localization. For the M197 and L244 mutants, the change in + charge localization seems to correlate with longer P* decay times. At present these effects are not completely understood, yet it is tempting to suggest that subtle changes in the + charge localization in P^+ affects the P* decay kinetics and primary electron transfer. Clearly more data and further study are required to examine this question thoroughly.

As well, there seems to be a correlation between the decrease in initial electron transfer rate and an increase in P/P^+ redox midpoint potential (see Table III). A similar correlation has been observed in *Rb. sphaeroides* RCs bearing point mutations near P where initial electron-transfer rates decreases with increasing P/P^+ redox midpoint potential (Williams *et al.*, 1992). These observations were rationalized as resulting from a decrease in driving force of the electron transfer since an increase in the P oxidation potential should result in a decrease in energy between P* and the initial charge-separated state. However, such a correlation between the free energy difference and the initial transfer rate in the RC may represent an oversimplified view as it does not consider the possible role of the accessory BChl molecule.

RHF-INDO/SP molecular orbital calculations by Plato *et al.* (1988b, 1991) on the *Rps. viridis* primary donor structure suggest that the major contribution to the spin density asymmetry favoring P_L in $P^{\bullet+}$ stems from the strong dipole moment of the protein backbone atoms. Another major contribution to the spin density asymmetry in *Rps. viridis* is predicted to originate from Tyr M195 (Phe M197 in *Rb. sphaeroides*) (Plato *et al.* (1988b, 1991) which is H-bonded to the C_2 acetyl carbonyl of P_M (Deisenhofer & Michel, 1989). In the Phe M197 → Tyr mutant studies in this work, the tyrosine residue is now H-bonded to the C_2 acetyl carbonyl of P_M just like in *Rps. viridis*, and the FTRR data on $P^{\bullet+}$ are consistent with an increase in the localization of the + charge on P_L but this increase is minor and does not seem to be a major factor as predicted. Parson *et al.* (1990) have reported electrostatic interaction energy calculations on *Rps. viridis* and concluded that Tyr M195 does not play a dominant role in the localization of the + charge on one BChl molecule in P (they propose that the + charge should reside on P_M). Thus with these two different weightings on the importance of the Tyr M195 in affecting the + charge localization on one BChl molecule in P from *Rps. viridis*, the FTRR data presented in this work indicate that, at least for the mutation in *Rb. sphaeroides*, there is an effect of increasing the localization on P_L.

The lack of observation of sizable intensity of the ca. 1717- and ca. 1743-cm^{-1} bands in the P^+ FT Raman spectra of the Met L248 → Thr and Ser L244 → Gly mutants is interesting. It signals a drastic decrease in the resonance Raman scattering of the P_L^+ species in these two mutants at 1064 nm excitation, indicating that the electronic structure of P^+ has been significantly altered. The exact nature of the complex region between 900 and 1300 nm in oxidized RC absorption spectra is not completely understood (Olson *et al.*, 1985) but probably involves many contributions from one-electron promotions, from the lower filled dimer molecular orbitals, to the half-filled "a_{1u}"-like dimer HOMO as is predicted for monomeric Chl $a^{\bullet+}$ and BChl $a^{\bullet+}$ (Hanson, 1991); these many one-electron promotions result in the observation of low-intensity, continuous absorption in (B)Chl $a^{\bullet+}$ species in the NIR region. As we have discussed before (Mattioli *et al.*, 1991b), excitation of the resonance Raman spectrum of $P^{+\bullet}$ from wildtype *Rb. sphaeroides* R26 using 1064 nm excitation (which is ca. 1400 cm^{-1} above in energy from the 1248 nm absorption maximum of $P^{\bullet+}$ results in the enhancement of P_L^+ modes over those of $P_M^{(+)}$. It must be noted that this observation of P_L^+ is not necessarily due to the predominant localization of the + charge on P_L but is due to the nature and composition of the electronic absorption at 1064 nm and the resulting resonance condition; the degree of localization is obtained by an analysis of band shifts in this observed P_L^+ spectrum compared to P_L^0. The small degree of P_L^+ resonance Raman contributions in the two mutant RCs excited at 1064 nm could be signalling a significant change in the nature and/or composition of the electronic absorption at 1064 nm. This could manifest itself either by (i) a loss of *y*-polarization character in the region of 1064 nm, which would then not result in significant resonance enhancement of the C_2 and C_9 carbonyls (Mattioli *et al.*, 1990), or (ii) a loss of P_L^+ character in the 1064-nm region and thus a decrease of the resonant scattering enhancement of this species; this in turn could be reflecting a change in the + charge localization, one which is less localized on P_L. Since the nature of the near-IR absorption region at ca. 1250 nm is not fully understood at present, further work is required to prove or disprove these hypotheses, perhaps via a Raman excitation profile of $P^{\bullet+}$.

A stimulating observation in this work has been that the redox midpoint potentials of these three mutants are all quite similar to that of the WT. This is particularly interesting for the M197 mutant where a new H-bond donated by a tyrosine residue is formed on a conjugated acetyl carbonyl of the primary donor and results in only a 20–30-mV increase in oxidation potential. Apparently, the formation of a H-bond by the genetically-introduced tyrosine residue to the C_2 acetyl carbonyl of P_M in *Rb. sphaeroides* does not sizably affect the redox properties of P. This is in striking contrast to the recent work of Williams *et al.* (1992) who have reported mutants of *Rb. sphaeroides* where leucine residues at positions L131 and M160 have been replaced by histidine residues in order to introduce H-bond donors near the C_9 keto carbonyls of P_L and P_M, respectively. As well, a Phe M197 → His mutation of *Rb. capsulatus*, similar to the one described in this work, has been reported (Stocker *et al.*, 1992). For these mutants, an increase in the redox midpoint potential of P, with respect to that of wild type has been associated with the introduction of histidine residues which likely result in the formation of H-bonds. As well, Murchison *et al.* (1993) have observed a 80-mV decrease in redox potential of P when His L168, which is H-bonded to the C_2 acetyl carbonyl of P_L, was replaced by a phenylalanine residue not capable of H-bonding. These

same authors have also shown that the replacement of Phe L167 to a leucine residue increases the redox potential of P by 25 mV, a change similar to what is seen in the Phe M197 → Tyr mutant reported here. These observations clearly indicate that the presence of histidine residues near P, and which most likely H-bond with the conjugated carbonyls of P, sizably affect the redox potential of P. Comparing these results with ours, it would seem that a change in H-bond interaction is not necessarily sufficient to alter the redox potential of P. In view of the different chemical natures of a tyrosine and a histidine residue, further factors which must be considered in order to fully understand the relation between H-bonding and redox properties, are the strength of the H-bond, the chemical nature of the H-bond donor, and the presence of nearby, possibly charged amino acid residues which may or may not be a potential H-bond donor. We are currently exploring these aspects.

CONCLUSIONS

The Phe M197 → Tyr mutation in *Rb. sphaeroides* results in the formation of a new H-bond between the tyrosine residue and the C_2 acetyl carbonyl of P_M in the primary donor. This mutation results in only minor changes in various physical properties of the primary donor. The initial electron transfer kinetics to the acceptor $BPhe_L$ molecule is slower by less than a factor of 2, yet the redox midpoint potential (to within ± 30 mV) of $P \rightarrow P^+$ is not sizably changed as are not the zero-field splitting parameters of ^3P. As well, the localization of the + charge in P^+ on P_L is altered to a very small degree suggesting that localization is slightly increased in the P^+ state (from ca. 80% to 90%).

The results of this investigation further demonstrate that the point mutation of a single residue in the bacterial reaction center does not drastically affect RC function. Despite the fact that a significant modification in the structure of the primary donor (i.e. the formation of a new H-bond on a conjugated carbonyl of P) has occurred, there is relatively little change in electron-transfer kinetics nor is there any dramatic change in the localization of the + charge, or the redox potential. It would seem that it is the collective effect of many amino acid residues which is influencing not only the photochemistry and directionality of the electron transfer in the bacterial RC, but also the physicochemical properties of the primary donor itself.

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