Resonance Raman Characterization of *Rhodobacter sphaeroides* Reaction Centers Bearing Site-Directed Mutations at Tyrosine M210†

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ABSTRACT: Resonance Raman (RR) spectroscopy and low-temperature absorption spectroscopy have been used to investigate the structural changes in the reaction centers (RCs) of *Rhodobacter sphaeroides* induced by site-specific mutations on the tyrosine (Y) M210 residue. RCs in which Y M210 has been genetically replaced with phenylalanine (F) or leucine (L) exhibit a 5-fold decrease in their primary electron-transfer kinetics (Finkele et al., 1990). The general similarity of RR spectra of the wild-type RCs as compared to those of the two mutant RCs indicates that no significant global structural changes occur upon mutation at the level of any of the six bacteriochlorin pigments. In the RR spectra of the two mutant RCs there is a conspicuous absence of contributions from the $BPhe_M$ prosthetic group, which is interpreted in terms of a change in the resonance enhancement conditions of this chromophore. Low-temperature absorption spectroscopy reveals marked shifts in the Q_x absorption band of $BPhe_M$. This shift is interpreted as arising from a destabilization of the protein in the vicinity of $BPhe_M$ and accounts for the change in resonance condition for this chromophore in its RR contributions. As well, there is a 3-nm red shift of the Q_y absorption band of the BChls from 803 to 806 nm for the mutant RCs. Difference RR spectra yielding structural information concerning, selectively, the primary donor (P) indicate that the structure of the P binding pocket is conserved for these mutant RCs. The tyrosine M210 is not observed to be engaged in a hydrogen bond with either of the acetyl or keto carbonyls of P.

The primary events in bacterial photosynthesis in which light energy is converted to chemical potential energy occur in the membrane-bound protein complex known as the reaction center (RC).[1] High-resolution X-ray crystallographic structures of the RC of *Rhodopseudomonas* (*Rps.*) *viridis* (Deisenhofer et al., 1984, 1985; Deisenhofer & Michel, 1989; Michel et al., 1986; Michel & Deisenhofer, 1985) have elucidated the arrangement of the chromophores within the protein. Furthermore, the X-ray structures of *Rps. viridis* (Deisenhofer et al., 1984; Deisenhofer & Michel, 1989) and that of *Rhodobacter* (*Rb.*) *sphaeroides* (Allen et al., 1987a,b; Chang et al., 1986; Tiede et al., 1988) have identified specific amino acid residues that could likely interact with the chromophores. These chromophores are positioned in two symmetrically arranged protein subunits called L and M. Along with the H subunit, these three polypeptides form the core of the RC structure. The isolated RC of *Rps. viridis* possesses a fourth, tetraheme cytochrome subunit. The L and M subunits constitute a dimeric complex in which the prosthetic groups [i.e., four bacteriochlorophylls (BChl), two bacteriopheophytins (BPhe), two quinones (Q), and a non-heme ferrous atom (Fe)] are arranged in pairs related by a pseudo-C_2-symmetry axis. This axis runs from the special pair of BChl molecules (P_L and P_M, where P designates the primary donor) to the Fe atom. Despite the apparent symmetric arrangement of the chromophores in the RC, electron transfer seems to follow only one of the two possible pathways, namely the L branch. Once P is excited by a photon or singlet exciton, primary charge separation occurs in about 3 ps (Fleming et al., 1988; Holzapfel et al., 1989; Kirmaier & Holten, 1988). Wasteful back-reactions leading to charge recombination are several orders of magnitude slower than the forward reactions, thus ensuring high quantum efficiency of charge separation [for reviews see Kirmaier and Holten (1987) and Parson (1987)].

One of the primary goals of structure–function studies of the RC is attempting to explain the physicochemical basis for such rapid, efficient, and highly directional electron transfer. Studies concerning the function of the RC have concentrated on spectroscopic properties and electronic structure of the chromophores, which have been shown to be dependent on the interactions between the chromophores and the protein and among the chromophores themseleves (Kirmaier & Holten, 1987).

Site-directed mutagenesis permits the introduction of specific alterations in the protein sequence, thereby opening new avenues in structure–function studies. In principle, one may test hypotheses concerning the role of the protein residues in binding the prosthetic groups and in modifying the spectroscopic and possibly the electron-transfer properties of the RC (Coleman & Youvan, 1990).

In this contribution we investigate some spectroscopic properties of the RC from *Rb. sphaeroides* Ga strain bearing mutations at the tyrosine M210 residue (Gray et al., 1990). The rationale for site-directed mutation of this particular residue is multifold. First, tyrosine M210 and phenylalanine L181 of *Rb. sphaeroides* form a pair of symmetry-breaking

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[1] Abbreviations: RR, resonance Raman; *Rb.*, *Rhodobacter*, *Rps.*, *Rhodopseudomonas*; *Rsp.*, *Rhodospirillum*; RC, reaction center; P, primary donor; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; Y, tyrosine (M210); F, phenylalanine; L, leucine; WT, wild type.

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residues that are related by the C_2-symmetry axis previously mentioned; this pair is conserved for *Rps. viridis*, *Rhodobacter capsulatus*, and *Rhodospirillum* (*Rsp.*) *rubrum* (Komiya et al., 1988, and references therein). Both residues are aromatic, but only tyrosine is polar and is capable of forming a hydrogen bond. In the *Rb. sphaeroides* RC, tyrosine M210 is in van der Waals contact with P_L, P_M, $BChl_L$, and $BPhe_L$, while phenylalanine L181 is in van der Waals contact with P_L, P_M, $BChl_M$, and $BPhe_M$ (Tiede et al., 1988). The conservation of this pair of residues in at least four species of the bacterial RC as well as their location between P and BPhe molecules suggests that they may play a role in the electron-transfer process. Furthermore, the possible role of tyrosine M210 in hydrogen bonding to one of the acetyl carbonyls of P has remained controversial (Allen et al., 1987a; Robert & Lutz 1988).

Recent room temperature femtosecond kinetic studies (Finkele et al., 1990) reveal that the electron transfer from P to $BPhe_L$ is significantly slower for the RC mutant *Rb. sphaeroides* bearing a phenylalanine (Y → F) or leucine (Y → L) at the M210 position as compared to the kinetics of the wild-type (WT) RC. For a system such as the bacterial RC, which undergoes significant charge redistribution, it can be expected that the course of the reaction will be sensitive to the polarity of the solvent (i.e., the protein residues). It is important in this case to determine if the changes in electron-transfer kinetics are caused by the replacement of the tyrosine residue itself or whether they are caused by "secondary" or indirect effects where the replacement of the tyrosine residue results in a tertiary and/or quaternary structural change in the RC. Clearly, the resolution available from X-ray methods is too low to assess the above considerations.

Resonance Raman (RR) spectroscopy provides detailed information concerning the structure and conformations of several types of chromophores in the RC as well as their interactions with the protein (Lutz, 1984; Lutz & Robert, 1988; Robert, 1990; Robert et al., 1989; Robert & Lutz, 1986, 1988). From conclusions drawn from RR spectroscopy, some key questions concerning the structure of P (Robert & Lutz, 1986) could be answered. As a vibrational spectroscopy, RR is capable of revealing the state of ligation of the BChl chromophores as well as the existence and strengths of other protein interactions suggested by the X-ray crystallographic structure, such as the hydrogen bonding of certain carbonyls with amino acid residues. This type of submolecular information is important in structure–function studies because it will indicate with very high sensitivity if states of ligation or hydrogen bonding have changed upon mutation and if global structural changes (i.e., protein unfolding) have occurred. RR spectral band intensities are also very sensitive to changes observed in the electronic absorption bands of the moiety being studied.

In conjunction with RR spectroscopy, low-temperature absorption spectroscopy will reveal subtle changes not observable at room temperature because spectral bands tend to sharpen at cryogenic temperatures. These changes may indicate if any possible electronic couplings between the chromophores have changed upon mutation and which of these may be related to the observed electron-transfer kinetics. As well, analysis of resonance Raman spectra is incomplete without knowledge of the positions of absorption bands giving rise to resonance condition. The second-derivative analysis of the low-temperature absorption spectra complements the low-temperature linear dichroism (LD) work earlier reported (Gray et al., 1990), which showed no significant changes in the relative orientations of the chromophores in the mutant RC with respect to the wild type.

Experimental Procedures

Materials

The mutants were constructed and their RCs were prepared 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; the optical density was 20, measured at 800 nm.

Since the mutants were grown semiaerobically, it is important to compare the mutant RC to the WT RC isolated from cells grown under the same conditions. Under these conditions of growth, the physicochemical properties of the RC are essentially the same as for those grown under photosynthetic conditions, the only difference being in carotenoid content (Farchaus & Oesterhelt, 1989). This control is essential in analyzing Raman intensity enhancement patterns between WT and mutants.

Methods

Low-Temperature Absorption Spectroscopy. The RC preparations were diluted in glycerol resulting in a 60/40 (v/v) glycerol/water mixture and placed in a 1-mm path-length cell held in an optical cryostat cooled with flowing, cold helium gas. Absorption spectra were recorded with a Cary 17 spectrophotometer and digitally stored by using a Tracor TN 1710 signal averager. Room temperature absorption spectra were recorded before and after freezing by using the same apparatus. In all cases, these two spectra were identical, demonstrating that no damage was observable during a freeze–thaw cycle. The second-derivative spectra were calculated by using the Tracor system data processing unit.

Resonance Raman Spectroscopy. Resonance Raman spectra were excited with 363.8-nm laser radiation from an Ar^+ laser (Coherent Innova 100 Series) and recorded at 15 K. The experimental apparatus has been described elsewhere (Robert & Lutz, 1986). The spectra presented here represent the sum of 20–40 individual spectral scans.

The greatest information content in Raman spectra of bacteriochlorins in the RC is obtained with Soret resonance. All six bacteriochlorin pigments present in the RC have overlapping Soret absorption bands and, therefore, the Raman scattering of all pigments should be resonance-enhanced and contribute to the observed spectrum, although to different extents. Specific vibrational information concerning only the primary donor is selectively obtained according to a RR difference method (Robert & Lutz, 1986, 1988). This difference technique exploits the actinic effect of the 363.8-nm excitation radiation, which can produce a significant steady-state population of $P^+Q_A^-$. The resonance enhancement of the P^+ species scattering is very much weaker than that of the ground-state neutral P at 363.8 nm, so that the difference of RR spectra recorded under different actinic levels yields a spectrum arising from P in its neutral, ground state. The different actinic levels are produced by adjusting the laser irradiance on the sample. The technique and its applications are fully described elsewhere (Robert & Lutz, 1986, 1988).

In "high-irradiance" conditions the estimated laser power penetrating the sample is less than 2 mW. The laser spot was moved after each individual spectral scan. No laser photodamage was observed under these conditions, and the high-irradiance spectra appeared to be completely reversible when the laser irradiance was reduced. Irradiance levels were changed by adjusting the beam focus and the laser power so

that an estimated difference factor of ca. 500 exists between low- and high-irradiance conditions.

The P^+ species may also be produced chemically by treating the RC with potassium ferricyanide [200 μM $K_3Fe(CN)_6$]. The RR difference spectra obtained from treated and untreated RCs were the same as those obtained with low and high laser irradiance.

The 1591-cm^{-1} band in the RR spectra of the RC, which is almost entirely due to the BPhe molecules under these resonance conditions, was used for normalization purposes (Robert & Lutz, 1986, 1988). It is important to note that variations of ±10% in the weighting factors do not affect the positions of maxima observed in the calculated difference spectra. This variation in the weighting factor is well outside the limits of uncertainty.

Results

Low-Temperature Absorption Spectroscopy. The absorption spectra at 14 K for WT and the two mutant (Y → F and Y → L) RCs are shown in Figures 1 and 2. Analysis of these spectra verifies that the relative ratios of the integrated intensities of the major absorption bands remain constant when all three spectra are compared. This demonstrates that all chromophores are present and are in their neutral ground states in the resting, untreated mutant RC.

At 14 K, the broad P absorption band of WT shows a maximum at 889 nm that appears significantly red-shifted for the two mutants. The P band maximum for the Y → F and the Y → L mutant is observed at 894 and 892 nm, respectively. Such variations in the position of the P band have been previously discussed for the site-directed mutant RC, and it was suggested that they were arising from detergent and preparation factors (Breton et al., 1989).

It is seen in Figure 1 that the main Q_y band of the BChl molecules at 803 nm in the WT spectrum shifts to 806 nm for both the Y → F and Y → L mutants. The 815-nm component that appears as a shoulder in the WT spectrum is, accordingly, less well resolved in the mutant RC spectra, but the second-derivative spectra clearly show that the position of this component remains constant. The 757-nm Q_y absorption band corresponding to the two BPhe molecules does not exhibit any significant shift for the two mutant RCs but does show a broadening; this band has a 19-nm half-bandwidth (FWHM) for the WT spectrum as opposed to 23 and 24 nm for the Y → F and Y → L mutant spectra, respectively.

In the BChl Q_x spectral region (Figure 2) the 597-nm band in the WT spectrum shifts to 601 nm for the Y → F mutant and to 603 nm for Y → L mutant. The shoulder in the WT spectrum that appears as a component centered at 606 nm cannot be resolved in the second-derivative spectra of the mutants because of overlap with the main band.

Also shown in Figure 2 are changes in the Q_x bands of the BPhe molecules. At 14 K, the Q_x bands of $BPhe_L$ and $BPhe_M$ in the WT spectrum are spectrally resolved with two distinct bands at 546 and 533 nm, respectively. In the second-derivative spectrum they are present as components at 547 and 533 nm. The second derivatives of the mutant spectra clearly show that the 547-nm component ($BPhe_L$) remains unshifted while the 533-nm component ($BPhe_M$) red-shifts to 536 nm for the Y → L mutant and to 538 nm for the Y → F mutant. These spectral characteristics are consistent with those found in Gray et al. (1990).

Resonance Raman Spectra. The maximum information content regarding coordination states and conjugated carbonyl group interactions of bacteriochlorin molecules is contained in the high-frequency region (1550–1750 cm^{-1}) of their RR

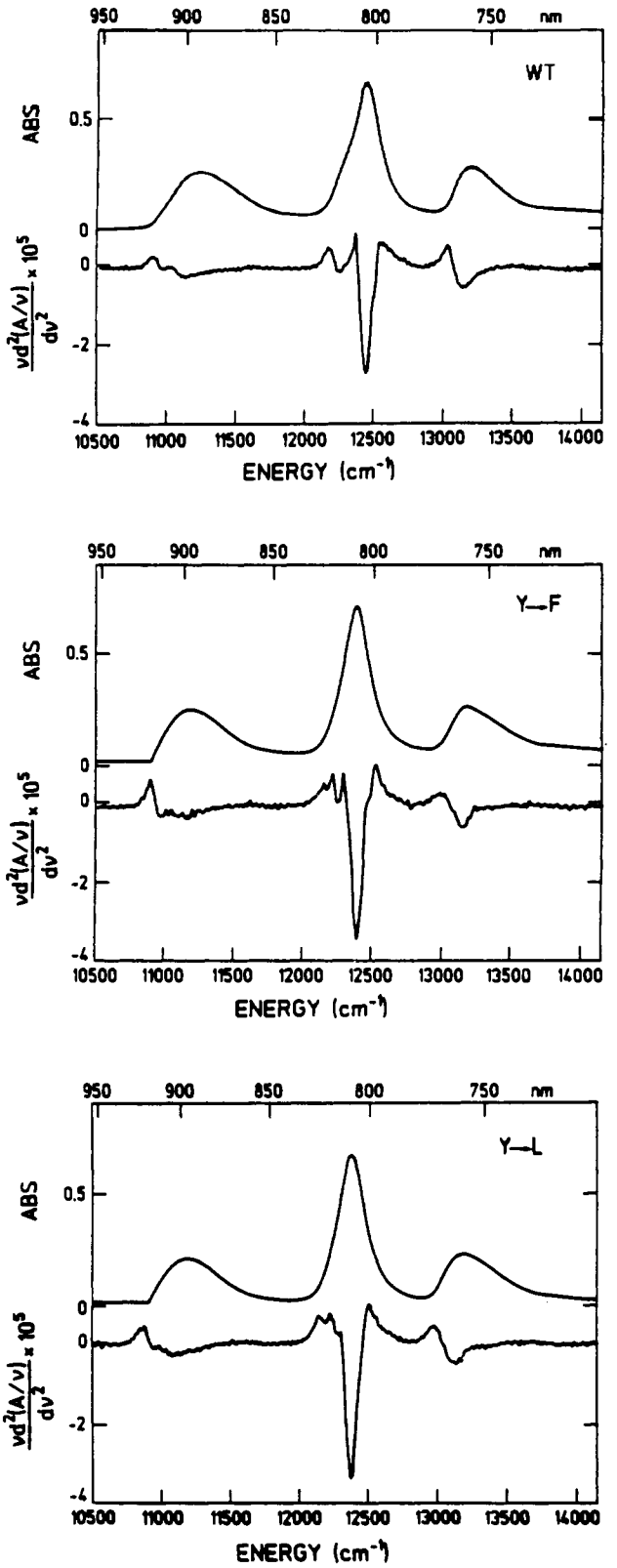

FIGURE 1: Absorption and second-derivative spectra in the Q_y region of the *Rb. sphaeroides* RC in 60% glycerol/water solution at 14 K for wild type (top), Y → F mutant (middle), and Y → L mutant (bottom).

spectra. This spectral region is dominated by a strong ca. 1615-cm^{-1} band that largely arises from a methine bridge stretching mode. The frequency of this band is sensitive to the coordination state of the central Mg atom in BChl, being ca. 1615 cm^{-1} when pentacoordinated and ca. 1600 cm^{-1} when hexacoordinated (Cotton & Van Duyne, 1981; Robert & Lutz, 1985). In the same spectral region, the stretching modes of

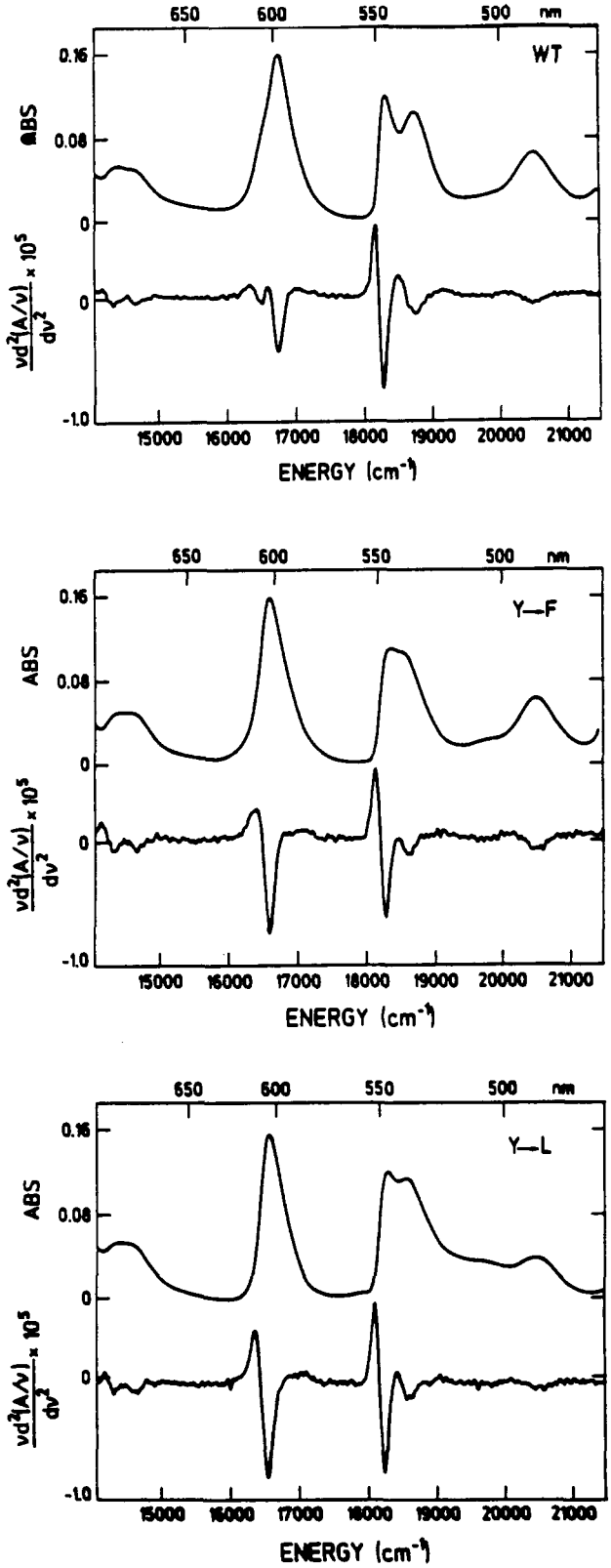

FIGURE 2: Absorption and second-derivative spectra in the Q_x region of the *Rb. sphaeroides* RC in 60% glycerol/water solution at 14 K for wild type (top), Y → F mutant (middle), and Y → L mutant (bottom).

the C_9 keto and C_2 acetyl carbonyl groups of BChl and BPhe are also observed. For BChl *a* these modes appear at ca. 1700 and 1660 cm^{-1}, respectively, when these carbonyls are free from intermolecular interactions (Lutz, 1984 & Robert, 1988). The frequencies of these modes downshift according to the strength of the interaction in which they are engaged. In vitro

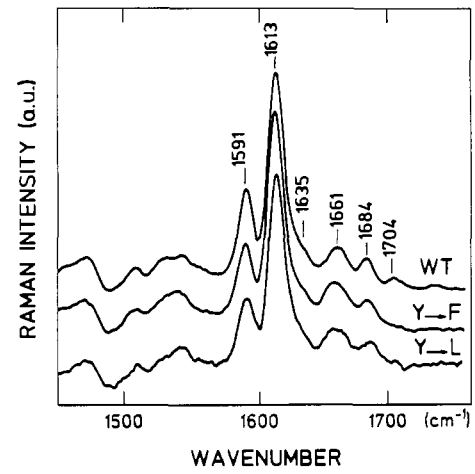

FIGURE 3: Resonance Raman spectra (1450–1750-cm^{-1} region) of the *Rb. sphaeroides* RC at 14 K for wild type (top), Y → F mutant (middle), and Y → L mutant (bottom), with 363.8-nm excitation and 8-cm^{-1} spectral resolution.

studies have shown that, typically, the frequency of the C_2 acetyl carbonyl mode is 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).

Figure 3 shows the RR spectra of the WT and mutant RCs at 15 K with 363.8-nm excitation. In principle, each one of these spectra contains information from all six bacteriochlorin chromophores in the RC, and thus we are probing six different sites of the RC. In general, the three spectra appear quite similar in the 1450–1750-cm^{-1} region, strongly suggesting no major structural changes in the RC as a whole. The similarity of the frequencies of the main carbonyl bands indicates that no hydrogen bonds to the carbonyls are broken nor are any new ones formed upon the mutations. The 1613-cm^{-1} band in the WT spectrum, which is sensitive to the Mg-coordination state, is unchanged for the mutant spectra, indicating that the BChl molecules in the mutants remain coordinated with one axial ligand.

Despite the similarities there are some specific differences that are exhibited by the mutant spectra. The intensity ratio of the 1613-cm^{-1} band to the 1591-cm^{-1} band is 2.44 for the WT, while for the Y → F and the Y → L mutants it is 2.84 and 2.80, respectively (see Discussion). As well, there is a weakening or absence of the 1704-cm^{-1} band that is clearly present in the WT spectrum. For the Y → F mutant this band is completely absent, while for the Y → L mutant it is barely, if at all, present above background. This band has been previously assigned to the free keto carbonyl of $BPhe_M$ (Robert & Lutz, 1988). Still another, subtle difference between the WT and mutant RR spectra is the apparently broader half-bandwidth of the 1660-cm^{-1} band. This band is ca. 2 cm^{-1} broader for both mutants as measured full width at half-maximum. The apparent differences in the relative intensities of the 1660-cm^{-1} and the 1685-cm^{-1} bands are not considered significant and in no way affect our conclusions.

Resonance Raman Difference Spectra of P. Specific information concerning the primary donor (P) may be directly obtained from the low- minus high-irradiance difference RR spectra. These difference spectra are shown in Figure 4 for WT and mutant RCs. In general, all three difference spectra appear quite similar. Again, as in the case of the RR spectra, the half-bandwidth of the 1660-cm^{-1} band is slightly larger for the mutants. There is a possible upshift of the 1634-cm^{-1} band to 1638 cm^{-1} for the Y → L mutant. The relative weakness of the difference spectrum for this mutant as well

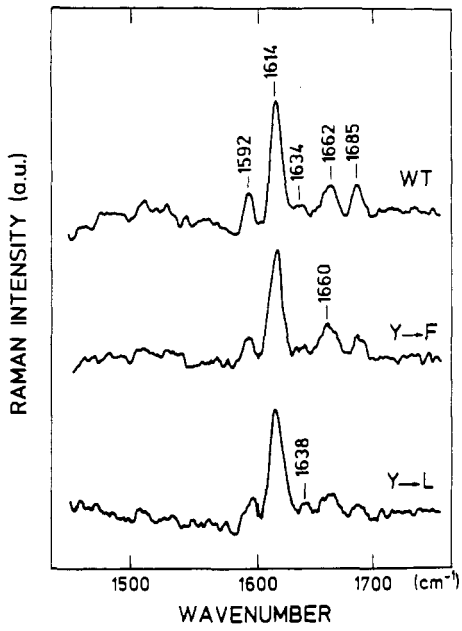

FIGURE 4: Difference resonance Raman spectra (1450–1750-cm^{-1} region) obtained from the low-irradiance minus high-irradiance resonance Raman spectra of the *Rb. sphaeroides* RC at 14 K for wild type (top), Y → F mutant (middle), and Y → L mutant (bottom).

as the weakness of the 1634-cm^{-1} band results in a poorer signal-to-noise ratio as compared to WT and Y → F spectra. Examination of several difference spectra has shown that the position of the 1634-cm^{-1} band may vary from 1634 to 1638 cm^{-1}. Because of the poor signal-to-noise ratio the significance of the shift is uncertain.

The difference RR spectra between untreated RC and those treated with potassium ferricyanide were identical with the low- minus high-irradiance difference spectra. The similarity of the mutant RC difference spectra compared to that of WT strongly suggests that no major structural change of P or its protein-binding environment has occurred upon mutation. Specifically, no hydrogen bonds between the conjugated carbonyls of P and the protein are seen to be broken nor are any new ones formed. As well, the Mg-coordination states of both BChl constituents of P remain pentacoordinated.

Discussion

Absorption Spectra. The variation in the peak maximum of the main Q_y transition of P seems to be due to preparation factors and may not be directly attributable to the mutations. Room temperature, steady-state photobleaching studies (Gray et al., 1990) revealed that the position of this P absorption band was unchanged for the mutant chromatophores as compared to WT, strongly suggesting that the variations of the P band seen in the RC occur after RC isolation and purification. Similar phenomena were observed in the mutant RC of *Rb. capsulatus* (Breton et al., 1989). Indeed, the position of this band is sensitive to both the nature and concentration of detergent used as well as to the presence or absence of Q_A (Debus et al., 1985). The origin of this shift observed in the RC preparations could be due to a slight change in the geometry of P caused by the detergent. Parson and Warshel (1987) have calculated that a 0.1-Å variation in the distance between the BChl molecules or a 15° rotation of the acetyl carbonyl group of either molecule may result in a ca. 20-nm shift of the 960-nm P band of *Rps. viridis*.

Minor perturbations are also seen for $BChl_L$ that are not thought to be due to detergent effects (Gray et al., 1990). The shift of the 803-nm component to 806 nm seen in the second-derivative spectra (Figure 1) has been consistently observed in the (low-temperature) absorption spectra of several independent RC preparations thus appearing insensitive to detergent. The same shift was observed in the room temperature, steady-state photobleaching experiments for the mutant chromatophores (Gray et al., 1990) and thus is not a consequence of the RC purification process. By contrast, variations in the Q_x spectral region are not rigorously reproducible for different preparations.

The shift of the 803-nm absorption band has been interpreted as arising from a change in the Q_y transition of $BChl_L$ (Gray et al., 1990). In general, the spectral region in the vicinity of 800 nm is quite complex. The main 800-nm band involves the Q_y transitions of $BChl_L$ and $BChl_M$. For the RC of *Rb. sphaeroides* some of this band's intensity can be associated with interactions of these BChl chromophores with the upper-state exciton component of P (Knapp et al., 1986; Scherer & Fischer, 1986). The 815-nm component has been assigned to one of the accessory BChl Q_y transitions (Kirmaier et al., 1985) and more specifically to $BChl_M$ (Holten et al., 1987; Breton, 1985, 1988). Recent photochemical trapping experiments (Robert et al., 1985) where either $BPhe_L$ or $BPhe_M$ has been selectively reduced suggest that $BChl_L$ exhibits an absorption maximum at ca. 803 nm while that of $BChl_M$ is comparatively red-shifted at ca. 806 nm. These trapping experiments have been extended into the low-temperature regime where these components were more accurately determined (Zhou et al., 1990); the $BChl_L$ component is found at 802 nm, while the 814-nm component quite exclusively arises from $BChl_M$. The 815-nm component seen in the WT second-derivative spectrum does not shift in the mutant spectra and indicates that there is no similar effect on the $BChl_M$ transition. The perturbation on $BChl_L$ alone could be arising from a direct dipole interaction of the tyrosine M210 residue, which is removed upon mutation, or from a change in van der Waals interaction. Also likely is the possibility that the site-specific mutation of tyrosine M210 with a nonpolar residue induces slight modifications in the conformation of the more polarizable residues nearby in the $BChl_L$ vicinity.

The absorption spectra in Figure 2 show that the $BPhe_M$ Q_x absorption bands are red-shifted for the mutants as compared to the WT RC. Variations in the BPhe Q_x spectral region have been previously observed for mutants of *Rb. capsulatus* (Breton et al., 1989; Bylina et al., 1988), where the glutamic acid residue L104 has been specifically mutated but no significant changes in electron-transfer kinetics were observed. This residue is presumably hydrogen-bonded to the C_9 keto carbonyl of $BPhe_L$, and the shift of its Q_x absorption band from 548 to 534 nm was interpreted as arising mainly from the breaking of this hydrogen bond. Thus, the position of the Q_x absorption band may be quite sensitive to the hydrogen-bonding state of the carbonyls.

It is interesting to note that the $BPhe_M$ molecule is situated near the exterior of the protein subunit (Deisenhofer & Michel, 1989), making it very susceptible to various chemical treatments and/or perturbations [see, for example, Agalidis et al. (1984) and Debus et al. (1985)]. The mutant preparations in this work are known to be intact with the H subunit present (Gray et al., 1990), and thus the changes in the Q_x band of $BPhe_M$ do not result from its absence (Agalidis et al., 1984). It seems more likely that the observed changes arise from the destabilization of the M subunit in a manner similar to that when the H subunit is removed (or denatured) or when the Q_A binding site is damaged, resulting in RCs that are depleted in Q_A (Agalidis et al., 1984). The observation of perturbations

on a chromophore far removed from the specific mutation site may arise from a partial destabilization of the M protein subunit. The mutation of M210 residue, which is situated on the membrane-spanning D helix of the M subunit, also results in a modification of the Q_A binding site, which itself is found on this helix. This modification is observed as a loss of bound quinone at this site in the RC preparations but not in the chromatophores (Gray et al., 1990). These observations suggest that the integrity of the isolated mutant RC is slightly affected.

Resonance Raman Spectra. The overall RR spectra of the mutant RCs are very similar to that of the WT RC, indicating that no significant changes have occurred at the level of H-bonding and Mg coordination for the six bacteriochlorin–protein sites. However, one important difference between the WT RR spectrum and the mutant RR spectra is the absence or dramatic weakening of the 1704-cm^{-1} band of the $BPhe_M$ C_9 keto carbonyl. The observed shift of the $BPhe_M$ Q_x absorption band indicates that there is some perturbation on the electronic energy levels of this molecule as compared to the WT RC. Although it was not possible to record a low-temperature absorption spectrum in the Soret region, it may be presumed that a similar perturbation in the Soret transition(s) of $BPhe_M$ has also occurred, thus changing the resonance condition that enhances the Raman scattering of this molecule.

There is strong evidence to suggest that, indeed, the contributions of the $BPhe_M$ to the RR spectra of the mutant RC are weakened. The intensity ratio of the 1613-cm^{-1} band to the 1591-cm^{-1} band is smaller for the WT than for the two mutants. With 363.8-nm excitation, the 1591-cm^{-1} band arises mainly from the BPhe pigments while the 1613-cm^{-1} band arises from both BChl and BPhe pigments. Thus, a decrease in the intensity of the 1591-cm^{-1} band relative to the 1613-cm^{-1} band is consistent with a loss of BPhe contribution in the RR spectrum; this is indeed observed in the mutant RR spectra. The loss of the 1704-cm^{-1} band and the observed changes in intensity ratios are not due to the absence of $BPhe_M$ because the absorption spectrum and pigment analysis of these RCs (Gray et al., 1990) both show that two bacteriopheophytins are present. From these observations we conclude that the vanishing of the 1704-cm^{-1} band is due to a general decrease in the Raman scattering cross section of $BPhe_M$ at 363.8 nm, most likely due to changes in its electronic energy levels.

In order to investigate the carbonyl-stretching frequencies of $BPhe_M$ alone, RR spectra were excited at 530.9 nm (data not shown), a diagnostic wavelength for this chromophore. Unfortunately, the significant red shift of the Q_x band of $BPhe_M$ resulted in a poor enhancement of its RR spectrum, and the weak keto carbonyl could not be observed above background. Thus, no information concerning the frequency of this band could be obtained. However, the C_2 acetyl carbonyl mode, which appears as a strong shoulder of the 1613-cm^{-1} band, was observed and exhibited no significant change in its state of hydrogen bonding upon mutation.

Resonance Raman Difference Spectra of P. As seen in Figure 4, the difference spectra of WT and the two mutants are quite similar and indicate that for the primary donor the conjugated carbonyl–protein interactions and the coordination state of the central Mg atoms remain largely unchanged after the mutation. The possible shift of the 1634-cm^{-1} band to 1638 cm^{-1} could be reflecting a change in conformation of the H-bond between the C_2 acetyl carbonyl of P_L with histidine L168 (Zhou et al, 1989; Robert, 1990). The fact that this 4-cm^{-1} shift is most apparent for the leucine mutant may indicate that greater protein conformational changes could be present for this mutant since the leucine residue is less rigid and smaller than tyrosine and phenylalanine.

The bands at 1660 cm^{-1} in the difference spectra of the two mutants are slightly broader by ca. 2 cm^{-1} (FWHM) than for the WT difference spectrum. This band has been assigned as consisting of two modes (nearly degenerate in frequency) of different P carbonyls, one of the free acetyl of P_M and one of a bonded keto and P_L (Robert & Lutz, 1986; Zhou et al., 1987). The observed broadening could reflect a partial lifting of the degeneracy of these two frequencies. The estimated shift of one of the components in the 1660-cm^{-1} band is about 5 cm^{-1}, although no clear resolution is observable. This small shift cannot be explained in terms of the breaking of a hydrogen bond or the formation of a new one, which would result in much larger band shifts. Indeed, for the removal of a hydrogen bond from a carbonyl group of BChl, one would expect to observe a 20–50-cm^{-1} upshift of the corresponding band, as predicted from in vitro studies (Lutz, 1984). For example, from the X-ray crystallographic structure of the RC from *Rps. viridis* (Michel et al., 1986), tyrosine M195 (which is replaced by a phenylalanine residue in *Rb. sphaeroides*) is less than 3.4 Å from this acetyl carbonyl and in optimum geometry for a hydrogen bond. From the RR study of the primary donor in *Rps. viridis* (Zhou et al., 1989), the frequency of the C_2 acetyl carbonyl of P_M, which is engaged in a hydrogen bond with tyrosine M195 (Deisenhofer & Michel, 1989), is observed at 1628 cm^{-1}; the frequency of the same carbonyl, which is free from hydrogen-bonding interaction, is observed at 1668 cm^{-1} for BChl *b* (Zhou et al., 1989). Thus, the shift for such a hydrogen bond is about 40 cm^{-1}. The tyrosine M210 residue is in close proximity to the acetyl carbonyl of P_M and could be regarded as a possible H-bond donor to this carbonyl. However, in the present difference spectra of the RC of *Rb. sphaeroides*, we see no evidence for a large shift indicative of the removal of a hydrogen bond in the mutants. It may thus be concluded that tyrosine M210 is not hydrogen-bonded to the acetyl carbonyl of P_M.

The other component of the 1660-cm^{-1} band, namely the keto carbonyl of P_L, is observed to be engaged in a hydrogen bond for WT as well as the mutants. From the X-ray structure of *Rps. viridis* (Deisenhofer & Michel, 1989), this carbonyl is likely to be hydrogen-bonded to threonine L248. For *Rb. sphaeroides* the corresponding residue is methionine, whose side chain is not capable of hydrogen bonding, and the question of possible candidates has been raised previously (Allen et al., 1987a; Zhou et al., 1989). These include interaction with the backbone of the protein, a water molecule of crystallization, or possibly another unidentified group.

Drastic alterations in the protein structure that would be quite obvious in a RR study but may not be resolved in the X-ray structure would be (i) the breaking or formation of H-bonds with the conjugated carbonyls of the bacteriochlorin chromophores and (ii) the change in coordination state of their central Mg atom. Vibrational Raman data, however, will readily reflect changes in bonds that do not even approach these "drastic" cases. For example, shifts of 5 cm^{-1} of any carbonyl-stretching mode are easily detected in the present spectra. According to Badger-type rules (Zadorozhnyi & Ishchenko, 1965), such shifts correspond to ca. 0.8 kcal/mol energy change in bond strength. These changes could correspond to H-bond (if present) length changes of about 0.15 Å (Page, 1984), which would probably not be detected by X-ray crystallography, keeping in mind that X-ray structure coordinates at 2.8-Å resolution could be in error by about ±0.2 Å. Of course, with frequency shifts of this order of magnitude,

it is often difficult to ascertain their origin as arising from the small change in a bond length or from changes in the local dielectric permittivity. From the fact that the observed H-bond interactions are conserved in the P binding pocket and in the other pigment–protein pockets, it is very unlikely that sizable secondary structure changes occur at these sites.

Role of Tyrosine M210 during Primary Charge Separation. It was previously suggested that tyrosine M210 could be involved in an interaction with the keto carbonyl of the accessory $BChl_L$ during formation of P^+ (Robert & Lutz, 1988). The hypothesis arises when studying *Rb. sphaeroides 26*, a carotenoidless mutant. For this RC the contributions of P to the RR spectrum are extremely low and thus do not appear in the low- minus high-irradiance RR difference spectrum. What does appear, however, is a clear 14-cm^{-1} downshift of the RR keto carbonyl band of $BChl_L$ upon P^+ formation. The shift is most likely due to the hydrogen bonding of a water molecule to the C_9 keto carbonyl of $BChl_L$, but the possibility that it may also arise from an interaction of tyrosine M210 with the keto carbonyl of the accessory $BChl_L$ during formation of P^+ could not be discounted (Robert & Lutz, 1988; Lutz et al., 1988). From the refined crystal structure of *Rps. viridis* (Deisenhofer & Michel, 1989), there exists a water molecule in the vicinity of $BChl_L$ that appears to be hydrogen-bonded to histidine M200, the axial ligand of P_M. Thus, this water molecule can act as a bridge between P_M and $BChl_L$. Conceivably, a slight motion of the histidine M200 residue during photoexcitation or primary charge transfer would induce a similar motion to the hydrogen-bonded water and thus change the strength of the water molecule hydrogen bond with the keto carbonyl of $BChl_L$. In the resting RC this water–carbonyl distance is 2.9 Å (J. Deisenhofer, personal communication), i.e., very near to the optimal distance for such a hydrogen bond.

In the present difference spectra, this shift is masked because of the dominating contributions of P. It is thus difficult to assess whether the shift is present and/or changed. However, on the basis of the great similarities of the WT and mutant RC difference spectra, it seems reasonable to presume that the shift is still present [see, for example, Zhou et al. (1989)]. This would mean that tyrosine M210 is not responsible for the observed shift in the difference spectra of R26 and, thus, that the water molecule remains the most likely candidate.

Because of the proximity of tyrosine M210 to the acetyl carbonyl of P_M that is not H-bonded, the estimated 5-cm^{-1} shift in one of the components under the 1660-cm^{-1} Raman band in the RR difference spectra could likely be due to the influence of the tyrosine on the P_M acetyl carbonyl. Interestingly, molecular orbital calculations on the primary donor of the *Rps. viridis* RC (Plato et al., 1988) predict for P^* an unequal charge density distribution of the excited electron in P favoring the P_M molecule. If this hypothesis were correct, then the observed shift in the RR difference spectrum could have implications concerning the influence of tyrosine M210 on directional, primary charge separation. For example, in the absence of tyrosine a more equal distribution of the excited electron between P_L and P_M could result in a longer lived P^* state.

In summary, the RR spectra presented here indicate that the genetic replacement of tyrosine M210 in the RC of *Rb. sphaeroides* with phenylalanine or leucine does not result in any observable alteration of the vibrational structures of the local pigment–protein environments of the six bacteriochlorin chromophores throughout the RC, strongly suggesting that there is no drastic alteration of the overall physical structure of the RC. More specifically, the binding pocket of P is largely unaffected by the mutation, yet this genetic modification influences the electron-transfer kinetics (Gray et al., 1990; Finkele et al., 1990).

It is still possible that minor protein structural changes, not perceptible by RR spectroscopy, have occurred. These structural changes would involve displacements of residues forming the pigment pockets on a much smaller scale than those stated above, or they would occur far removed from the chromophores. In this case, these structural changes may be reflected by the observed shifts of the absorption maxima of P, $BChl_L$, and $BPhe_M$. The question now becomes which shifts are caused specifically by the removal of Y210 and which of these shifts are likely to be associated with the change in the initial electron-transfer kinetics?

From the photobleaching experiments on the WT and mutant chromatophores (Gray et al., 1990), there is strong evidence to suggest that the variations in the Q_y band of P are not a direct effect of the mutation but of the isolation of the RC and perhaps subsequent loss of Q_A. As well, the variations in the $BPhe_M$ Q_x band may also be due to preparation factors and loss of Q_A, which has been demonstrated to occur only after RC isolation. It is interesting to note that the variation of the P band in the WT and mutant RCs used in this work, as a result of detergent exchange, does not seem to influence the initial electron-transfer kinetics (Finkele et al., 1990) nor does the absence of Q_A (Stilz et al., submitted). Thus, these phenomena due to RC isolation and Q_A loss need not necessarily influence the initial electron-transfer kinetics.

The shift of the $BChl_L$ Q_y component, however, seems to be genuinely resulting from the tyrosine mutation and could very well influence the electron-transfer kinetics. This shift (46 cm^{-1}), which is not sensitive to detergent and is not consistent with loss of Q_A (Debus et al., 1985), could arise from an electrostatic effect of the phenolic group (Parson et al., 1990) directly on the chromophore or indirectly via the polarizable residues surrounding $BChl_L$. At present it is uncertain whether this shift may be correlated with the change in initial electron-transfer kinetics and possibility the quantum efficiency (Finkele et al., 1990). Taken together, the results of this study and those of the femtosecond kinetics are extremely interesting because they demonstrate that the electron-transfer rates in the RC can be affected by the replacement of a single, polar, aromatic residue in the absence of detectable secondary protein structural changes around the active sites.

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