Temperature-Dependent Behavior of Bacteriochlorophyll and Bacteriopheophytin in the Photosynthetic Reaction Center from *Rhodobacter sphaeroides*†

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ABSTRACT: We have reexamined the temperature dependence of resonance Raman (RR) spectra of the bacteriochlorin cofactors bound to reaction centers from *Rhodobacter sphaeroides*. Three types of resonant excitations were performed, namely, Soret band, bacteriopheophytin Q_x-band, and near-infrared, Q_y-band (pre)resonances. Sample temperature was varied from 300 to 10 K. In both Soret-resonant and Q_y-preresonant Raman spectra, the *ca*. 1610-cm^{-1} band corresponding to a bacteriochlorophyll C_aC_m methine bridge stretching mode is observed to increase in frequency by 4−6 cm^{-1} as temperature is decreased from 300 to 15 K. This upshift is interpreted as arising from a change in conformation of the bacteriochlorophyll macrocycles. It may be nonspecific to the protein-bound cofactors, since a similar 4-cm^{-1} upshift was observed in the same temperature range for BChl *a* in solution. Q_x-resonant Raman spectra of either of the two bacteriopheophytin (BPhe) cofactors were obtained selectively using excitations at 537 and 546 nm. No significant frequency shift was observed for the C_aC_m stretching mode of $BPhe_L$ between 200 and 15 K. We conclude, at variance with a previous report, that the macrocycle of the $BPhe_L$ primary electron acceptor does not undergo any significant conformational change in the 200−15 K temperature range. Q_y-preresonant excitation of RCs at 1064 nm provided selective Raman information on the primary electron donor (P°). The stretching frequencies of the two conjugated keto and acetyl carbonyl groups of the M-branch primary donor BChl cofactor (P_M) did not significantly change between 300 and 10 K. In contrast the keto carbonyl stretching frequency of cofactor P_L was observed to upshift by 5 cm^{-1}, while its acetyl carbonyl frequency downshifted by 2 cm^{-1}. The latter shift indicated that the strong H-bond between the acetyl group of P_L and His L168 may have slightly strengthened at 10 K. Excitation at 1064 nm of chemically oxidized RCs selectively provided RR spectra of the primary donor in its radical $P^{\bullet+}$ state. These spectra can be interpreted as a decrease of the localization of the positive charge on P_L from 78% to 63% when the temperature decreased from 300 to 10 K resulting in a more electronically symmetric dimer. Possible origins of the temperature dependence of the positive charge delocalization in $P^{\bullet+}$ are discussed.

The photosynthetic reaction center (RC)[1] of purple bacteria is a membrane protein consisting of three polypeptide subunits (named L, M, and H) wherein the primary light reactions and subsequent stable charge separation take place [reviewed in Parson, (1991), Kirmaier and Holten (1993), and Woodbury and Allen (1995)]. In addition to these three subunits, the isolated RC from *Rhodopseudomonas viridis* possesses a tightly bound tetraheme cytochrome subunit. The protein cofactors of the isolated RC from *Rhodobacter sphaeroides* consist of six bacteriochlorin pigments (four bacteriochlorophyll and two bacteriopheophytin molecules), one carotenoid, two quinones, and one non-heme iron atom. The bacteriochlorins and quinones are arranged in pairs within the L and M subunits, along a pseudo-C_2 symmetry axis, forming two symmetric possible electron transfer branches. However, this transfer predominantly occurs via the branch of chromophores more closely associated with the L subunit. 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 from P 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; Holzapfel *et al*., 1990; Kirmaier & Holten, 1991) via a monomeric accessory BChl molecule on the same branch, $BChl_L$, either through a superexchange mechanism or through the formation of the distinct radical pair intermediate $P^+BChl_L^-$ [see recent reviews DiMagno and Norris (1993), Kirmaier and Holten (1993), and Zinth and Kaiser (1993)]. Although the X-ray crystal structures of the bacterial RCs from *Rps. viridis* (Deisenhofer & Michel, 1989; Deisenhofer *et al*., 1995) and from *Rb. sphaeroides* (Yeates *et al*., 1988; El-Kabbani *et al*., 1991; Chirino *et al*., 1994; Ermler *et al*., 1994; Arnoux & Reiss-Husson, 1996) have been solved to atomic resolution, many questions remain open concerning the driving forces which are responsible for the speed, efficiency,

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[1] Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; ENDOR, electron nuclear double resonance; ESEEM, electron spin−echo envelope modulation; FT, Fourier transform; FWHM, full width at half-maximum; HOMO, highest occupied molecular orbital; INDO, intermediate neglect of differential overlap; LDAO, lauryldimethylammonium oxide; LUMO, lowest unoccupied molecular orbital; OEP, octaethylporphyrin; P, primary electron donor; Q_A, primary quinone; RC, reaction center; RR, resonance Raman; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane.

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directionality, and temperature dependence of the initial light-induced electron transfer.

The temperature dependence of certain electron transfer reactions occurring in the RC has been studied by several groups. Fast optical absorption studies have shown that the initial light-induced electron transfer from P to $BPhe_L$, which occurs in about 3 ps at room temperature (Woodbury *et al*., 1985; Martin *et al*., 1986; Holzapfel *et al*., 1989) is faster by a factor of 2−4 (depending on the species) as the temperature is lowered to 10 K (Fleming *et al*., 1988; Nagarajan *et al*., 1990, 1993). The subsequent electron transfer from $BPhe_L$ to the primary quinone (Q_A) has also been observed to proceed faster at low temperature (Kirmaier *et al*., 1985; Kirmaier & Holten, 1988). These spectroscopic data in conjunction with the crystal structure have led to the development of a number of theoretical models for the mechanism of electron transfer in the RC [see review by Friesner and Won (1989)]. The increase in electron transfer rate as temperature is lowered cannot be simply accounted for only by an increase in the cofactors' electronic couplings due to decreases in distances resulting from contraction of the protein matrix (Bixon & Jortner, 1986; DiMagno & Norris, 1993). Kirmaier and Holten (1990) have proposed that the observed increase in the primary electron transfer kinetics is the result of the shifting of a distribution of RCs favoring those with inherently faster electron transfer rates. Very recently Ortega *et al*. (1996) have reinvestigated the temperature dependence of the $P^+Q_A^-$ charge recombination reaction in a series of mutant reaction centers of *Rb. sphaeroides* where the $P/P^{\bullet +}$ redox midpoint potential was systematically altered. The increase in the charge recombination rate with decreasing temperature was specifically attributed to a temperature-dependent change in the reorganization energy (Franzen & Boxer, 1993; Dutton & Moser, 1994).

Decrease of the temperature could influence the electron transfer rate and kinetics through small protein conformational changes around the redox components or through conformational changes of the chromophores themselves. In addition, these conformational changes could alter H-bonding interactions between the chlorophylls and the protein. Vibrational spectroscopy, and in particular resonance Raman (RR) spectroscopy, because of its selectivity, is a powerful method to probe possible conformational changes occurring at the level of the bacteriochlorin molecules of the RC. Bocian and co-workers (Peloquin *et al*., 1990), using RR spectroscopy, reported the observation of a temperature-dependent conformational change of one of the two BPhe molecules in the RC of *Rb. sphaeroides*. They proposed that the BPhe molecule concerned is the photoactive $BPhe_L$ molecule and that these changes could in part be responsible for the observed temperature dependence of the electron transfer rates. This proposal is of particular importance for electron transfer theories dealing with the bacterial RC. However, the interpretation given by Peloquin *et al*. (1990) of their experimental data were conflicting with previously reported RR spectra of $BPhe_L$ and of $BPhe_M$ (Agalidis *et al*., 1984) that did not exhibit any significant frequency difference between the $\nu(C_aC_m)$ bands of the two cofactors at low temperature.

In this contribution we have reexamined the temperature dependence (300−10 K) of the observed RR spectra from the reaction center of *Rb. sphaeroides* to determine if there are specific temperature-dependent chromophore conformational or structural changes occurring in the RC which could account for the observed differences in the electron transfer kinetics. Under Soret excitation conditions, the behavior of the Raman band corresponding to the collective contributions of the C_aC_m stretching modes of the six bacteriochlorin pigments indicates that no significant conformational changes occur between the temperatures 200 and 15 K. We have also recorded RR spectra of these RCs excited at 545 and 537 nm to preferentially select the RR spectra of $BPhe_L$ and $BPhe_M$, respectively, as was described previously by Lutz (1980) and Agalidis *et al*. (1984). Previous work which drew conclusions concerning the temperature-dependent conformational changes of $BPhe_L$ relied on RR spectra excited at 530.9 nm (Peloquin *et al*., 1990), a wavelength which is not expected to preferentially enhance the RR spectrum of $BPhe_L$ over that of $BPhe_M$. We have also studied temperature-dependent changes in the vibrational spectrum of the primary electron donor in both its neutral ($P°$) and cation radical ($P^{\bullet +}$) states using Fourier transform resonance Raman spectroscopy to selectively obtain the vibrational spectrum of the primary donor in these two redox states. This technique also permits the recording of excellent room-temperature Raman spectra of reaction centers, which is not always possible with Soret resonant excitation.

MATERIALS AND METHODS

RCs from *Rb. sphaeroides* R26 were prepared as described in Robert and Lutz (1986). RCs of *Rb. sphaeroides* strain Y were the kind gift of Dr. F. Reiss-Husson. For resonance Raman experiments the final RC concentrations were *ca*. 100 μM in 25 mM Tris-HCl (pH = 8.0) and 0.05% LDAO or 0.1% cholate for strain Y and R 26 RCs, respectively. For the Fourier transform Raman experiments, the R26 RCs were concentrated to *ca*. 400 μM. The pigments BChl *a* and BPheo *a* were purified as described in Berger *et al*. (1990). Solutions of 10^{-3} M in argon-purged tetrahydrofuran (THF), which was dried and kept over molecular sieves, were prepared under nitrogen atmosphere and sealed in a 1-mm path length quartz cuvette.

Resonance Raman spectra were excited using 363.8-nm radiation from an Ar^+ laser (Coherent Innova 100) or the 530.9-nm line of a Kr^+ laser (Coherent Innova 90). Excitation wavelengths in the 535−548-nm region were provided by a dye laser (Spectra Physics Model 375), containing the dye coumarin 450 in ethylene glycol and benzyl alcohol, and pumped with the 514.5-nm line of the Ar^+ laser. The RC samples were placed in a gas-flow cryostat (SMC-TBT, France) in which cold helium gas was circulated. The temperature at the sample was monitored and controlled using a SMC-TBT Model 200 temperature regulator. Raman spectra were collected using a 60° grazing incidence geometry. The scattered light was collected and dispersed through a Jobin-Yvon double monochromator (Ramanor HG2S-UV) equipped with an EMI 9635/158 photomultiplier tube for the near-UV experiments and with an EMI 9558A photomultiplier tube for the visible experiments. With 363.8-nm excitation no more than 2 mW, typically, was used at the cryostat window. For visible excitation in the Q_x absorption region, no more than 15 mW of laser power was used. In order to avoid any possible photochemistry or photodegradation at higher temperatures, especially at 363.8

nm, the laser beam was defocused. It was shown that, under the conditions used in our experiments, more than 90% of the RCs remained in their resting ground state (Robert & Lutz, 1986). For RCs measured at room temperature in a quartz cuvette, the sample was thoroughly purged with, and sealed under, argon. Sample integrity was checked using visible absorption spectroscopy; after 20 min of exposure to 2 mW of 363.8-nm radiation, there was no observable degradation. Typical spectral resolution at 1000 cm^{-1} Raman shift was *ca*. 6 cm^{-1}. Signal-to-noise ratios were improved by averaging several spectra. The spectra reported here represent the sum of 10−40 individual scans, depending on the intensity of the RR signal, which varies both with excitation wavelength and with temperature.

Near-infrared Fourier transform (pre)resonance Raman spectra excited at 1064 nm were recorded using a Bruker IFS 66 interferometer equipped with a FRA 106 Raman module as described elsewhere (Mattioli *et al*., 1991, 1994). The same cryostat described previously was used to record the low-temperature FT Raman spectra. Typically, 180 and 200 mW of laser power was used for the room and low temperatures, respectively. Spectra were recorded for 10 different temperatures between 300 and 10 K. In order to ensure that the RCs were fully reduced or oxidized, the samples were treated with ascorbate or ferricyanide, respectively (Mattioli *et al*., 1991, 1993).

In some experiments, the RC samples were frozen using two different methods referred to as "rapid" and "slow" freezing. The "rapid" freezing method consisted of quickly immersing the RC sample into liquid nitrogen and then transferring it to the cryostat held at the desired temperature. "Slow" freezing consisted of introducing the RC sample into the cryostat which was at room temperature and then decreasing slowly to the desired temperature, for example, at 10 K during a time of 90 min. Reproducibility of the temperature conditions was checked by varying the temperature randomly; the spectra were verified to be reproducible to within 1 cm^{-1}. For experiments where the aqueous RC protein samples were mixed with cryoprotectants, spectrophotometric-grade glycerol (Aldrich) and 2 M sucrose reagents were pretreated with ascorbate or ferricyanide prior to mixing to a final RC sample-to-cryoprotectant ratio of 50% (v/v).

RESULTS

Isolated Pigments in Vitro. For purposes of comparison and reference, we have measured the Soret resonance Raman spectra, excited at 363.8 nm at room and low temperatures, of isolated BChl *a* and BPhe *a* dissolved in dry tetrahydrofuran (THF) (Figure 1). In this solvent the central Mg atom of the BChl *a* molecule is axially coordinated with two THF molecules. As the temperature is lowered, no change in the number of axial ligands is expected for BChl *a* dissolved in THF (Lutz *et al*., 1982).

In Figure 1, the intense 1597-cm^{-1} band (at 300 K) arises from a C_aC_m methine bridge stretching mode (Cotton & Van Duyne, 1981; Lutz, 1984; Donohoe *et al*., 1988). The frequency of this band is sensitive to the number of axial ligands on the central Mg atom and downshifts by *ca*. 10 cm^{-1} as the number of axial ligands increases from 1 to 2 (Cotton & Van Duyne, 1981; Callahan & Cotton, 1987). An

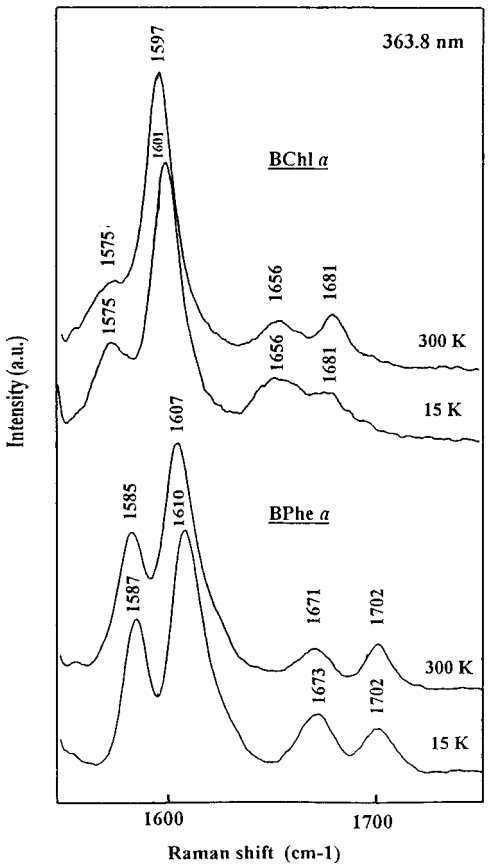

FIGURE 1: Soret resonance Raman spectra of isolated BChl *a* and BPhe *a* in dry THF (10^{-3} M) at 300 and 15 K. Excitation was at 363.8 nm.

analogous band is observed for BPhe *a* at 1607 cm^{-1} at 300 K.

The νC_aC_m vibrations of porphyrin molecules are sensitive to core size and extent of ruffling (Callahan & Cotton, 1987; Shelnutt *et al*., 1991). We have observed a 3-cm^{-1} upshift of the BPhe *a* C_aC_m stretching mode (1607 cm^{-1}) upon cooling THF solutions from 300 to 15 K (Figure 1) and a similar 4-cm^{-1} upshift is observed for the corresponding band of BChl *a* (1597 cm^{-1}) upon cooling to 15 K. There is also a *ca*. 1-cm^{-1} narrowing of both these bands upon cooling to 15 K. For the case of BChl *a*, this upshift should not be reflecting a change in the number of Mg axial ligands as the temperature is lowered because it already possesses two axial ligands. Moreover, a similar upshift is observed for BPhe *a*, which does not possess a central Mg atom and thus is unable to undergo changes in coordination. Therefore, these upshifts most likely signal a change in conformation of the macrocycle skeleton of both BChl *a* and BPhe *a*.

Figure 1 also shows that the C_2 acetyl and C_9 keto carbonyl groups of BChl *a* (BPhe *a*) vibrating at 1656 cm^{-1} (1671 cm^{-1}) and 1681 cm^{-1} (1702 cm^{-1}), respectively (Lutz, 1984), do not significantly shift as the temperature is lowered. It is noteworthy that previous observations of small shifts (Lutz *et al*., 1982) were most probably due to the lower quality of the room-temperature spectra.

Soret-Excited Resonance Raman Spectra of RCs. The Soret RR spectra of RCs from *Rb. sphaeroides* Y excited at 363.8 nm at various temperatures are shown in Figure 2. Under these conditions of Raman excitation, all six bacteriochlorin pigments contribute, in various degrees, to the RR spectrum, whose general features have been extensively

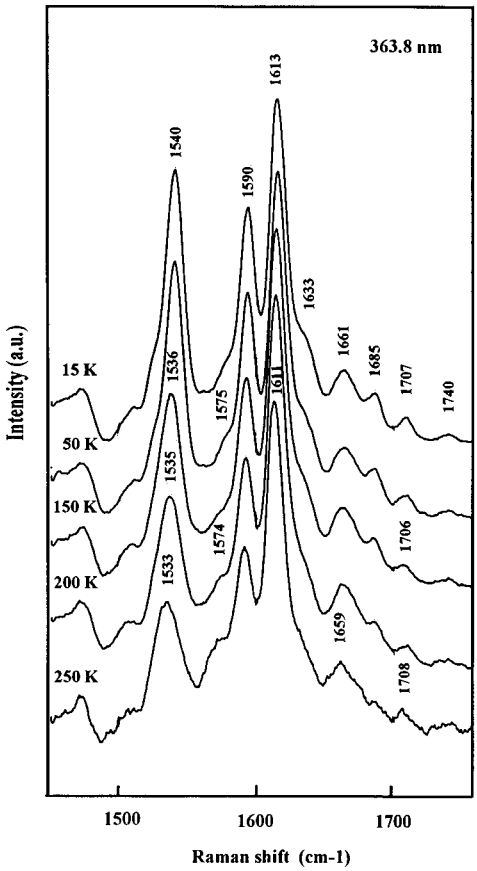

FIGURE 2: Soret resonance Raman (363.8-nm excitation) spectra of reaction centers from *Rb. sphaeroides* Y at various temperatures (between 250 and 15 K).

discussed elsewhere (Robert & Lutz, 1986, 1988). The *ca.* 1613-cm^{-1} band primarily arises from the above-mentioned C_aC_m stretching modes of the BChl and BPhe molecules, and the *ca.* 1590-cm^{-1} band arises largely from the BPhe molecules under these excitation conditions. The spectral region 1620−1710 cm^{-1} contains Raman bands arising from the stretching modes of the C_2 acetyl and C_9 keto carbonyls of all the BChl and BPhe molecules; their assignments have been discussed previously (Robert & Lutz, 1988; Robert, 1990).

Resonance Raman spectra of RCs were studied as the temperature was varied in the range from 250 to 15 K (Figure 2). At 250 K there is an apparent 2-cm^{-1} downshift of the bacteriochlorin νC_aC_m band, whereas at room temperature it is downshifted by 4 cm^{-1} (data not shown) as compared to the 15 K spectrum. Thus, the observed frequency of this collective band is seen to be most sensitive between 200 K and room temperature. Relative to that at 1613 cm^{-1}, the 1590-cm^{-1} band arising from a bacteriochlorin C_bC_b mode (Lutz, 1984; Donohoe *et al.*, 1988) decreases in intensity but does not appear to shift even at 250 K. There seems to be no significant change in relative intensity of the strongest carbonyl band, namely, the 1661-cm^{-1} band, with respect to the 1613-cm^{-1} band at 15 K (Figure 2). A downshift not larger than 2 cm^{-1} is observed at 250 K with respect to the 15 K spectrum. This observation is in disagreement with the 6-cm^{-1} downshift of the 1660-cm^{-1} band reported by Peloquin *et al.* (1990).

O_x-Excited Resonance Raman Spectra of the RC BPhe Molecules. At 77 K and lower temperatures, the Q_x absorption bands of the BPhe molecules are resolved as two

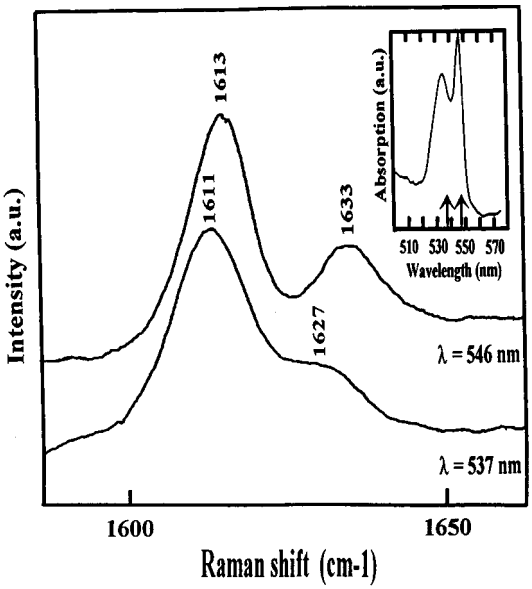

FIGURE 3: Bacteriopheophytin *a* Q_x resonance Raman spectra of reaction centers from *Rb. sphaeroides* R26 at 30 K excited at 546 and 537 nm. These selected wavelengths preferentially enhance the contributions of $BPhe_L$ and $BPhe_M$, respectively, as indicated by the Q_x electronic absorption spectrum of BPhe *a* (15 K) in the inset.

distinct components at 547 nm and a broader one at 535 nm (inset in Figure 3), corresponding to the photoactive $BPhe_L$ (Parson *et al.*, 1975; Rockley *et al.*, 1975) and to the $BPhe_M$ cofactors, respectively [see review by Kirmaier and Holten (1987)]. Excitation of bacterial RCs at low temperature in this range (i.e., 530−550 nm) permits selective observations of RR spectra of the two BPhe cofactors (Lutz, 1980, 1984; Agalidis *et al.*, 1984).

Figure 3 shows the low-temperature (15 K) RR spectra of the BPhe molecules in the RC from *Rb. sphaeroides* R 26 excited at 537 and 546 nm; these excitation wavelengths result in the preferential resonance enhancement of the $BPhe_M$ and $BPhe_L$ molecules, respectively (Lutz, 1980, 1984; Agalidis *et al.* 1984). These two RR spectra differ significantly, indicating that the two BPhe molecules are experiencing different interactions with the protein.

This is illustrated by the different frequencies of a band observed at 1627 cm^{-1} for $BPhe_M$ (537-nm excitation) and at 1633 cm^{-1} for $BPhe_L$ (546-nm excitation). Whatever the vibrational origin of this band, these observed differences are not likely due to a difference in resonance conditions, as this would primarily affect the relative band intensities but not their frequencies. This band has been assigned to the stretching mode of the C_2 acetyl carbonyl group of BPhe *a* engaged in a H-bond (Lutz, 1980, 1984; Agalidis *et al.*, 1984). Other workers, however, have recently proposed a skeletal origin for bands they observed in the 1620−1640-cm^{-1} region in similar conditions (Palaniappan *et al.*, 1993). A weak, *ca.* 1630-cm^{-1} shoulder is actually apparent in RR spectra excited in the Q_x band of isolated BPhe *a* in non-H-bonding solvents (Lutz *et al.*, 1976). The weakness of this feature in the *in vitro* RR spectra, however, as well as the absence of other candidates in the RC Q_x RR spectra, led us to the $\nu C_2{=}O$ assignment in interpreting the much stronger bands seen in the RC $BPhe_L$ and $BPhe_M$ spectra.

As discussed earlier, the selective observation of each of the two BPhe cofactors using 536- and 547-nm excitations

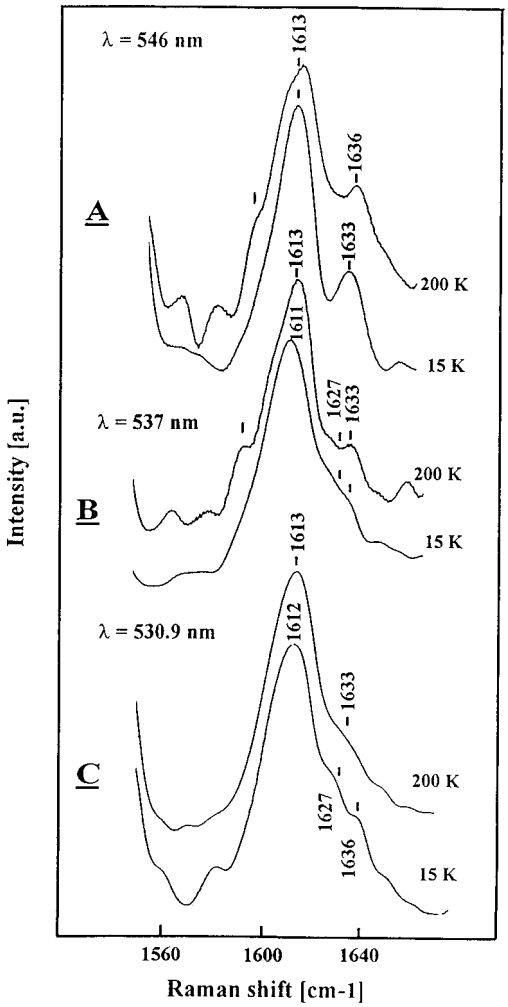

FIGURE 4: Bacteriopheophytin *a* Q_x resonance Raman spectra of reaction centers from *Rb. sphaeroides* Y excited at (A) 546, (B) 537, and (C) 530.9 nm at 15 and 200 K. The spectra in this figure were smoothed using an 11-point Savitsky−Golay routine.

is, however, not perfect, even at 15 K, inasmuch as both spectra each exhibit two bands at 1678 and 1703 cm^{-1} attributed to the stretching mode of the keto carbonyls of $BPhe_L$ and $BPhe_M$, respectively (Lutz, 1980, 1984; Agalidis *et al*., 1984).

Another difference between the RR spectra of Figure 3 is the width of the 1613-cm^{-1} band which appears broader in the spectrum excited at 537 nm (22 cm^{-1} FWHM) as compared to that excited at 546 nm (15 cm^{-1} FWHM). The maxima of these bands are measured to be 2 cm^{-1} apart.

Panels A and B of Figure 4 show the temperature dependence of the RR spectra of RCs from *Rb. sphaeroides* Y excited at 546 and 537 nm. Because of the weaker resonance enhancement in the Q_x spectral region as compared to that in the Soret region, these Q_x RR spectra are not of as high quality. For this reason, we have denoted the frequencies of the Raman band at their center and estimate the uncertainty in these band positions to be *ca*. 2 cm^{-1}. For the RR spectra excited at 546 nm, which correspond to the preferential enhancement of $BPhe_L$, there is no observable shift in the position of the 1613-cm^{-1} band when the RC is cooled from 200 to 15 K. There appears to be a broadening of this band from 15 cm^{-1} to *ca*. 22 cm^{-1} (FWHM) upon warming. The presence of a well-resolved 1636-cm^{-1} band at 200 K shows that even at this temperature, when the $BPhe_L$ and $BPhe_M$ Q_x absorption bands are no longer resolved, excitation with 546-nm light still preferentially enhances $BPhe_L$ contributions. These experiments clearly show that in the temperature range 200−15 K there is no observable shift of the C_aC_m band of the photoactive $BPhe_L$ molecule (only a broadening) and thus no observable conformational changes under the experimental conditions used. This conclusion is at odds with those of Peloquin *et al.* (1990).

With 537-nm excitation the RR spectrum of the $BPhe_M$ molecule, a modest 2-cm^{-1} upshift of the 1611-cm^{-1} band is observed at higher temperatures. There is also an apparent decrease of the bandwidth at 200 K (from 22 cm^{-1} at 15 K to 19 cm^{-1} FWHM at 200 K); this trend is in contrast to that observed for $BPhe_L$ at 546-nm excitation but similar to what was reported by Peloquin *et al.* (1990) with 530.9-nm excitation. We note that the 2-cm^{-1} shift and the 3-cm^{-1} broadening are at the limit of our estimated uncertainty. Again the appearance of the 1625 cm^{-1} shoulder indicates that $BPhe_M$ contributions are still being sizably enhanced at these higher temperatures using 537-nm radiation; however, the appearance of a feature at 1633 cm^{-1} indicates that $BPhe_L$ contributions are also more enhanced at higher temperatures at this wavelength.

Figure 4C shows the low-temperature RR spectra of RCs excited with 530.9-nm light from a Kr^+ laser. This wavelength lies on the short-wavelength side of the low-temperature-resolved $BPhe_M$ Q_x absorption band. As seen by the presence of two partially resolved shoulders at 1627 and 1636 cm^{-1} in the 15 K spectrum, this excitation enhances the RR spectra of both $BPhe_M$ and $BPhe_L$; at 200 K a 1633-cm^{-1} feature is seen which apparently is an unresolved envelope of the 1627- and 1636-cm^{-1} bands of relative intensities similar to those observed at 15 K. In general, for both *Rb. sphaeroides* Y and R26, there is no evidence of significant shifting of the 1613-cm^{-1} band upon warming, only a 4-cm^{-1} narrowing of the half-bandwidth. This latter trend is similar to that reported by Peloquin *et al.* (1990).

Near-Infrared Fourier Transform Raman Spectra: The Primary Electron Donor. (Pre-)resonant Raman spectra of the primary donor in either its reduced (P°) or cation radical state ($P^{\bullet+}$) can be selectively obtained using near-infrared Fourier transform Raman spectroscopy excited with 1064-nm light (Mattioli *et al*., 1991). The assignments of the observed carbonyl vibrational frequencies in the preresonance Raman spectrum of P° and the resonance Raman spectrum of $P^{\bullet+}$ on RC from *Rb. sphaeroides* obtained under these experimental conditions have been discussed elsewhere (Mattioli *et al*., 1991, 1993, 1994). Thus, using 1064-nm light, we can selectively monitor structural and/or conformational changes of the primary electron donor as a function of temperature. Such changes may be revealed by frequency shifts of the stretching modes of the methine bridges or of the conjugated carbonyl groups of the two BChl molecules constituting the primary donor.

Figure 5A shows the FT Raman spectra of reduced RCs (poised in 10 mM ascorbate) from *Rb. sphaeroides* R 26 at 300, 150, and 10 K. The assignments of the bands in the higher frequency region (1600−1750 cm^{-1}) have been discussed elsewhere (Mattioli *et al*., 1991, 1994). In the room-temperature FT Raman spectrum, the band at 1607 cm^{-1} corresponds to the C_aC_m stretching modes of the two BChl *a* molecules constituting P. The 1620- and 1652-cm^{-1} bands correspond to the stretching modes of the C_2 acetyl

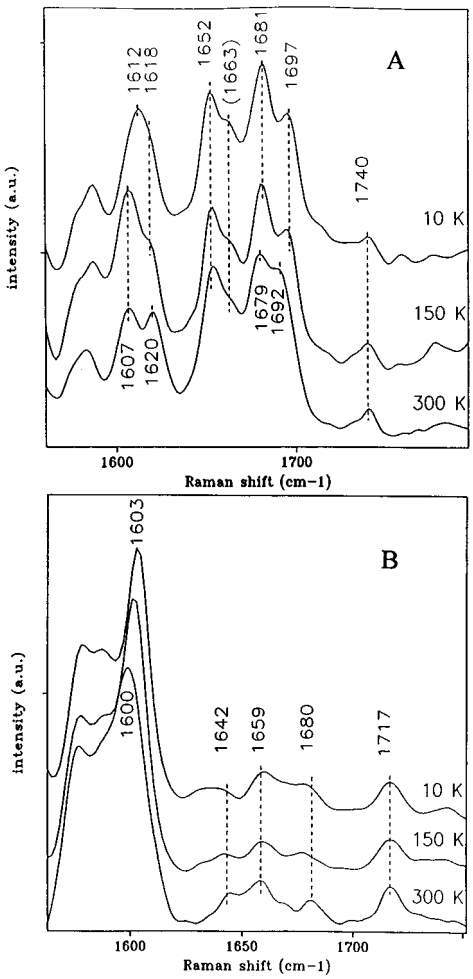

FIGURE 5: Near-infrared Fourier transform (pre)resonance Raman spectra (1064-nm excitation) of reaction centers from *Rb. sphaeroides* R 26 at 10, 150, and 300 K, in the presence of (A) ascorbate (neutral P state) and (B) ferricyanide ($P^{\bullet+}$ cation radical state). The RCs were rapidly frozen in liquid nitrogen before being transfered to the cryostat (see Materials and Methods). The frequencies indicated were obtained by spectral deconvolution and second-derivative analyses. The 1663-cm^{-1} band (in parentheses) does not arise from P° (Mattioli *et al*., 1991). Six thousand interferograms were co-added; resolution was 4 cm^{-1}.

carbonyl groups of P_L and P_M, respectively. The C_2 carbonyl of P_L is strongly H-bonded (to His L168), while that of P_M is free of H-bond interactions. The 1679- and the 1692-cm^{-1} bands correspond to the stretching modes of the C_9 keto carbonyl groups of P_M and P_L, respectively. Both these latter groups are free from H-bond interactions.

For the 10 K FT Raman spectrum, spectral deconvolution and second-derivative analyses of the broad band centered at *ca*. 1614 cm^{-1} indicate that it is composed of two bands at 1612 and 1618 cm^{-1} (Figure 5A), which correspond to the 1607- and 1620-cm^{-1} bands, respectively, in the room-temperature FT Raman spectrum. In general, no major changes were induced in the FT Raman spectra by either fast or slow cooling of the RC down to 10 K (see Materials and Methods), with or without the cryoprotectants glycerol or sucrose (data not shown). The general pattern of H-bonds reflected by the carbonyl frequencies is conserved upon cooling: there is, indeed, no formation of any new H-bond nor is the existing one ruptured upon cooling to 10 K.

However, the low-temperature (10 K) FT Raman spectrum (Figure 5A) reveals significant differences as compared to the room-temperature spectrum. Similar to what is observed for BChl *a in vitro*, the band corresponding to the C_aC_m stretching mode of the two BChl *a* molecules constituting P upshifts 5 cm^{-1}, from 1607 to 1612 cm^{-1}, as the temperature is lowered to 10 K. The stretching frequency of the P_L C_9 keto carbonyl is observed to significantly upshift, from 1692 to 1697 cm^{-1} upon cooling to 10 K. Similarly, the P_L C_2 acetyl carbonyl band at 1620 cm^{-1} (at room temperature) downshifts 2 cm^{-1} to 1618 cm^{-1} at 10 K; as the temperature is gradually lowered, this 2-cm^{-1} downshift appears to occur already at 150 K, at a temperature higher than that corresponding to the observation of the upshift of the 1607-cm^{-1} band (C_aC_m mode). In contrast to these observations that the C_2 and C_9 carbonyl groups of P_L downshift in vibrational frequency as the temperature is lowered, those of P_M remain essentially unchanged (Figure 5A). This latter behavior is similar to that observed for BChl *a in vitro* (Figure 1). These results show that (i) at the level of the conjugated C_2 and C_9 carbonyl groups, P_L responds to temperature changes significantly differently than P_M and than BChl *in vitro*; this observation is indicative of a change in the specific protein environment of P_L; and (ii) a possible strengthening of the H-bond on the P_L C_2 acetyl carbonyl group may occur on cooling the RC sample to 10 K. Using the empirical Badger-type relations given in Zadorozhnyi and Ishchenko (1965), the 2-cm^{-1} downshift of the 1620-cm^{-1} band is estimated as a 0.3 kcal/mol increase in H-bond enthalpy of the existing 4.8 kcal/mol H-bond (taking 1653 cm^{-1} as representing the frequency of the P_L C_2 acetyl carbonyl stretching mode when free from H-bonding; Mattioli *et al*., 1994, 1995).

Figure 5B shows the FT Raman spectra for oxidized R26 RCs at different temperatures; these spectra are essentially those of $P^{\bullet+}$ (Mattioli *et al*., 1991). For the 300 K spectrum, the bands at 1600, 1642, and 1717 cm^{-1} have been assigned to the C_aC_m, the C_2 acetyl carbonyl, and the C_9 keto carbonyl stretching modes of the cation radical $P^{\bullet+}$ species, respectively. The 1642- and 1717-cm^{-1} bands have been specifically assigned to the C_2 and C_9 carbonyl groups, respectively, of the P_L cofactor in the $P^{\bullet+}$ cation radical dimer (Mattioli *et al*., 1991, 1993, 1994). The 10 K FT Raman spectrum indicates that, as observed for P° but to a lesser extent, there is a modest upshift of the C_aC_m band upon cooling (3 cm^{-1}). There is no observable shift of the 1717-cm^{-1} band as the temperature decreases to 10 K; only a small broadening of 2 cm^{-1} could be observed.

To study the possible effects of cryoprotectants on the temperature dependence of the FT Raman spectra of the primary donor, we recorded spectra of reaction centers in glycerol and aqueous sucrose mixtures. Under our experimental conditions, neither glycerol nor sucrose contributed sizable bands in the 1600−1800-cm^{-1} region of the FT Raman spectrum (data not shown). Figure 6 presents the FT preresonance Raman spectra of reduced RCs from *Rb. sphaeroides* R26 in 50% (v/v) glycerol and in 2 M sucrose compared to those in aqueous buffer, all at 10 K. Upon comparing the three spectra in Figure 6 there appears to be no effect of these cryoprotectants on the vibrational spectrum of P°. Similar results were obtained for $P^{\bullet+}$, at different temperatures and using different freezing methods (data not shown). Thus, the addition of cryoprotectants does not result in any observable conformational distortions near the primary donor at cryogenic temperatures.

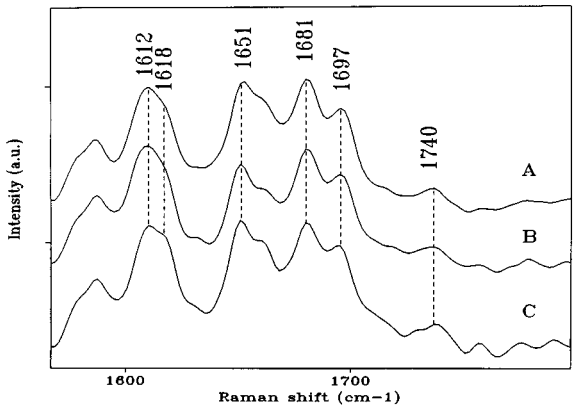

FIGURE 6: Comparison of the 1550−1880-cm^{-1} region of the Fourier transform preresonance Raman spectra (1064-nm excitation) of reaction centers from *Rb. sphaeroides* R 26 poised with ascorbate (P°) in 0.1% cholate and 10 mM Tris-HCl (pH = 8.0) buffer solution (A) and those mixed with 50% (v/v) glycerol (B) or 50% (v/v) sucrose (C), all at 10 K. The samples were rapidly frozen.

Figure 7 graphically summarizes the temperature dependence of the observed frequencies of the FT Raman bands arising from the *ca*. 1607-cm^{-1} C_aC_m stretching mode and from the stretching modes of the C_2 acetyl and C_9 keto carbonyl groups of the primary donor of R 26 reaction centers, in either its neutral or radical cation state. The C_aC_m stretching mode frequency is significantly temperature-dependent, both in the P° and $P^{\bullet+}$ states; from 300 to 10 K it indeed upshifts by 5 cm^{-1} in P° and by 3 cm^{-1} in $P^{\bullet+}$. In the P° state, the stretching frequency of the C_9 keto carbonyl group of P_L also upshifts by 5 cm^{-1}, while in the $P^{\bullet+}$ it is essentially temperature-insensitive (1717 cm^{-1}, Figure 5B). The stretching frequencies of both carbonyl groups of P_M also are essentially temperature-insensitive in the P° state.

We have previously shown that the observed upshift of the P_L C_9 keto carbonyl stretching frequency upon oxidation provides an estimation of the degree of localization of the resulting positive charge in the dimer radical cation $P^{\bullet+}$ (Mattioli *et al*., 1991, 1992, 1993, 1994). In our model, the 32-cm^{-1} upshift of the keto carbonyl stretching frequency of monomeric BChl *a in vitro* upon its one-electron oxidation (Cotton *et al*., 1981; Mäntele *et al*., 1988; Heald & Cotton, 1990; Diers & Bocian, 1994) represents 100% localization of the positive charge on one BChl *a* cation molecule; we assume that the positive charge localization is linear with the observed upshift and that no other protein-induced BChl structural changes affecting the C_9 keto carbonyl stretching frequency occurs as a result of P oxidation. Thus, the 25-cm^{-1} upshift observed for the P_L C_9 keto frequency upon oxidation of P at room temperature (compare 1692 cm^{-1} in Figure 5A with 1717 cm^{-1} in Figure 5B) reflects an estimated localization of *ca*. 78% on P_L. The magnitude of the oxidation-induced upshift of the P_L C_9 keto stretching frequency upon P oxidation decreases as temperature is lowered (Figure 8). This decrease in upshift mainly arises from the temperature dependence of the keto stretching frequency in the P neutral state (Figure 7) since the frequency of the same carbonyl band in the $P^{\bullet+}$ oxidized state remains constant at 1717 cm^{-1} at any temperature (see Figure 5B). The observed upshifts of the P_L C_9 keto stretching frequency upon P oxidation for different temperatures and the resulting percentage localization of the positive charge on P_L are shown in Figure 8. Our analysis indicates that the positive charge localization on P_L decreases from 78% to 63% as the temperature is lowered from 300 to 10 K.

DISCUSSION

Q_x-Resonant Raman Spectra of the RC BPhe a Molecules. On the basis of temperature effects observed in the RR spectra of RCs excited at 530.9 nm, it has been suggested that the skeletal mode at *ca*. 1610 cm^{-1} of one of the BPhe *a* molecules upshifts by *ca*. 4 cm^{-1} as the temperature is lowered (Peloquin *et al*., 1990). It has been furthermore suggested that this upshift can be attributed to a flattening of the macrocycle, at low temperatures, most likely of the $BPhe_L$ molecule and that this structural change could be in part responsible for the temperature dependence of electron transfer rates in the bacterial RC (Peloquin *et al*., 1990). The 4-cm^{-1} upshift of the 1610-cm^{-1} C_aC_m stretching mode of the $BPhe_L$ molecule was estimated from a curve-fitting of the broadened 1610-cm^{-1} band excited with 530.9-nm light at 200 K. However, this observation was made using an excitation wavelength which does not predominantly enhance the $BPhe_L$ RR spectrum over that of $BPhe_M$.

In this work, we have obtained RR spectra selectively from $BPhe_M$ and from $BPhe_L$ at 15 K using the excitation wavelengths of 537 nm (or 530.9 nm) and 546 nm, respectively. The data presented here clearly show that the *ca*. 1610-cm^{-1} C_aC_m stretching modes of these two cofactors do not differ by 4 cm^{-1} (see Figure 3) at 15 K but by only 2 cm^{-1} at most, a value which falls within our experimental error. Thus, on the basis of the Raman data presented here, it cannot be concluded that one BPhe molecule undergoes significantly different structural changes with respect to the other at 15 K. The most conclusive piece of evidence that $BPhe_L$, specifically, does not undergo significant conformational changes as the temperature is lowered comes from the temperature dependence of the RR spectrum excited at 546 nm which preferentially enhances the $BPhe_L$ contribution: we observed no significant shift of the *ca*. 1610-cm^{-1} C_aC_m band as the temperature was lowered from 200 to 15 K (Figure 4A). The narrowness of the 1613-cm^{-1} band (15 cm^{-1} FWHM at 15 K) is indicative of one population of similar BPhe *a* molecules predominantly contributing to the RR spectrum. The broadening of this band as the temperature is raised is consistent with a loss in homogeneity; it is not consistent with an increase in relative enhancement of the contribution from another molecule which would have a significantly different *ca*. 1610-cm^{-1} C_aC_m stretching frequency, because no shift is observed with the broadening. It is also, in particular, not consistent with a change in relative enhancement of the $BPhe_M$ molecule contribution since the 1636-cm^{-1} band is still clearly present at 200 K with no indication of a 1627-cm^{-1} feature (Figure 4A). We thus conclude that in the temperature range 200−15 K there is no observable shift of the *ca*. 1610-cm^{-1} C_aC_m mode of the photoactive $BPhe_L$ molecule and thus no observable conformational changes occur for this cofactor in this temperature range.

The observation that the *ca*. 1610-cm^{-1} C_aC_m band of $BPhe_M$ excited at 537 or 530.9 nm broadens as the temperature is lowered is interesting. In comparison with the $BPhe_L$ spectrum excited at 546 nm, the above-mentioned broadening appears as extra intensity on the low-frequency side of the 1613-cm^{-1} band and thus should not be the result of

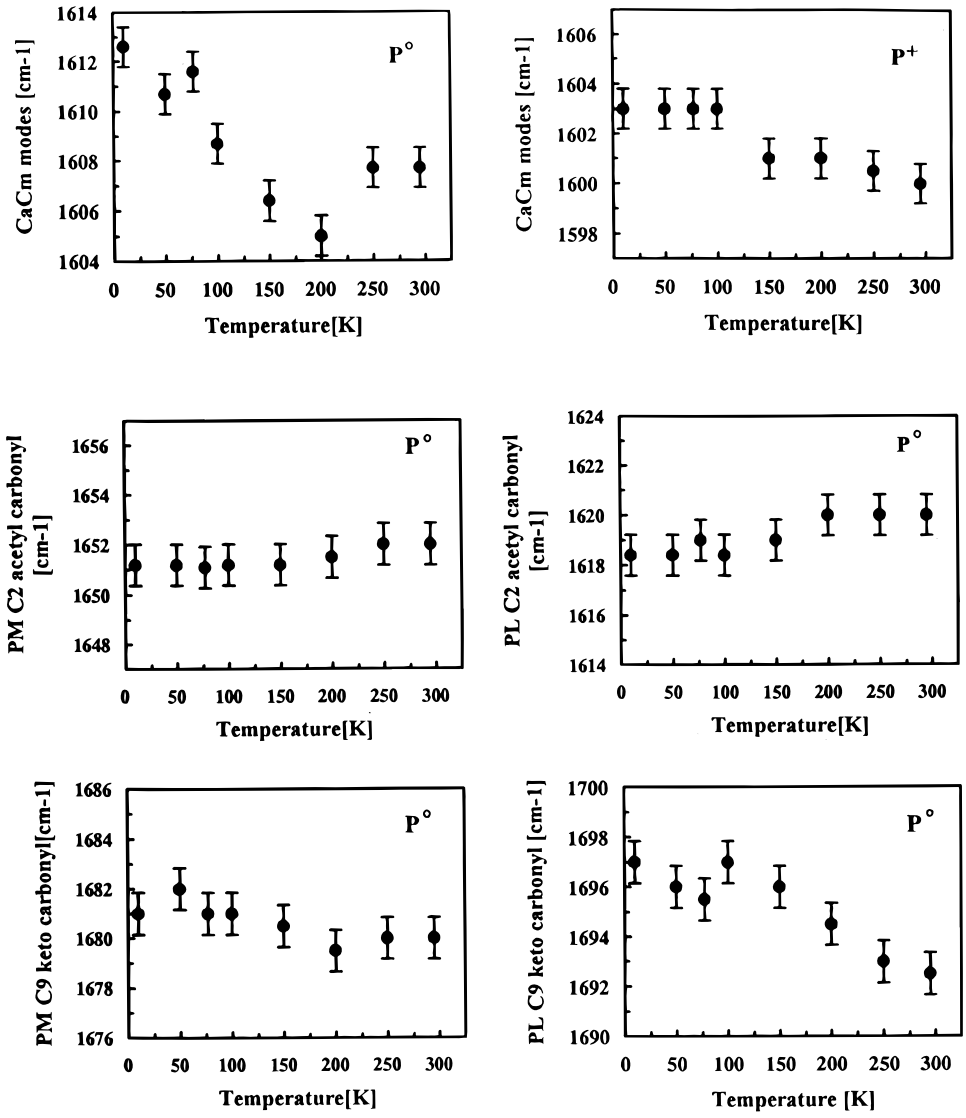

FIGURE 7: Observed vibrational frequencies of the FT Raman bands assigned to the C_2 acetyl and C_9 keto carbonyl groups of neutral P° from *Rb. sphaeroides* R 26 RCs as a function of temperature. Also shown are the variations of the *ca*. 1610-cm^{-1} C_aC_m methine bridge stretching mode assigned to P° and P$^{\bullet+}$ as a function of temperature.

interference from the 1627-cm^{-1} shoulder (Figure 4B,C). This 3-cm^{-1} broadening could result from a heterogeneity of structural states of $BPhe_M$ which would increase at low temperature. Such a heterogeneity could also account for the larger width of the Q_x transition of $BPhe_M$, compared to that of $BPhe_L$, which is observed at low temperature (see inset, Figure 3). Crystallographic studies indicated that in RCs of both *Rps. viridis* and *Rb. sphaeroides* at room temperature, the M side appears less ordered than the L one, in particular around the BPhe sites, as manifested by much larger *B*-factors (Deisenhofer *et al*., 1995; Ermler *et al*., 1994; Arnoux & Reiss-Husson, 1996). The possibility that this heterogeneity may increase upon freezing samples of isolated RCs due to increased external constraints is further suggested by several biochemical and spectroscopic data which indicated that $BPhe_M$ is particularly sensitive to various treatments affecting the environment of the RC (Agalidis *et al*., 1984; Debus *et al*., 1985).

BChl C_aC_m Stretching Modes and Conformational Changes. The Soret RR spectra (363.8-nm excitation) presented in this work indicate that all six bacteriochlorin molecules in the RC could collectively undergo a temperature-dependent conformational change involving the methine bridges and affecting *ca*. 1610-cm^{-1} C_aC_m stretching mode frequencies between room temperature and 15 K. However, specific Q_x excitation of the Raman spectra of the BPhe molecules in the RC show that their *ca*. 1610-cm^{-1} C_aC_m modes do not significantly shift between 200 and 15 K (see above). Thus one may conclude that only the BChl cofactors are undergoing significant conformational changes. The FT Raman spectra of the primary donor specifically show that conformational changes involving the *ca*. 1610-cm^{-1} C_aC_m modes occur for the P_L and P_M BChl cofactors (see below).

The temperature dependence of the resonance Raman spectra of isolated BChl *a* and BPhe *a in vitro* is useful in interpreting the RC protein data. Soret-resonant Raman spectra of monomeric BChl *a* and BPhe *a* in the polar solvent THF exhibited a single sizable temperature-induced shift, affecting the *ca*. 1605-cm^{-1} band. This mode, which has a predominant C_aC_m stretching character (Cotton & Van Duyne, 1981; Lutz, 1984; Donohoe *et al*., 1988), was upshifted by 4 and 3 cm^{-1} in the BChl *a* and BPhe *a* spectra, respectively, when the temperature was decreased from 300 to 15 K.

The 1610- and 1601-cm^{-1} bands (Figure 1) of BPhe *a* and BChl *a*, respectively, correspond to modes which arise

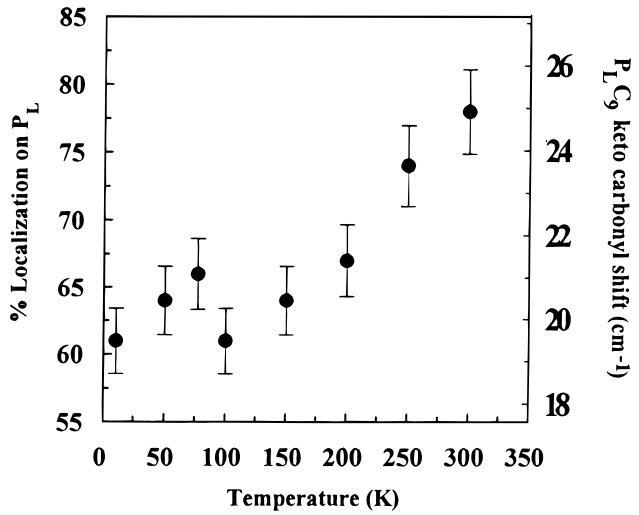

FIGURE 8: Observed upshift of the vibrational frequency of the C_9 keto carbonyl group of P_L from *Rb. sphaeroides* RCs upon $P^{\bullet+}$ formation and the corresponding estimated percentage of localization of the resulting positive charge on P_L as a function of temperature, for the range 10−300 K.

mainly from C_aC_m stretching modes and are expected to possess significant ν_{10} character of NiOEP (Donohoe *et al*., 1988) and will therefore be sensitive to changes in core size as well as in the planarity of the macrocycle ring. Asher and Murtaugh (1983) have discussed the temperature dependence of ν_{10}-like modes of porphyrins and proposed that the increase in frequency they observed for these modes as the temperature was decreased was due to anharmonic coupling with lower frequency modes. For Ni octaethylporphyrin (NiOEP), the ν_{10} C_aC_m mode was seen to upshift as the macrocycle conformation changed from a ruffled or puckered to a more planar conformation (Alden *et al*., 1989). On this basis, our observations of 3−4-cm^{-1} upshifts of the *ca*. 1610-cm^{-1} C_aC_m mode frequencies of BChl *a* and BPhe *a* upon cooling may be interpreted as resulting from changes in core size due to a conformational change of the macrocycle, most likely adopting a more planar geometry at low temperatures (Alden *et al*., 1989; Peloquin *et al*., 1990).

Theoretical calculations (Fajer *et al*., 1990) suggest that conformational variations of the macrocycles would shift the HOMOs and LUMOs of BChl molecules and thus their redox and photophysical properties [see also Watanabe and Kobayashi (1991)]. For instance, INDO calculations (Fajer *et al*., 1990) predicted that a puckered porphyrin ring will be more easily oxidized by *ca*. 120 meV, whereas reduction was predicted to be insensitive to such distortions.

Modifications of Existing Protein Interactions with the Primary Donor. The temperature-dependent shifts observed for the primary donor carbonyl bands are, in general, more pronounced for P_L than for P_M. The vibrational frequencies of both conjugated carbonyl groups of P_L were affected as temperature was lowered: the C_2 acetyl carbonyl vibrational frequency downshifted by 2 cm^{-1}, while that of the C_9 keto carbonyl group upshifted by 5 cm^{-1}. The resonance Raman data of BChl *a* in THF solution exhibited no significant shift in the vibrational frequency of either carbonyl group as temperature was lowered (see Figure 1); thus, the alterations of the vibrational frequencies of the P_L conjugated carbonyl groups seem to be arising from specific changes in interactions of the P_L cofactor with the protein.

The 2-cm^{-1} downshift of the P_L C_2 acetyl carbonyl vibrational frequency reflects a modest *ca*. 0.3 kcal/mol increase (see Results) in the strength of the H-bond between this carbonyl group and His L168, as the temperature is lowered. This strengthening probably arises from a repositioning of His L168 with respect to P_L or from a change in the conformation of the C_2 acetyl carbonyl itself resulting in a more favorable H-bond geometry at 10 K. On the basis of a recent study where a linear correlation was found between the $P°/P^{\bullet+}$ redox midpoint potential and the number and strengths of histidine-donated H-bonds to P (Mattioli *et al*., 1995), this 0.3 kcal/mol increase in the H-bond strength should amount to only a minor *ca*. 8-mV increase in the $P°/P^{\bullet+}$ redox potential, assuming the same correlation holds at 10 K. Such a small predicted change in the $P°/P^{\bullet+}$ redox potential is not expected to sizably affect the electron transfer rates (Lin *et al*., 1994).

The 5-cm^{-1} upshift of the (free) P_L keto carbonyl 1692-cm^{-1} band to 1697 cm^{-1} at low temperature could arise from (i) a decrease in the dielectric permittivity of the keto carbonyl local environment due to the change in temperature or (ii) from a conformational change of the BChl ring V, containing this keto carbonyl group, with respect to the macrocycle, due to a non-hydrogen-bonding interaction with the protein.

(i) The frequency of the keto carbonyl stretching mode of chlorophylls is known to be sensitive to the dielectric properties of the medium (Lutz *et al*., 1982; Koyama *et al*., 1986; Krawczyk, 1989). Frequency downshifts of up to 14 cm^{-1}, attributed to electrostatic or dielectric effects, of the keto carbonyl of a BPhe molecule in mutant RCs of *Rb. capsulatus* have been reported (Palaniappan & Bocian, 1995). As well, Wachtveitl *et al*. (1993) reported changes in the FT Raman spectrum of a mutant RC of *Rb. sphaeroides* where methionine L248 was replaced with a threonine residue near the C_9 keto carbonyl of P_L; although no H-bond was formed, a 4-cm^{-1} upshift and an apparent decrease in intensity of the 1691-cm^{-1} band was observed. This was attributed to dielectric or electrostatic effects changing the polarizability of the C_9 keto carbonyl of P_L in the mutant RC. For *Rb. sphaeroides* RCs in thin poly(vinyl alcohol) films, Steffen *et al*. (1994) have reported that the dielectric constant is approximately 2-fold lower in the protein at cryogenic temperatures (1.5 and 77 K) as compared to room temperature, specifically in the region of the BPhe cofactors. A decrease in local dielectric permittivity would result in an upshift in the frequency of the stretching vibration of the carbonyl group (Krawczyk, 1989). The observed upshift of the P_L keto carbonyl vibrational frequency as temperature was lowered could be consistent with a decrease in the local dielectric constant at low temperature (Steffen *et al*., 1994); however, the observed 2-cm^{-1} downshift of the P_L acetyl carbonyl would not be consistent with the decrease in dielectric constant. Since only the P_L keto carbonyl is significantly affected by a 5-cm^{-1} upshift and not the other conjugated carbonyl groups of P, this purported dielectric change should be extremely localized with respect to this keto carbonyl group. Such a localized temperature-dependent dielectric change near the single keto carbonyl group may be explained by a change in the local structure of its environment where a change in orientation of dipoles or a change in the distance of a polarizable group is brought about by a temperature-dependent conformational change [see point

(ii) below]. Interestingly, for the $P^{\bullet+}$ species, no upshift is observed for the P_L keto carbonyl at low temperatures, which should experience the same temperature-dependent change in dielectric permittivity in its local environment (Figure 5B). As well, no shift of any one of the conjugated carbonyl groups of either BChl or BPhe in THF was observed as the temperature was lowered (Figure 1). These latter two observations suggest that a possible change in dielectric permittivity near P is not entirely responsible for the observed upshift of the P_L keto carbonyl vibrational frequency.

(ii) In discussing a recent X-ray crystallographic structure of the *Rb. sphaeroides* RC, Ermler *et al*. (1994) have pointed out that ring V of P_L is bent toward helix C whereas the homologous ring V in *Rps. viridis* is bent in the opposite direction toward helix D. This significant conformational difference appears to be largely due to the residues Cys L247 and Met L248 found in *Rb. sphaeroides*, which are replaced by the smaller residues Gly and Thr, respectively, in *Rps. viridis*. Thus, primarily, the conformation of ring V of P_L in *Rb. sphaeroides* probably results in an energetically unfavorable contact between the C_9 keto carbonyl of P_L and Met L248. The genetic replacement of Met L248 in *Rb. sphaeroides* to a threonine residue resulted in a significant decrease in intensity as well a 4-cm^{-1} upshift of the FT Raman band arising from the C_9 keto carbonyl of P_L (Wachtveitl *et al*., 1993), which could be reflecting this contact. Thus, the temperature dependence of the 1692-cm^{-1} band of the C_9 keto group of P_L could be reflecting a change in the ring V conformation as the temperature is lowered, possibly helices C and D repositioning with respect to each other and thereby changing the Met L248 steric crowding of the C_9 keto carbonyl of P_L.

In summary, it appears that the observed 5-cm^{-1} upshift of the P_L C_9 keto carbonyl vibrational frequency largely originates from a specific temperature-dependent protein conformational change around P_L (the common element in the above points), largely implicating Met L248. However, other factors may be involved. For instance, we have observed that the 1691-cm^{-1} band had concomitantly upshifted to 1694 or 1696 cm^{-1} in some mutant RCs of *Rb. sphaeroides* for which a similar 2-cm^{-1} downshift of the P_L C_2 acetyl carbonyl band had occurred (Mattioli *et al*., 1994, 1995). These observations could be signalling that a perturbation (e.g., H-bond or change in orientation) on the C_2 acetyl carbonyl group of BChl could influence the vibrational frequency of the other C_9 carbonyl group. Resonance Raman studies (Willems & Bocian, 1984) have indicated that two conjugated carbonyl groups on the same porphyrin macrocycle can be coupled through its π-system and that one of the carbonyl groups can influence the electron-withdrawing capabilities of the second carbonyl group. At present it is not clear to what degree a change in the orientation of the C_2 acetyl carbonyl group with the macrocycle could also influence the vibrational frequency of the C_9 keto carbonyl.

Macrocycle Conformational Changes of the Primary Donor. The Raman data presented here lead us to conclude that the *ca*. 1610-cm^{-1} C_aC_m stretching modes of the four BChl molecules in the RC undergo an upshift in frequency as the temperature is lowered to 10 K which may be interpreted as the macrocycles adopting a more planar conformation at low temperatures. This same behavior is directly and specifically observed for the *ca*. 1610-cm^{-1} C_aC_m stretching modes of the two BChl molecules, P_L and P_M, constituting the primary donor. This is shown by the FT Raman spectrum of reduced P in which the 1607-cm^{-1} band, which corresponds to the C_aC_m methine bridge stretching modes of both P_L and P_M, upshifts by 5 cm^{-1} at 10 K as compared to room temperature.

Can the observed bacteriochlorophyll conformational changes deduced from the present data influence the electron transfer rate in the bacterial RC? Lowering the temperature should result in a contraction of the RC protein which in principle could decrease distances between the chromophores P, $BChl_L$, and $BPhe_L$. This contraction has not been determined for the bacterial RC but it has been for myoglobin. Frauenfelder *et al*. (1987) have determined a 3% (volume) shrinkage of myoglobin as the temperature was lowered to 80 K. DiMagno and Norris (1993) have argued that the changes in the $\pi-\pi$ contact distances between P and $BPhe_L$ estimated from a comparable 3% shrinkage for the RC would not be enough to explain the increase in electron transfer rate in the RC at cryogenic temperatures, assuming all other parameters influencing electron transfer rate were unchanged. The resulting BChl macrocycle flattening, deduced from our Raman data, as temperature is decreased could also be expected to decrease the distances between the chromophores participating in electron transfer. However, these changes in distance should be sizably less than 0.5 Å [see Barkigia and Fajer (1993) and references therein] and hence they still cannot account for the observed increase in electron transfer rate at low temperatures. The macrocycle conformational changes, however, have been calculated to affect the spectral and redox properties of the BChl molecules on the order of *ca*. 100 meV [Fajer *et al*., 1990; Barkigia and Fajer (1993) and references therein]. This could be an important factor in changing the energy of the $P^+BChl_L^-$ state as a function of temperature and thus the role of $BChl_L$ in a two-step or superexchange mechanism at room and low temperatures.

The reorganization energy is an important parameter in electron transfer theories (Marcus & Sutin, 1985) which describes how much the reactants and their environment change during the electron transfer reaction. It can be decomposed into an "inner" or "intramolecular" reorganization energy of the atoms of the reactants and an "outer" reorganization energy of the atoms of their surrounding medium. It is generally accepted that the "outer" contributions are often dominating. Inner reorganization energies of up to 200 meV have been measured for the transformation of aromatic phenylenediamine derivatives from their neutral to anionic states (Grampp & Jaenicke, 1984).

Recently, Ortega *et al*. (1996) have studied the $P^+Q_A^- \rightarrow PQ_A$ charge recombination reaction as a function of temperature for a series of mutant *Rb. sphaeroides* RCs where the $P^\circ/P^{\bullet+}$ redox midpoint potential was systematically altered. The increase in electron transfer rate as temperature decreased was modeled as principally arising from a decrease of *ca*. 300 meV in reorganization energy as temperature was lowered from 295 to 10 K. These authors were able to account for their temperature-dependent data using a Marcus surface where two vibrational modes, one at 150 cm^{-1} and another one at 1600 cm^{-1}, are coupled to this reaction. Similar values have been reported by other groups (Gunner & Dutton, 1989; Franzen & Boxer, 1993). Since high-frequency modes at, for example, *ca*. 1600 cm^{-1} are not

Boltzmann-populated at room temperature, their contributions to the reorganization energy are expected to be independent of temperature unless there is a specific temperature-dependent mechanism affecting these vibrational modes.

Ortega *et al*. (1996) did not propose a specific mechanism by which a mode at *ca*. 1600 cm^{-1} should be temperature dependent. In this work, we have identified a *ca*. 1610-cm^{-1} C_aC_m stretching vibrational mode of the BChl molecules in the reaction center that undergoes a temperature-dependent shift in frequency. This shift can be interpreted as a change (most likely flattening) in the BChl macrocycle conformation and thus could contribute to the temperature dependence of the reorganization energy. The carbonyl groups of the RC quinones and of the protein amide I mode vibrate at *ca*. 1660 cm^{-1} [see review by Mäntele (1995) and references therein] could also contribute; however, the temperature dependence of these modes has not been scrutinized.

One way the observed temperature-dependent BChl conformational changes could contribute to the decrease in reorganization energy is if they were similar to changes in the macrocycle conformation induced by oxidation (or reduction) of the BChl molecule. Not much is known concerning changes in macrocycle conformation of BChls in the RC upon oxidation (or reduction). However, if the redox-induced distortion of the BChl molecules in the RC during electron transfer is somehow mimicked by the temperature-dependent flattening we ascribe to the 4−6-cm^{-1} upshift of the *ca*. 1610-cm^{-1} C_aC_m modes, then the low-temperature flattening is expected to effectively reduce the "inner" reorganization energy of the BChl reactants (P and the accessory $BChl_L$). Thus the temperature-dependent conformational changes of the BChl macrocycles described in this work may in part contribute to the decrease in inner reorganization energy.

Macrocycle Conformational Changes of the Oxidized Primary Donor. Interestingly, the vibrational frequency of the C_9 keto carbonyl band of P_L at 1717 cm^{-1} (Figure 5B) does not shift sizably as a function of temperature when P is oxidized. Since the 1600−1603-cm^{-1} band corresponding to the νC_aC_m vibration of $P^{\bullet+}$ upshifts to a lesser degree as temperature is lowered as compared to the situation for neutral P, it appears that the BChl macrocycle for $P^{\bullet+}$ may be, as a whole, less susceptible to temperature-dependent conformational changes than the neutral species.

Positive Charge Localization on P_L. On the basis of our analysis of the upshift of the C_9 keto carbonyl of P_L as P is oxidized, we find that the degree of charge localization on P_L decreases as the temperature is lowered, and thus, the oxidized primary donor becomes more electronically symmetric at low temperatures.

This increase in electronic symmetry of the dimer P as temperature is lowered may be consistent with the picture that an increase in $\pi-\pi$ interaction of the P_L and P_M macrocycles has occurred at low temperature, either due to protein contraction moving the two macrocycles closer together or due to the purported flattening of the two macrocycles, extending the $\pi-\pi$ overlap, or a combination of both. An increase in the $\pi-\pi$ interaction/overlap would facilitate the sharing of the unpaired electron of $P^{\bullet+}$ over the two macrocycles. A thermal expansion model of the RC was proposed by Friesner and Won (1989) to account for the large red shift of the P absorption band as the temperature is lowered. In this model, P_L and P_M are trapped in a single potential well in which these two molecules are in their closest proximity (and, thus, most strongly interacting) at low temperatures.

^{1}H ENDOR measurements of the cation radical $P^{\bullet+}$ in RC single crystals from *Rb. sphaeroides* R26 at room temperature (288 K) revealed a spin density distribution of 2:1 (i.e., 66% localization) in favor of P_L (Lendzian *et al*., 1993). Recent ENDOR and ESEEM studies suggest that two distinct populations of $P^{\bullet+}$ states, probably due to two different protein conformational states, exist in bacterial RCs solubilized in the detergent LDAO, one which results in 66% and the other in 80% localization of unpaired electron spin density on P_L (Rautter *et al*., 1994); at room temperature, the 66% population should be dominant, while at low temperatures, upon freezing without cryoprotectants, the 80% population was observed (Rautter *et al*., 1994; Kass *et al*., 1995). This interpretation is not in agreement with our Raman observations in two important aspects: (i) We do not observe two $P^{\bullet+}$ populations with different positive charge localizations on P_L; FT Raman evidence for $P^{\bullet+}$ populations with 66% and 80% positive charge localization on P_L would be the observation of both a 1712- and a 1717-cm^{-1} band in the same spectrum, which is not the case at any temperature (Figure 5). (ii) The trend and behavior of charge localization on P_L as the temperature is lowered are both different; we interpret our Raman data as a *gradual* decrease in positive charge localization on P_L of a single population as temperature is lowered while the ENDOR data are interpreted as the abrupt increase in the population of $P^{\bullet+}$ states with increased unpaired spin density on P_L at temperatures below 273 K (Rautter, 1995). At present, the origin of this discrepancy is not known. It should be noted that, on the basis of the complex spectral signature in the 1700-cm^{-1} region in the light-induced $P^+Q_A^-/P°Q_A$ FTIR difference spectra of *Rb. sphaeroides* RCs and chromatophores, Morita *et al*. (1991, 1993) have interpreted their data as reflecting multiple $P^{\bullet+}$ structures which should differ in the degree of delocalization of the unpaired electron and that the degree of localization seemed to change with decreasing temperature (Morita *et al*., 1991). Again, in the FT Raman work reported here, we find no explicit evidence for multiple states of $P^{\bullet+}$ at any given temperature (see above).

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