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Temperature-Dependent Conformational Changes in the Bacteriopheophytins of *Rhodobacter sphaeroides* Reaction Centers†

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ABSTRACT: Resonance Raman (RR) spectra are reported for the photosynthetic reaction center (RC) protein from *Rhodobacter sphaeroides* 2.4.1. The spectra were obtained with a variety of excitation wavelengths, spanning the UV, violet, and yellow-green regions of the absorption spectrum, and at a number of temperatures ranging from 30 to 270 K. The RR data indicate that the frequencies of certain vibrational modes of the bacteriochlorin pigments in the RC shift with temperature. These shifts are reversible and do not depend on external factors such as solvent or detergent. The acetyl carbonyl bands exhibit the largest shifts with temperature. These shifts are attributed to thermal effects involving the torsional vibrations of the acetyl groups of several (or all) of the bacteriochlorins rather than to specific pigment-protein interactions. The frequency of the structure-sensitive skeletal mode near 1610 cm^{-1} of one of the two bacteriopheophytins (BPhs) in the RC is also sensitive to temperature. In contrast, no temperature sensitivity is observed for the analogous modes of the bacteriochlorophylls or other BPhs. Over the range 160-100 K, the skeletal mode of the BPh upshifts by $\sim$4 cm^{-1}. This upshift is attributed to a flattening of the macrocycle at low temperatures. It is suggested that the BPh active in the electron-transfer process is the pigment whose structure is temperature dependent. It is further suggested that such structural changes could be responsible in part for the temperature dependence of the electron-transfer rates in photosynthetic RCs.

The X-ray crystal structures recently reported for the reaction center (RC)[1] proteins from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* elegantly elucidate the macromolecular arrangement of the pigments in the protein matrix (Deisenhofer et al., 1984, 1985; Michel et al., 1986; Chang et al., 1986; Allen et al., 1987, 1988). These data delineate the distances between and relative orientations of the bacteriochlorophyll (BChl) and bacteriopheophytin (BPh) pigments which constitute the active sites for the initial steps of light-induced charge separation (Okamura et al., 1982). They also provide insight into the structure of the individual chromophores and the nature of the interactions between these species and the amino acid residues of the protein. Spectroscopic techniques provide other means for probing the structure of RCs [for reviews, see Parson (1982), Hoff (1984), Kirmaier and Holten (1987), and Friesner and Won (1989)]. In addition, these techniques provide information regarding the time scales of the electron-transfer events. Along these lines, optical absorption studies have shown that the initial light-induced transfer of an electron from the special pair donor $BChl_2$ to the primary acceptor BPh_L occurs in $\sim$3 ps at room tem-

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[1] Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; C_aC_m and C_bC_b, stretching modes of the carbon-carbon bonds in the methine bridges and β positions of the pyrrole rings, respectively, of the bacteriochlorin macrocycle; LDAO, lauryldimethylamine *N*-oxide; L and M, light and medium polypeptides, respectively, of the reaction center; Q_A, quinone; RR, resonance Raman; RC, reaction center; Tris, tris(hydroxymethyl)aminomethane.

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perature (Woodbury et al., 1985; Breton et al., 1986; Martin et al., 1986; Wasielewski & Tiede, 1986; Kirmaier & Holten, 1988a). This transfer occurs only through the chromophores in the L subunit despite the approximate 2-fold rotation symmetry which relates these pigments to those in the M subunit (Kirmaier et al., 1985b). Other spectroscopic studies have shown that the rate of electron transfer from $BChl_2$ to BPh_L increases by a factor of 2–4 (depending on the bacterial species) as the temperature is lowered from 300 to 10 K (Martin et al., 1988; Fleming et al., 1988). The rate of the next electron-transfer step, BP_L to the primary quinone (Q_A), has also been shown to increase as the temperature is lowered (for *Rb. sphaeroides*) (Kirmaier et al., 1985a; Kirmaier & Holten, 1988b). These spectroscopic data in conjuction with the crystal structures have led to the development of a number of theoretical models for the electronic structure of the pigments and the mechanism of electron transfer in RCs [for reviews, see Hanson (1988) and Friesner and Won (1989)].

In addition to optical absorption techniques, vibrational spectroscopy and in particular resonance Raman (RR) spectroscopy have been used to probe the structural features of photosynthetic RCs (Lutz, 1984; Lutz & Robert, 1985, 1988; Robert & Lutz, 1986, 1988; Zhou et al., 1985; Bocian et al., 1987; Schick & Bocian, 1987). The focal point of most of the RR studies has been the nature of the interactions (primarily hydrogen bonding) between the carbonyl groups on the BChls and BPhs (C_2 acetyl and/or the C_9 keto) and the protein matrix. On the basis of such studies, Lutz and co-workers have proposed assignments for the C_2 and C_9 carbonyl stretches, ν(C=O), for all six bacteriochlorins in the RC and made predictions concerning the extent of hydrogen bonding of these carbonyl groups to amino acid residues of the protein (Lutz & Robert, 1985, 1988; Robert & Lutz, 1986, 1988). These workers have also used the frequencies of the carbonyl modes as an indicator of structural changes which occur upon charge separation (Robert & Lutz, 1988).

Although the sensitivity of ν(C=O) to hydrogen bonding makes carbonyl modes useful probes of pigment–protein interactions, ν(C=O) is not particularly sensitive to the structure of the bacteriochlorin macrocycle. The macrocycle structure is more accurately reflected in the frequencies of the ring skeletal vibrations (Cotton & Van Duyne, 1981; Callahan & Cotton, 1987; Fujiwara & Tatsumi, 1986; Boldt et al., 1987; Schick & Bocian, 1987; Donohoe et al., 1988; Fonda & Babcock, 1987). For example, Lutz and co-workers have assigned the coordination numbers of the Mg(II) ions in the various BChls in the RC on the basis of the frequencies of the core size sensitive vibrations ca. 1610 cm^{-1} (Lutz & Robert, 1985; Robert & Lutz, 1986; Zhou et al., 1987). To date, however, RR studies have not exploited the structural sensitivity of the skeletal modes in attempts to explore more subtle effects of the protein environment on the macrocycles.

In this paper, we report RR spectra of RCs from *Rb. sphaeroides* 2.4.1 obtained with a number of excitation wavelengths which span the UV, violet, and yellow-green regions of the absorption spectrum. The spectra are acquired at a variety of temperatures ranging from 30 to 270 K. The skeletal mode frequencies are used to assess structural differences among the bacteriochlorin pigments at various temperatures. Collectively, these data provide further insight into the factors which might contribute to the temperature dependence of the electron-transfer rates in RCs.

Materials and Methods

The RCs from *Rb. sphaeroides* 2.4.1 were prepared as described by McGann and Frank (1985). The proteins were solubilized either in 0.01 M Tris (pH 8.0), 0.15% Triton X-100, and 0.5 M NaCl or in 0.01 M Tris (pH 8.0), 0.1% LDAO, and 0.5 M NaCl. Reduced RCs were prepared by the addition of sodium dithionite or sodium ascorbate.

The RR spectra were acquired in a 90° scattering configuration on a computer-controlled Spex Industries 1403 double monochromator equipped with a photon counting detection system and a Hamamatsu R928P photomultiplier tube. The excitation wavelengths (λ_{ex} = 363.8, 406.7, 514.5, 528.7, 530.9, 545.2, and 568.2 nm) were provided by an argon ion (Coherent Radiation INNOVA-15UV) or krypton ion (Coherent Radiation INNOVA-K3) laser or by an ion laser pumped continuous-wave dye laser (Coherent Radiation CR-590-03). The incident power at the sample was typically 30 mW or less. Spectra were acquired at 0.5–1.0 cm^{-1} intervals with a count of 1 s/point. The spectral slit width was ~4 cm^{-1} for all excitation wavelengths.

The relatively low light levels and small slit widths used in the RR experiments necessitated spectral averaging. The spectra reported are typically the average of 20 scans. In order to prevent photodegradation during data acquisition, the samples were frequently repositioned in the laser beam. Even a minor amount of photodamage (undetectable by absorption spectroscopy) is readily discernible in the RR experiments because loss of sample integrity invariably leads to the production of highly fluorescent impurities. In addition, all spectra were acquired at reduced temperatures (270 K and below). Temperature control was achieved by mounting the samples, contained in capillary tubes, via a home-built holder onto the cold tip of an ADP Cryogenics DE-202 Displex closed-cycle refrigeration system. RR spectra were obtained of both neat samples (detergent/buffer only) and samples in ethylene glycol (1:3). Spectra of reduced RCs were obtained in the glycol mixture. The laser line and/or the Raman bands of glycol were used for frequency calibration.

Results

General Features of the RR Spectra. RR spectra of the RCs obtained with UV excitation (λ_{ex} = 363.8 nm) are shown in Figure 1 (top). The general features of these spectra are similar to those previously reported by Lutz and co-workers (Lutz et al., 1976; Lutz, 1984; Lutz & Robert, 1988). The excitation wavelength is near the Soret band maximum, and the RR spectra exhibit bands from all the bacteriochlorin pigments in the RC. This is evidenced by the appearance of a number of features in the 1630–1710 cm^{-1} region which are ascribable to ν(C=O) of the C_2 and C_9 carbonyl groups of the various pigments. Lutz and co-workers have identified and assigned all 12 of these carbonyl vibrations by using a variety of difference techniques (Lutz & Robert, 1985; Robert & Lutz, 1986, 1988). These techniques were used in part because all of the ν(C=O) bands are not resolved under the relatively low resolution conditions (8–10 cm^{-1} slit widths) used to acquire the spectra. In our spectra, which were recorded with a 4 cm^{-1} spectral slit width, a number of the individual ν(C=O) bands are apparent in the congested 1650–1665 and 1675–1690 cm^{-1} regions. Robert and Lutz (1988) have assigned seven separate carbonyl vibrations in this spectral region.

The UV excitation RR spectra also exhibit bands due to the ring skeletal vibrations of the various bacteriochlorin pigments. For example, the strong band observed near 1610 cm^{-1} is a characteristic C_aC_m vibration of both BChl and BPh (Lutz, 1984; Donohoe et al., 1988). This mode is the analogue of the ν_{10} band of porphyrins and is extremely sensitive to the core size and degree of ruffling of the macrocycle (Callahan

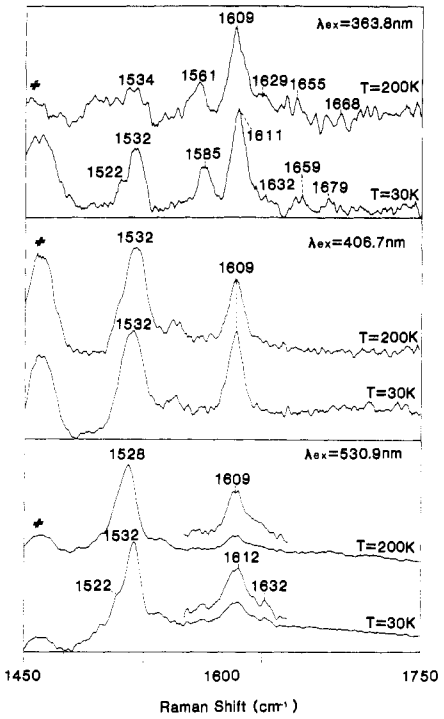

FIGURE 1: RR spectra of RCs from *Rb. sphaeroides* 2.4.1 in ethylene glycol glasses at 200 and 30 K with λ_{ex} = 363.8 nm (top), λ_{ex} = 406.7 nm (middle), and λ_{ex} = 530.9 nm (bottom). The band marked with the symbol (#) is due to the solvent.

& Cotton, 1987). Shoulders are observed on both the high-energy (~1620 cm^{-1}) and low-energy (~1602 cm^{-1}) sides of the 1610 cm^{-1} band. These features have not previously been observed in the RR spectra of RCs; however, they have been seen in the spectra of BChl model compounds (Donohoe et al., 1988). Normal coordinate calculations indicate that these bands are also due to C_aC_m vibrations. The relatively strong band in the 1580–1585 cm^{-1} region is due to scattering from another C_aC_m mode of the ring skeleton. This band is characteristically strong in BPh and weak in BChl. Bands assignable to other skeletal modes are observed near 1530 cm^{-1}. Normal coordinate calculations on BChl model compounds indicate that these bands correspond to C_bC_b vibrations of the ring skeleton (Donohoe et al., 1988). RR spectra of the RCs obtained with violet (λ_{ex} = 406.7 nm) excitation are shown in Figure 1 (middle). This excitation wavelength is on the red edge of the Soret band. The low-energy side of the composite Soret absorption should contain contributions primarily from the BChls because metalation of BPh results in a red shift of all the absorption maxima (Gouterman, 1978). The general features observed in the RR spectra are consistent with scattering only from these pigments. In particular, the characteristic BPh band in the 1580–1585 cm^{-1} region is absent while the ~1610 cm^{-1} band remains. There is no evidence of any splitting of this latter band which indicates that the frequencies of the ν_{10}-like skeletal modes of the BChls are essentially identical. The absence of BPh scattering should also be manifested in the carbonyl region. However, the overall Raman cross-section is quite low with λ_{ex} = 406.7 nm, and bands due to ν(C=O) are not clearly observed for any of the pigments. Bands due to the C_bC_b stretches are observed near 1530 cm^{-1}; however, carotenoid scattering is resonance-enhanced with violet excitation, and these pigments also exhibit a strong band near 1530 cm^{-1} (Lutz, 1984; Lutz et al., 1987).

Representative RR spectra of the RCs obtained with green excitation (λ_{ex} = 530.9 nm) are shown in Figure 1 (bottom). Excitation into the green region of the absorption spectrum

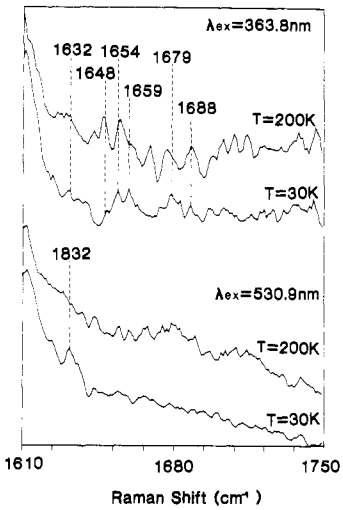

FIGURE 2: Expanded view of the carbonyl region RR spectra of RCs from *Rb. sphaeroides* 2.4.1 in ethylene glycol glasses at 200 and 30 K with λ_{ex} = 363.8 nm (top) and λ_{ex} = 530.9 nm (bottom).

allows selective resonance enhancement of the Raman bands of the BPhs (Lutz et al., 1976; Lutz, 1984). The band observed near 1610 cm^{-1} is due to scattering from the ν_{10}-like skeletal modes of these pigments. The 1580–1585 cm^{-1} RR band of BPhs is absent from the spectrum because this mode is not resonance-enhanced with Q_x excitation (Lutz et al., 1976). The general appearance of the 1610 cm^{-1} RR band observed with green excitation is somewhat different from those observed with the violet and UV excitation wavelengths (cf. Figure 1, top and middle panels). In particular, the band exhibits an asymmetric shape which could be due to slightly unequal contributions to the band contour by the ν_{10}-like modes of BPh_L and BPh_M. If so, the asymmetric appearance of the coutour would suggest that the ν_{10}-like modes of the two BPhs exhibit slightly different frequencies. In attempts to gain further insight into the nature of the composite RR band, spectra were acquired with other excitation wavelengths throughout the Q_x absorption bands of the two BPhs [Q_{max} = 531 nm for BPh_M and 546 nm for BPh_L (Dutton et al., 1978; Kirmaier et al., 1985b)]. Unfortunately, the relatively close proximity of the two absorptions precludes selective enhancement of the RR bands of an individual BPh. Scattering from the vibronic satellites of the Q_x bands further complicates the interpretation of the RR intensities in this region. This latter complication can be minimized by exciting to the red of the low-energy absorber; however, the RR cross-section in this region is extremely low. Consequently, we are unable to assign the individual ν_{10}-like modes of the BPhs. Because the RR cross-section with green excitation is quite low in general, there are not a large number of other features discernible in these spectra. The strong carotenoid band near 1530 cm^{-1} is the only exception.

Temperature Dependence of the RR Spectra. Representative RR spectra acquired at high (200 K) and low (30 K) temperatures are compared in Figure 1. As is evident, there are a number of differences between the spectra at the two temperatures. Frequency shifts and line shape changes are observed for the RR bands of the carbonyl and skeletal modes of the bacteriochlorin macrocycles as well as for the carotenoid vibrations. This is illustrated in Figure 2 which shows an expanded view of the RR scattering observed from the carbonyl modes at 200 versus 30 K. At 200 K, the frequencies of a number of the ν(C=O) bands appear to be shifted from those observed at 30 K. The effects are most pronounced for the bands of the C_2 acetyl groups which lie principally in the

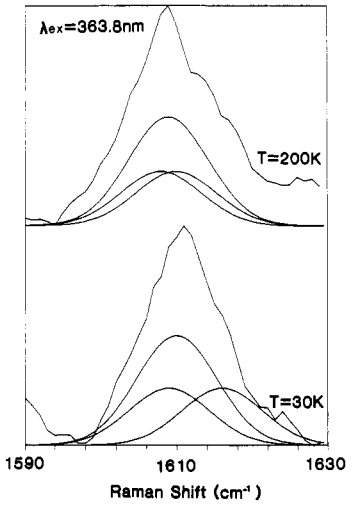

FIGURE 3: Expanded view of the 1610 cm^{-1} RR band of the RCs from *Rb. sphaeroides* 2.4.1 in ethylene glycol glasses at 200 and 30 K with λ_{ex} = 363.8 nm. The three Gaussian curves used to simulate the observed band are shown for both temperatures (see text).

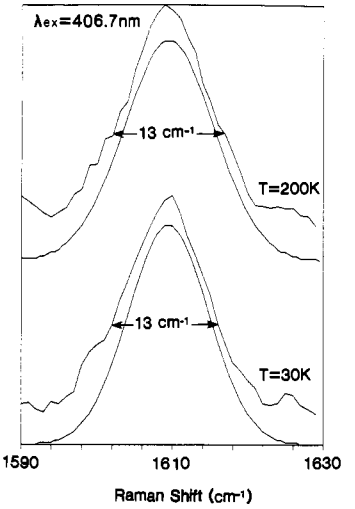

FIGURE 4: Expanded view of the 1610 cm^{-1} RR band of RCs from *Rb. sphaeroides* 2.4.1 in ethylene glycol glasses at 200 and 30 K with λ_{ex} = 406.7 nm. The Gaussian curve used to simulate the observed composite band is shown for both temperatures (see text). The observed and calculated curves are translated vertically with respect to one another for clarity.

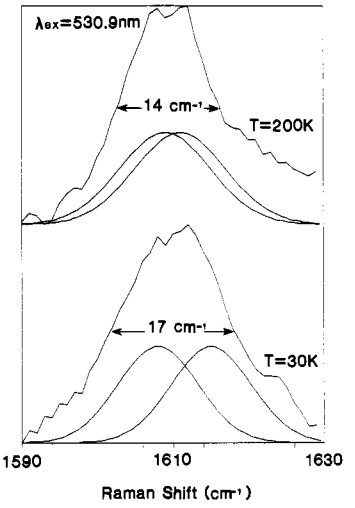

FIGURE 5: Expanded view of the 1610 cm^{-1} RR band of RCs from *Rb. sphaeroides* 2.4.1 in ethylene glycol glasses at 200 and 30 K with λ_{ex} = 530.9 nm. The two Gaussian curves used to fit the observed composite band are shown for both temperatures (see text).

1630–1660 cm^{-1} region. For example, the group of bands observed in the region 1650–1660 cm^{-1} at 30 K appears to be downshifted by ~6 cm^{-1} at 200 K. However, the complexity of the spectra and the relatively low signal-to-noise ratios in the carbonyl region preclude any detailed assessment of the effects of altering the temperature.

The effects of temperature on the bacteriochlorin skeletal vibrations are particularly noteworthy. Inspection of Figure 1 reveals that the low-frequency shoulder observed on the 1581 cm^{-1} BPh band at 200 K is absent at 30 K. In addition, the band maximum upshifts to 1585 cm^{-1} upon lowering the temperature from 200 to 30 K. Temperature-dependent shifts are also observed for the RR bands of the ν_{10}-like modes. An expanded view of the UV excitation scattering observed for this latter vibration is shown in Figure 3. The frequency of the band maximum observed at 30 K is approximately 2–3 cm^{-1} higher than that observed at 200 K (Figure 3).[2] In addition, the shoulders resolved at 200 K on the high-frequency side of the band appear to be filled in at 30 K.

The behavior of the 1581 and 1610 cm^{-1} bands indicates that the BPhs are at least in part responsible for the temperature-dependent spectral changes. The exact nature of this involvement is elucidated by examining the effects of temperature on the violet and green excitation RR spectra. Expanded views of the scattering observed for the ν_{10}-like vibrations at these two excitation wavelengths are shown in Figures 4 and 5, respectively. Inspection of Figure 4 reveals that the frequencies, line widths, and other features of the 1610 cm^{-1} bands observed with violet excitation are identical at 200 and 30 K. Inasmuch as this wavelength excites only the BChls (vida supra), it appears that these pigments are not responsible for the temperature-dependent spectral changes observed with UV excitation. In contrast, temperature-dependent spectral changes are again observed upon excitation of the BPhs with green excitation (Figure 5). With this excitation wavelength, the frequency of the ν_{10}-like band centroid is approximately ~3 cm^{-1} higher at 30 K than at 200 K (Figure 5). In addition, the width of the composite band is ~3 cm^{-1} larger at the lower temperature. Collectively, the appearance of the RR spectra suggests that the BPhs are exclusively responsible for the temperature-dependent spectral changes.

A variety of RR experiments were performed in attempts to determine factors that might influence the temperature-dependent spectral changes. First, the temperature was cycled repeatedly between 200 and 30 K. RR spectra were acquired at each temperature with both UV and green excitation. In all cases, the observed spectral features were essentially identical with those shown in the figures. The reproducibility of the spectral changes suggests that they reflect intrinsic changes in the pigment–protein complex. Next, RR spectra were compared for RCs prepared in Triton and LDAO. Both neat (detergent/buffer only) and ethylene glycol diluted samples were examined. Changing the detergent and/or the composition of the sample solution did not influence the spectral features at any temperature. Finally, RR spectra were obtained for RCs in the presence and absence of exogenous reductants in order to determine whether altering the concentration of oxidized $BChl_2$ influenced the temperature dependence of the spectral features. Again, this had no apparent influence on the observed features. In the course of these

[2] The RR frequencies we observe for the skeletal modes of the bacteriochlorin macrocycle at 30 K are uniformly ~4–5 cm^{-1} lower than those reported by Lutz and co-workers (Robert & Lutz, 1986, 1988). The origin of this descrepency is not known.

various experiments, approximately 20 separate data sets of 20 scans each were acquired. In all instances, the spectral features shown in the figures were reproduced. These observations further suggest that the temperature-dependent spectral changes reflect intrinsic changes in the pigment–protein complex and that these alterations are not governed by external factors such as solvent–protein and/or detergent–protein interactions.

In order to gain additional insight into the nature of the temperature-dependent frequency shifts of the RR bands, we performed computer simulations on the 1610 cm^{-1} contours. These bands were chosen because they exhibit the largest RR cross-sections at all excitation wavelengths. It should be emphasized that the objective of the simulations was only to determine whether a self-consistent picture of the temperature-dependent effects could be obtained for the UV, violet, and green excitation RR spectra. The simulations were not intended to obtain a set of unique or exact spectral parameters at a given excitation wavelength. In order to minimize the number of adjustable parameters, we assumed that only the ν_{10}-like skeletal modes contribute strongly to the composite band. This assumption in itself precludes an exact fitting of the band contours because RR scattering from other C_aC_m modes clearly contribute to the spectrum in the region of the 1610 cm^{-1} band (vida supra).

A representative set of bands whose sum reproduces the trends observed in the RR spectra is shown in Figures 3–5 along with the observed bands. These calculated curves were obtained by first simulating the ν_{10}-like bands observed in the violet and green excitation RR spectra. These simulations yield a set of spectral parameters for the BChls and BPhs which scatter exclusively in these two wavelength regions, respectively. The composite band observed for the various BChls at both 200 and 30 K is well reproduced by a single Gaussian curve with a full width at half-maximum of 13 cm^{-1} (Figure 4). This width is equal to that observed and is comparable to that observed for the ν_{10}-like band of model compounds (Donohoe et al., 1988). When 13 cm^{-1} is used as the intrinsic width of the ν_{10}-like RR band, the temperature-dependent behavior observed with green excitation can only be reproduced if two Gaussians of approximately equal or slightly unequal intensity are used in the simulation. Reasonable fits to the 200 K contour are obtained with one component centered near 1608–1609 cm^{-1} and the other near 1610–1611 cm^{-1} (Figure 5). The best fits to the 30 K RR spectra are obtained by maintaining one component near 1608–1609 cm^{-1} and shifting the other to 1614–1615 cm^{-1}. Good fits can also be obtained by fixing the position of the higher energy component and allowing the lower energy component to shift past it. Either of these shift patterns can reasonably account for the $\sim$3 cm^{-1} upshift of the centroid and the $\sim$3 cm^{-1} increase in width of the composite band. The fact that the qualitative features of the composite band contour can be reproduced in this fashion suggests that the ν_{10}-like mode of only one of the two BPhs might be sensitive to changes in temperature.

After the spectral parameters for the BChls and the BPhs from the violet and green excitation RR spectra were obtained, these parameters were fixed and used in attempts to reproduce the temperature dependence of the 1610 cm^{-1} band contour observed in the UV excitation spectra. The only adjustable parameter used in this fit was the relative contribution of the BChls and the BPhs to the total intensity of the RR band. There is no way of independently estimating this parameter. The peak position and general features of the composite band observed in the UV excitation spectra at both 200 and 30 K

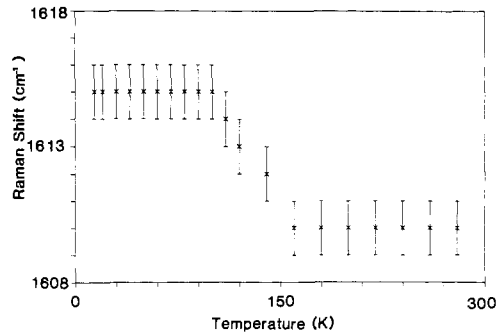

FIGURE 6: Temperature dependence of the frequency of the ν_{10}-like skeletal mode of the BPh. The points were determined by fitting the composite band observed with λ_{ex} = 363.8 nm (see text).

can be reproduced if the integrated intensity of the single Gaussian which represents the various BChls is assumed to be approximately equal to the total intensity of the two Gaussians which represent the BPhs (Figure 3). These relative intensities are reasonable inasmuch as the RR intensity of the 1610 cm^{-1} band is derived primarily from the two accessory BChls and the two BPhs. The $BChl_2$ moiety contributes only a small amount to the total intensity of the composite RR band (Robert & Lutz, 1986, 1988). (Under the illumination conditions used in our experiments, $BChl_2$ should be to a large extent photooxidized; thus, the contribution of this moiety to the RR scattering should be further diminished.)

In order to examine the detailed nature of the temperature dependence of the ν_{10}-like RR band of the BPhs, spectra were obtained at a variety of temperatures between 30 and 270 K. These spectra were obtained with UV rather than green excitation because the larger RR cross-section in the UV region affords a better spectral signal-to-noise ratio. The spectra were then simulated by varying the frequency of the same Gaussian component which was allowed to shift in the fits of the 200 and 30 K green excitation RR spectra. All other spectral parameters were held fixed at their previously determined values. The resulting plot of frequency versus temperature for the shifting Gaussian component is shown in Figure 6. As can be seen, there appears to be little or no shift in the RR band in the range 270–160 K. Between 160 and 100 K, the upshift occurs. The frequency then remains approximately constant as the temperature is lowered further. It should be noted that the shifting Gaussian component can be positioned anywhere in a 2 cm^{-1} window; thus, the actual temperature dependence may be considerably more or less steep than that shown in the figure. It is clear, however, that this component must shift on the order of 4–6 cm^{-1} between the lowest and highest temperatures in order to reproduce the frequencies observed for the composite RR band.

Discussion

The RR data indicate that the frequencies of certain vibrational modes of the bacteriochlorin pigments in the RC shift with temperature. These shifts are reversible and do not depend on external factors such as solvent or detergent. The temperature-dependent changes in the acetyl carbonyl region of the spectrum appear to involve several or all of the bacteriochlorins in the RC. This observation suggests that no specific structural change can be associated with the temperature-dependent shifts of these vibrations. Instead, these shifts most likely occur because the acetyl groups undergo significant torsional oscillations which freeze out at low temperature. Due to anharmonicity, the average torsional angles in the thermally populated states should be different; thus, the extent of conjugation of the C=O group into the π system

of the macrocycle will vary as a function of temperature. This would result in a distribution of frequencies for ν(C=O). The higher frequencies for ν(C=O) at 30 K versus 200 K suggest that the extent of conjugation is less at the lower temperature. In this regard, crystallographic studies on BChl indicate that the acetyl group is ~22° out-of-plane in the solid state (Barkigia et al., 1989).

Unlike the carbonyl groups, only the skeletal modes of the BPhs appear to be sensitive to changes in temperature. The spectral simulations of the ν_{10}-like band contour indicate that the simplest explanation for the trends observed in the RR spectra is that only one of the two BPhs in the RC is sensitive to temperature. Obviously, the spectra fits are not unique. Nevertheless, the possibility that only one of the two pigments is responsible for the RR shift patterns is intriguing. A possible alternative explanation for the doubled ν_{10}-like band is that the two BPhs in a given RC have identical frequencies and that there is a second form of the protein in which the frequencies of the ν_{10}-like modes of both BPhs are different. However, this seems unlikely because it requires that the relative populations of the two forms are approximately equal at all temperatures and that the RR bands of both BPhs in one form are sensitive to temperature while those in the other are not. Accordingly, it seems more reasonable that the doubled ν_{10}-like RR band is due to structural differences between BPh_L and BPh_M. This requires that the protein matrix exerts a unique influence on each of these pigments. The question remains as to the exact nature of the pigment–protein interaction and the resulting effect on the structure of the bacteriochlorin macrocycle.

The ν_{10}-like vibration is extremely sensitive to the core size and the extent of ruffling of the macrocycle (Callahan & Cotton, 1987). In the case of the BPhs, the absence of the Mg ion precludes modulation of the core size via changes in axial ligation. Consequently, the temperature-dependent frequency of the ν_{10}-like mode implies changes in the extent of out-of-plane distortion of the BPh macrocycle. The upshift of ~4 cm^{-1} which occurs upon lowering the temperature suggests that the ring is substantially flatter at low temperatures. Frequency shifts of this size cannot be attributed to subtle effects such as changes in extent of hydrogen bonding. In this regard, Callahan and Cotton (1987) have shown that the formation of hydrogen bonds to all the C=O groups of BChls in solution does not affect the frequency of the ν_{10}-like skeletal mode. It is difficult to envision the types of pigment–protein interaction that could lead to structural changes such as flattening of the macrocycle. This is particularly true in the case of the BPhs because the absence of the central metal ion precludes direct pigment–protein complex via an axial ligand to the metal. However, protein-induced structural changes similar to those we observe in RCs have been reported by Alden et al. (1990) in a recent study of hemoglobins reconstituted with Ni(II) porphyrins. In this system, the skeletal mode frequencies of the porphyrin in the protein are ~5 cm^{-1} higher than those in solution despite the fact that the Ni(II) ion is not axially ligated in either case. This observation provides additional evidence that long-range pigment–protein interactions can influence the degree of planarity of the macrocycle. It is possible that such interactions with one of the BPhs in the RC vary considerably as a function of temperature.

Conclusions

The RR spectra obtained for the RCs from *Rb. sphaeroides* indicate that the frequencies of a number of the vibrational modes are sensitive to temperature. In the case of the carbonyl stretches, the frequency shifts are not associated with specific pigment–protein interactions. In contrast, the simplest explanation for the skeletal mode shifts of the BPhs is that the conformation of only one of these two pigments in the RC is sensitive to temperature. The RR data presented herein cannot determine whether this pigment is BPh_L or BPh_M. However, recent RR studies on genetically modified RCs from *Rb. capsulatus* confirm that it is the active electron-transfer pigment BPh_L (J. M. Peloquin, E. J. Bylina, D. C. Youvan, and D. F. Bocian, unpublished experiments). It is interesting to speculate how protein interactions which influence the conformation of BPh_L could also influence the electron-transfer process in RCs. Structural changes in the macrocycle could alter both the electronic and Franck–Condon factors which mediate electron-transfer rates. Flattening of the macrocycle is one way in which the effective distance between BPh_L and both $BChl_2$ and Q_A could conceivably be reduced. Another way to shorten both distances is translation of the entire pigment in the protein pocket. This in turn could modify the pigment–protein interactions which influence the conformation. Such changes could in part account for the acceleration of the rates of transfer both from $BChl_2$ to BPh_L and from BPh_L to Q_A which occur in *Rb. sphaeroides* as the temperature is lowered (Martin et al., 1988; Fleming et al., 1988; Kirmaier et al., 1985a).

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Photoaffinity Labeling of Bacteriorhodopsin†

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ABSTRACT: ^{14}C-Labeled optically pure 3*S*- and 3*R*-(diazoacetoxy)-*all-trans*-retinals were incorporated separately into bacterioopsin to reconstitute functional bacteriorhodopsin (bR) analogues, 3*S*- and 3*R*-diazo-bRs. UV irradiation at 254 nm generated highly reactive carbenes, which cross-linked the radiolabeled retinals to amino acid residues in the vicinity of the β-ionone ring. The 3*S*- and 3*R*-diazo analogues were found to cross-link, respectively, to cyanogen bromide fragments CN 7/CN 9 and CN 8/CN 9. More specifically, Thr121 and Gly122 in fragment CN 7 were found to be cross-linked to the 3*S*-diazo analogue. The identification of cross-linked residues and fragments favors assignments of the seven helices A-G-F-E-D-C-B or B-C-D-E-F-G-A to helices 1-2-3-4-5-6-7 in the two-dimensional electron density map (Henderson et al., 1975, 1986; Mogi et al., 1987). The present results show that the chromophore chain is oriented with the ionone ring inclined toward the outside of the membrane (the 9-methyl group also faces the extracellular side of the membrane).

Bacteriorhodopsin (bR),[1] a light-driven proton pump, is the sole protein found in the purple membrane of *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971; Stoeckenius & Bogomolni, 1982). It consists of a polypeptide of 248 amino acids (Ovchinnikov et al., 1979; Khorana et al., 1979) and a chromophore, *all-trans*-retinal, covalently bound to Lys216 on helix G via a protonated Schiff base linkage (Bayley et al., 1981; Lemke & Oesterhelt, 1981; Mullen et al., 1981) (Figure 1). There are seven discrete hydrophobic segments in the bR

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[1] Abbreviations: bR, bacteriorhodopsin; CN *x*, fraction *x* obtained upon cyanogen bromide cleavage of bacteriorhodopsin; 3*R* (or 3*S*) bR, bacteriorhodopsin regenerated from 3*R* (or 3*S*)-(diazoacetoxy)-*all-trans*-retinal; PTH, phenylthiohydantoin; SE, size exclusion; bO, bacterioopsin.

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