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Accelerated Publications

Primary Donor Structure and Interactions in Bacterial Reaction Centers from Near-Infrared Fourier Transform Resonance Raman Spectroscopy†

Tony A. Mattioli,*,‡ Andreas Hoffmann,§ Bruno Robert,‡ Bernhard Schrader,§ and Marc Lutz‡

Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France, and Institut für Physikalische und Theoretische Chemie, Universität Essen, 4300 Essen 1, Germany

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ABSTRACT: Preresonance Raman and resonance Raman spectra of the primary donor (P) from reaction centers of the *Rhodobacter* (*Rb.*) *sphaeroides* R26 carotenoidless strain in the P and P^+ states, respectively, were obtained at room temperature with 1064-nm excitation and a Fourier transform spectrometer. These spectra clearly indicate that the chromophore modes are observable over those of the protein with no signs of interference below 1800 cm^{-1}. The chromophore modes are dominated by those of the bacteriochlorophylls (BChl *a*), and it is estimated that, in the P state, ca. 65% of the Raman intensity of the BChl *a* modes arises from the primary donor. This permits the direct observation of a vibrational spectrum of the primary donor at preresonance with the excitonic 865-nm band. The Raman spectrum of oxidized reaction centers in the presence of ferricyanide clearly exhibits bands arising from a BChl a^+ species. The magnitude of the frequency shift of a keto carbonyl of neutral P from 1691 to 1717 cm^{-1} upon P^+ formation strongly suggests that one BChl molecule in P^+ carries nearly the full +1 charge. Our results indicate that the unpaired electron in $P^{\bullet+}$ does not share a molecular orbital common to the two components of the dimer on the time scale of the resonance Raman effect (ca. 10^{-13} s).

The primary events in bacterial photosynthesis occur in membrane-bound proteins known as reaction centers (RCs).[1] The isolated RC consists of six bacteriochlorin pigments (four bacteriochlorophyll *a* and two bacteriopheophytin *a* molecules), two quinones, one non-heme iron, one carotenoid molecule, and approximately 850 amino acid residues contained in three polypeptide subunits named L, M, and H. Within the RC, electron transfer originates from the primary donor P, which consists of a pair of bacteriochlorophyll (BChl) molecules in mutual excitonic interaction. Although the X-ray crystallographic structures of the RC from *Rhodopseudomonas* (*Rps.*) *viridis* (Deisenhofer & Michel, 1989) and *Rhodobacter* (*Rb.*) *sphaeroides* (Allen et al., 1987a,b; Chang et al., 1986; Tiede et al., 1988) are resolved, the understanding of charge separation and stabilization requires a thorough characterization of the physicochemical properties of P and its cation radical, $P^{\bullet+}$.

The absorption spectrum of bacterial reaction centers exhibits a broad band in the near-infrared that corresponds to the first excited singlet state of the primary donor pair, ^1P. For bacteriochlorophyll *a* (BChl *a*) containing RCs, such as *Rb. sphaeroides*, this band appears at ca. 870 nm. The characterization of this band in an attempt to explain the asymmetric functioning of the RC has been the subject of recent intensive work [for a review, see Friesner and Won (1989)]. When P undergoes one-electron chemical or pho-

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* Author to whom correspondence should be addressed.
‡ CE Saclay.
§ Universität Essen.

[1] Abbreviations: RR, resonance Raman; NIR, near-infrared; FT, Fourier transform; RC, reaction center; *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; *Rsp.*, *Rhodospirillum*; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; EPR, electron paramagnetic resonance; THF, tetrahydrofuran.

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tochemical oxidation, this near-infrared P band bleaches and a new absorption band appears at 1240–1270 nm. Thus, the 1250-nm band in the oxidized RC of *Rb. sphaeroides* presumably belongs to the oxidized primary donor cation radical, $P^{\bullet+}$. Spin-density calculations compared with experimental ENDOR/TRIPLE data from the oxidized primary donors from *Rb. sphaeroides* and *Rps. viridis* (Plato et al., 1990, 1988) show spin distributions in P^+ that differ markedly from C_2 symmetry for both species. As well, the orientation of the triplet state, ^3P, of the primary electron donor as determined in single-crystal EPR studies of the RC reveals an asymmetric localization of this triplet state on the P_L component of P; this localization seems more pronounced in *Rps. viridis* than in *Rb. sphaeroides* (Norris et al., 1989). Resonance Raman spectroscopy, exciting in the Soret band of the RC, has also probed the asymmetry of the ground state of P at the level of the unequivalent bonding pattern of the conjugated carbonyls of P_L and P_M for both *Rb. sphaeroides* (Robert & Lutz, 1986) and *Rps. viridis* (Zhou et al., 1989). Such structural asymmetry has been observed in the crystal structure of the *Rps. viridis* RC (Deisenhofer & Michel, 1989).

Resonance Raman (RR) spectroscopy has been successfully used for studying protein–(bacterio)chlorin interactions and, in particular, the vibrational structure of P [for reviews, see Lutz (1984), Lutz and Robert (1988), and Robert (1990)]. However, obtaining the RR contributions of P has been, up to now, achieved through Soret-excited difference Raman techniques (Robert & Lutz, 1986; Robert, 1990). Indeed, direct excitation within the lowest lying P absorption band has encountered serious technical difficulties. RR spectra of *Rb. sphaeroides* RCs were recently reported (Donohoe et al., 1990) with excitation wavelengths spanning the 870-nm absorption band; however, as they contain vibrational frequencies of up to 250 cm^{-1} only, their structural information content remains limited. Similarly, although RR spectroscopy has been used for studying monomeric BChl a^+ cations in vitro (Lutz & Klëo, 1979; Cotton et al., 1980), its application to the study of P^+ has proven technically difficult (Lutz & Klëo, 1979). Thus, until now, vibrational Raman information concerning P has been limited while that of its cation radical has remained scarce.

With the recently introduced technique of near-infrared (NIR) Fourier transform (FT) Raman spectroscopy, the Raman effect is excited by using NIR laser radiation, generally the 1064-nm emission from the Nd:YAG laser, and the analysis of the spectrum is accomplished by interferometry, which compensates for the loss in the Raman scattering cross section at this wavelength (Hirschfeld & Chase, 1986; Schrader & Simon, 1988; Schrader et al., 1990). This technique has already been applied to biological systems such as bacteriorhodopsin (Sawatski et al., 1990), and its use for obtaining Raman spectra of bacterial RCs has recently been reported (Mattioli et al., 1990; Johnson & Rubinovitz, 1990). In this work, we demonstrate that the use of NIR FT Raman spectroscopy with excitation at 1064 nm results in the direct observation of a preresonance Raman spectrum of P or a resonance Raman spectrum of P^+, according to the redox potentials at which the RCs are poised.

Experimental Procedures

Materials. *Rb. sphaeroides* R26 RCs were isolated as described in Robert and Lutz (1986). The sample concentration was 640 μM in 5% cholate and 50 mM Tris, pH 8.6. Under these conditions the P band of the RC samples showed an absorption maximum at 865 nm. The pigments BChl *a* and BPhe *a* were purified as described in Berger et al. (1990).

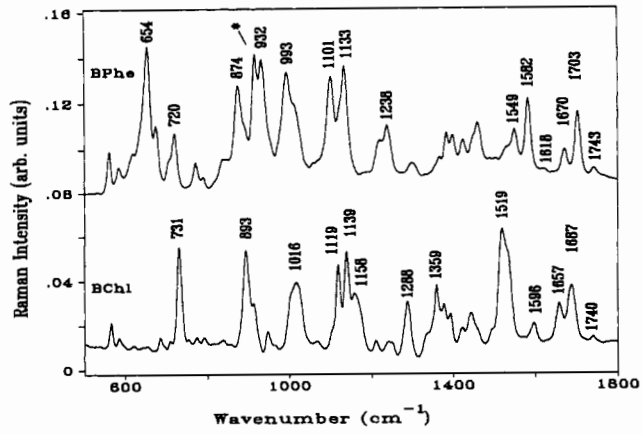

FIGURE 1: Fourier transform preresonance Raman spectrum of 10^{-2} M (a) BPhe *a* and (b) BChl *a* in dry THF excited at 1064 nm. The asterisk denotes the THF solvent band. The spectral resolution is 4 cm^{-1}. Conditions: room temperature; 150-mW laser power; coaddition of 500 scans.

Solutions of 10^{-2} M in dried, argon-purged tetrahydrofuran (THF) were used.

Methods. FT Raman spectra were recorded with a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous, diode-pumped Nd:YAG laser. Typical laser powers used were 150–200 mW. Unless otherwise stated, biological samples were contained in a sapphire cell (Schrader et al., 1990) while in vitro pigment samples were contained in a quartz tube approximately 5 mm in diameter.

Raman photons were collected with a 180° backscattering geometry. All spectra were recorded at room temperature and were the result of the coaddition of 1000 interferograms within a total recording time of about 50 min. The spectra presented were corrected for luminescent background by using a polynomial fit to the spectra. Corrections to the different backgrounds led to difference spectra whose baselines were not perfectly flat, thus making a zero point difficult to obtain accurately. However, by careful comparison of the difference spectrum with the P^+ and P spectra, the disappearance and appearance of most bands may be unambiguously noted.

Results and Discussion

BChl a and BPhe a in Vitro. Figure 1 shows the room temperature FT Raman spectra of monomeric BChl *a* and BPhe *a* at ca. 10^{-2} M in THF. We have already discussed in some detail the FT preresonance Raman spectrum of BChl *a* excited at 1064 nm at room temperature as compared to that excited in Soret resonance at 363.8 nm at 15 K. The different intensity patterns that are observed are indicative of a preresonance condition with the lowest lying Q_y absorption band (Mattioli et al., 1990). The C_aC_m stretching mode of BChl is sensitive to the number of axial ligands coordinated to the central Mg atom (Cotton & Van Duyne, 1981). In the Soret-excited RR spectrum of BChl *a*, this most intense band is observed at ca. 1610 cm^{-1} or ca. 1600 cm^{-1}, depending if one or two axial ligands, respectively, are present. In the FT preresonance Raman spectrum of BChl *a* in THF where the central Mg atom possesses two axial ligands, the C_aC_m mode appears as a weak band at 1596 cm^{-1}.

Both FT Raman spectra show intense bands arising from the π-conjugated carbonyl stretching modes. The C_2 acetyl and C_9 keto carbonyl stretching bands of BChl *a* in THF, a solvent that is not a H-bond donor, appear at 1658 and 1687 cm^{-1}, respectively, while for BPhe *a* in THF they appear at 1670 and 1703 cm^{-1}, respectively. The stretching mode of the

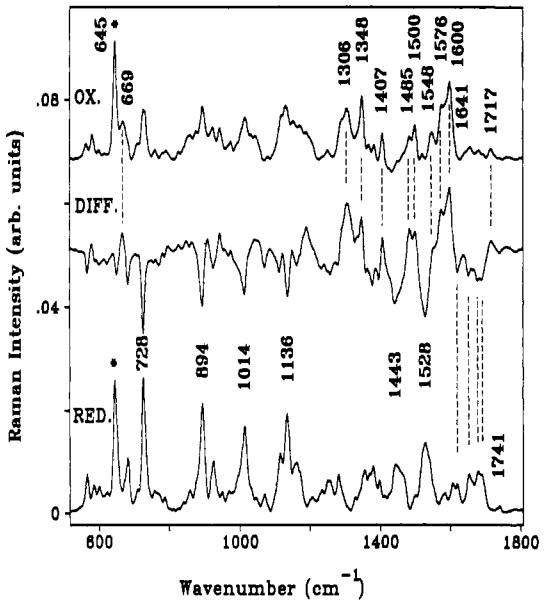

FIGURE 2: (Bottom) Fourier transform preresonance Raman spectrum of the RC from *Rb. sphaeroides* R26 in the presence of ascorbate (reduced RC) with 180-mW laser power. (Top) Fourier transform Raman spectrum of the RC in the presence of ferricyanide (oxidized RC) with 280-mW laser power. (Middle) Difference spectrum P^+ minus P calculated by subtracting the bottom spectrum from the top spectrum, normalizing on the C–H band at ca. 2900 cm^{-1}. In the difference spectrum, negative peaks represent the preresonance Raman bands of P while the positive bands represent the resonance Raman band of P^+. Asterisks denote the 645-cm^{-1} sapphire band. The spectral resolution is 4 cm^{-1}. Conditions: room temperature; coaddition of 1000 scans.

C_{10a} carbomethoxy ester carbonyl is very weakly active in many RR spectra of BChls (Andersson et al., 1989; Lutz & Mäntele, 1991). However, this mode is clearly active in the FT Raman spectra of BChl *a* and BPhe *a* at 1740 and 1743 cm^{-1}, respectively (Figure 1).

Comparison of the FT Raman spectra of BChl *a* and BPhe *a* indicates certain intense modes that could be used for identification of each of these species: BChl *a* shows strong 730- and 1519-cm^{-1} bands while for BPhe *a* obvious bands appear at 645 and 1584 cm^{-1}.

Reduced Reaction Centers. Figure 2 shows the room temperature FT Raman spectrum of RCs from *Rb. sphaeroides* in the presence of ascorbate. It remarkably resembles the FT Raman spectrum of a BChl *a* species, as signaled by the characteristic 728-, 894-, 1014-, 1163-, and 1528-cm^{-1} bands (compare with Figure 1). On the other hand, it is markedly different from that reported by Johnson and Rubinovitz (1990), which was obtained at much higher laser powers (1 W compared to 180 mW in this work). The number of carbonyl bands in the 1620–1740-cm^{-1} region clearly indicates that more than one bacteriochlorin species is observed. In this region, the protein is expected to contribute mainly around 1655 cm^{-1}, the region of the amide I band (Yager & Gaber, 1987). Comparing Figure 2 with the FT Raman spectra of the isolated pigments (Figure 1) leads to the conclusion that BChl modes dominate the FT Raman spectrum of the RCs. At 2900 cm^{-1}, a complex band (not shown) arising from C–H stretching modes is present that does not exist in the FT Raman spectra of isolated pigments in CCl_4. However, subtraction of the FT Raman spectrum of the buffer/detergent solution from that of the RC reveals that there is no significant contribution from the protein C–H modes to the complex 2900-cm^{-1} band. Because this band is expected to be not weaker than the amide I Raman band, we thus conclude that

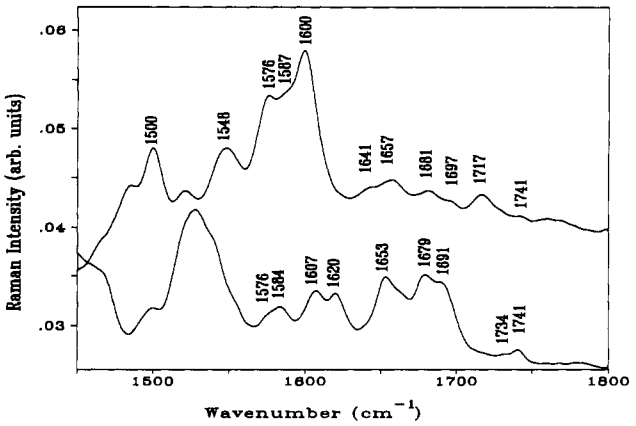

FIGURE 3: Blowup of the carbonyl stretching region of Figure 2 for the FT Raman spectra of oxidized RCs (above) and reduced RCs (below).

the contributions of the RC protein, if sizable at all in the present FT Raman spectrum, should be extremely weak as compared to those of BChl *a*.

If spectra are recorded from a quartz cell, with no interference from the 645-cm^{-1} sapphire line, a weak 650-cm^{-1} band may be observed (data not shown), which indicates weak contributions of BPhe to the FT Raman spectra of reduced RCs. Similarly, the 1584-cm^{-1} band, which could possibly arise from BPhe molecules, appears to be also present with comparable intensity in the FT Raman spectra of antenna complexes containing only BChl *a* chromophores (Mattioli and Hoffmann, unpublished results). This confirms that the main contributions present in this spectrum are arising from BChl *a*. The C_aC_m stretching mode, observed at 1607 cm^{-1}, unambiguously indicates the presence of BChl species with a single axial ligand. The 1620-cm^{-1} frequency matches that of a H-bonded acetyl carbonyl only, while the 1653-cm^{-1} band can arise from either a H-bonded keto carbonyl group or a free-from-interaction acetyl carbonyl (Lutz, 1984). The 1691- and 1683-cm^{-1} bands correspond to free keto carbonyl groups in different environments. There is a weak band observed at 1663 cm^{-1} (about one-third the intensity of the other carbonyl bands, as estimated by a simple curve-fitting routine). Because of its weakness, and also because it seems to be present in the oxidized RC spectrum (see Figure 3), we conclude that this band arises from a BChl other than P. Its frequency matches well those reported for either of the acetyl C=O of accessory BChls, i.e., 1660 and 1663 cm^{-1} (Robert, 1990). The C_{10} carbomethoxy C=O groups likely contribute at 1732 and 1741 cm^{-1}.

Oxidized Reaction Centers. In the absorption spectrum of oxidized reaction centers, there are at least two distinct bands (Woodbury et al., 1985, and references cited therein) in the 900–1300-nm region exhibiting maxima at 1150 and 1250 nm. Relative to the 1250-nm absorption band of P^+, 1064-nm excitation falls ca. 1400 cm^{-1} above this lowest lying observable transition in a spectral region where there is sizable absorption intensity (Woodbury et al., 1985, and references cited therein). Although the exact resonance condition is not known at present, excitation at 1064 nm should possibly promote enhanced scattering of P^+ through resonance (i) with the 1150- or 1250-nm (cf. below) bands if they correspond to the electronic origin of an electronic state of P^+ (since in this case 1064 nm would be resonant with a vibronic sublevel of one of these possible states), (ii) with an absorption band near 1064 nm where there is sizable absorption intensity but which is not distinctly observed in the absorption spectrum, or (iii) with a higher lying absorption band of P^+ in a strong preresonance

condition. Indeed, as shown in Figure 3, the FT RR spectrum of RCs poised in the P^+ state by ferricyanide treatment markedly differs from that of reduced RCs. Normalizing the spectra of Figure 2 either on the 2900-cm^{-1} complex band or on the 645-cm^{-1} sapphire line reveals a significant loss of intensity (65%, as estimated from the 728-cm^{-1} band of BChl *a*) for almost all Raman bands present in Figure 2 upon oxidation of P and the appearance of new components, in particular at 669, 1306, 1348, 1407, 1485, 1500, 1548, 1576, 1600, and 1717 cm^{-1}. This 65% bleaching is consistent with the preresonance enhancement of P modes with the 865-nm P absorption band. Bands that have been bleached should primarily correspond to those preresonantly enhanced modes associated with the 865-nm absorption band (which bleaches upon oxidation), while the new bands are associated with P^+ formation (cf. below). Indeed, the addition of either ferricyanide or ascorbate is not expected to induce any drastic change of the Raman cross sections of chromophores other than P. The extent of the bleaching is such that it is clear from the computed oxidized-minus-reduced spectrum (Figure 2) that the bands arising from P are readily observable in the reduced spectrum itself. It may thus be concluded that the NIR FT Raman spectrum of RCs from *Rb. sphaeroides* in the presence of ascorbate gives direct vibrational information about P in its neutral, ground state.

The oxidized RC spectrum exhibits new 1348- and 1407-cm^{-1} modes that closely match two RR bands at 1340 and 1414 cm^{-1} which have been previously suggested to constitute reliable marker bands for the formation of BChl a^+ (Cotton et al., 1980). As well, a new 1717-cm^{-1} mode appears in the carbonyl stretching region. Its frequency is again very close to that of the keto carbonyl stretching mode of monomeric (B)Chl a^+ (Mäntele et al., 1988; Heald & Cotton, 1990). This unusually high frequency is an excellent marker for the oxidized species. Taken together, these results strongly suggest that a BChl a^+ species is observed in the NIR FT Raman spectrum of oxidized RC with 1064-nm excitation. A weak 1641-cm^{-1} mode is also present in that region which is not observed in the reduced RC spectrum; this frequency is also consistent with that of a H-bonded acetyl carbonyl of a BChl a^+ species (Mäntele et al., 1988).

In the carbonyl stretching region (Figure 3), five bands are observed to bleach upon P oxidation at 1620, 1653, 1679, 1691, and 1741 cm^{-1}, indicating that at least two BChl molecules are involved. These bands are assigned to P in its neutral ground state. In that same region, bands which are observed in the oxidized RC spectrum at 1657, 1681, and 1697 cm^{-1} cannot exclusively be assigned to P^+ formation. Indeed their frequencies correspond as well to those of the accessory BChl and BPhe molecules in the RCs (Robert, 1990), and their contributions may have been masked in the reduced spectrum because of the dominant P carbonyl modes. Moreover, the contribution of neutral BChls is signaled in other regions of the oxidized RC spectrum through the presence of the 728-, 894-, 1014-, and 1136-cm^{-1} modes.

Untreated Reaction Centers. Quite unexpectedly, it was observed, under the experimental conditions used, that the diode-pumped Nd:YAG laser produced a significant actinic effect on the RC preparation. Under continuous laser illumination, a spectrum of oxidized RC was recorded which is indistinguishable from that of RC which was chemically oxidized with ferricyanide. The photooxidation was fully reversed with the addition of ascorbate. Since the chemically oxidized RC (P^+Q_A) and the photooxidized RC ($P^+Q_A^-$ and/or $P^+Q_B^-$) spectra are indistinguishable, we may conclude that neither Q_A nor Q_A^- (Q_B^-) appreciably contributes to the FT Raman spectra of *Rb. sphaeroides* RCs (data not shown). Similarly, no difference was observed between ascorbate- (Q_A^-) and dithionite-treated (Q_A^- and possibly Q_A^{2-} after illumination; Okamura et al., 1979) RCs.

Primary Donor. Four distinct carbonyl bands of the present reduced RC spectrum have been attributed to the neutral ground state of the primary donor, namely the 1620-, 1653-, 1679-, and 1691-cm^{-1} bands. This indicates that more than one BChl molecule is involved, which is consistent with the oxidation of dimeric P. Furthermore, the similar intensities of these four bands reveal that P_L and P_M are preresonantly enhanced to the same degree: the excited state corresponding to the 865-nm absorption band is thus associated with these two molecules, and the excitation should be delocalized over the dimer on a time scale faster than the vibrational Raman effect (ca. 10^{-13} s). This is consistent with a supermolecule structure for P (Parson, 1982). The position (1607 cm^{-1}) and bandwidth (14 cm^{-1} FWHM) of the C_aC_m stretching mode band fully confirms that both P_L and P_M possess a single axial ligand on the central Mg atom (Robert & Lutz, 1986; Robert, 1990).

Because the 1679- and 1691-cm^{-1} frequencies are consistent only with keto carbonyl frequencies, we attribute the 1620- and 1653-cm^{-1} bands to the acetyl carbonyls of P, one of them being strongly H-bonded and the other one free from interactions. The 1679- and 1691-cm^{-1} bands correspond to a weakly interacting (or in a region of high dielectric constant; Krawczyk, 1989) and a free-from-interaction keto carbonyl group, respectively. This interaction pattern, as drawn from the results here, is in total agreement with X-ray crystallographic studies; indeed, it has been concluded for the RCs of *Rps. viridis* and *Rb. sphaeroides* that the acetyl carbonyl of P_L could be engaged in a H-bond with His L168, while the homologous group of P_M should be free from interaction (Deisenhofer & Michel, 1989; Tiede et al., 1988). On the other hand, no suitable H-bond acceptor has been found in the local environment of any keto groups of P in *Rb. sphaeroides*. These frequencies also closely match those obtained by Zhou et al. (1987) from the Soret-excited RR difference spectrum of P in *Rhodospirillum* (*Rsp.*) *rubrum* (1635, 1660, 1685, and 1703 cm^{-1}). However, in previous RR experiments on *Rb. sphaeroides* wild type (Robert & Lutz, 1986), only three bands have been described in the carbonyl stretching region, at 1635, 1660, and 1685 cm^{-1}. Comparing these results with those of *Rsp. rubrum*, and considering that the 1660-cm^{-1} mode was more intense in the difference spectrum of *Rb. sphaeroides* wild type than in that of *Rsp. rubrum*, Zhou et al. (1987) attributed part of the 1660-cm^{-1} band to a H-bonded keto carbonyl group in *Rb. sphaeroides* to account for the lack of the 1703-cm^{-1} band in the difference spectrum (see Table I). Recent sequencing of *Rsp. rubrum* (Bélanger et al., 1988), however, did not exhibit any amino acid replacement that would explain this difference in interaction, and these apparent discrepancies between the RR data and both the crystallographic and sequencing data have been recently underlined (Robert, 1990). From this work, it appears that both of the keto carbonyls of P are also free from interactions in *Rb. sphaeroides* R26. Differences in sequences between R26 and 2.4.1 are too few to explain this discrepancy and thus two hypotheses can be drawn: (i) because of the difference in preparation protocols (or RC stability) between 2.4.1 and R26, a nongenuine H-bond (with, e.g., a water molecule) is formed with one keto carbonyl group of P in 2.4.1 during its isolation; (ii) the free-from-interaction keto carbonyl

Table I: Observed Carbonyl Stretching Frequencies (cm^{-1}) of P and P^+

Rsp. rubrum S1,[a] P(Soret)	*Rb. sphaeroides* 2.4.1,[b] P(Soret)	*Rb. sphaeroides* R26,[c] P(Q_y)	*Rb. sphaeroides* R26,[c] $P^+(Q_y)$	carbonyl	molecule	H-bond donor
1634	1636	1620	1641	acetyl	P_L	His L168
1658	1660	1653		acetyl	P_M	none
1681	1684	1679		keto	P_M	none
1702		1691	1717	keto	P_L	none

[a] Data from Zhou et al. (1987). [b] Data from Robert and Lutz (1986). [c] This work.

corresponding to the 1703-cm^{-1} band is not appreciably enhanced in *Rb. sphaeroides*, at low temperature, due to, e.g., a strained conformation of P_L forcing this group slightly out of the porphyrin plane and thus reducing its degree of conjugation (Lutz et al., 1982). Current FT Raman experiments, conducted on R26 and 2.4.1 RCs at different temperature, will lead to an explanation of these results.

Structure of P^+. Using excitation within the 900–1300-nm absorption region of P^+, we have for the first time observed a detailed vibrational resonance Raman spectrum of the $P^{\bullet+}$ radical. The exact nature of this complex absorption region is not completely understood (Olson et al., 1985). The present results confirm that it involves at least one oxidized BChl *a* species, most likely of P^+. Moreover, most if not all of the RR bands that are unambiguously ascribed to P^+ in the present oxidized RC spectrum must arise from in-plane modes. This is necessarily true for the keto carbonyl stretching band at 1717 cm^{-1} and for the skeletal bands of the 1300–1600-cm^{-1} region (Lutz & Robert, 1988; Spiro et al., 1990). This observation constitutes strong indication that, whatever the A-type or B-type scattering mechanism(s) actually involved in the resonance, the electronic transition(s) that promote(s) P^+ resonant scattering at 1064 nm is (are) likely in-plane. Observing that the strongest RR bands ascribed to P^+ in the present spectra occur in the 1300–1600-cm^{-1} range (cf. difference spectrum in Figure 2), it could be possible that the 0-0 level of the lower lying transition might be located 1400–1500 cm^{-1} lower than 1064 nm, i.e., at 1250–1266 nm, within the 1250-nm absorption band. However, excitation profiles of P^+ RR bands in this region are needed to confirm this hypothesis.

The spectrum of P^+ is dominated by an intense band at 1600 cm^{-1}, which likely corresponds to the 1607-cm^{-1} band observed for the neutral species. The C_aC_m stretching mode thus downshifts by 7 cm^{-1} upon P oxidation. The downshift of this mode is observed upon the one-electron oxidation of metalloporphyrins and metallochlorins with a_{1u}-like redox orbitals (Spiro et al., 1990; Salehi et al., 1988) as well as for BChl *a* where the magnitude of the downshift has been evaluated at -8, -14, and -20 cm^{-1}, depending on excitation wavelength. From Tables II and III of Cotton et al. (1980) we calculate an average of -13 cm^{-1} [see also Lutz and Klêo (1979)]. The −7-cm^{-1} downshift observed in the present spectra seems to correspond to half of that resulting from the one-electron oxidation, in vitro, and thus might be interpreted as revealing the sharing of the positive hole by both of the molecules of P. However, the C_aC_m stretching mode is sensitive to the BChl core size and conformation; thus direct extrapolation of in vitro conclusions must be done extremely carefully. Fajer et al. (1986) have discussed the likelihood that porphyrins and chlorins undergo significant conformational change upon oxidation, and this has recently been discussed for the oxidation of Chl *a* as well (Heald & Cotton, 1990). Indeed, within the protein matrix of the RCs, either the core of the BChl molecules constituting P may be unable to undergo the same conformational change as those that may accompany the BChl oxidation, in vitro, or it may be strained into specific conformations due to the protein conformational changes that are induced by the creation of the positive hole (Robert & Lutz, 1986). Such events would also directly affect the spin redistribution in the radical as discussed by Renner et al. (1990). Unusual conformational structures in oxidized porphyrin dimers have been discussed in a recent review by Scheidt and Lee (1987). For these reasons as well as the apparent excitation-dependent observed shift of the C_aC_m mode upon oxidation of BChl *a* (Cotton et al., 1980), this mode may not constitute an accurate reflection of hole delocalization.

A better method to gauge the extent of hole delocalization is to use the keto carbonyl stretching frequency of BChl a^+. There is considerable structural evidence from X-ray crystallography and RR spectroscopy [for a review, see Lutz (1984)] that this group assumes little distortion from its normal in-plane conformation. In the carbonyl stretching region of the present FT Raman spectra, the 1641- and 1717-cm^{-1} bands have been unambiguously attributed to P^+ formation. The 1717-cm^{-1} band has been assigned to a free keto carbonyl group that has upshifted with respect to its neutral counterpart by 26 or 38 cm^{-1} (as the keto carbonyls of neutral P are observed at 1691 and 1679 cm^{-1}). This shift represents an upper limit observed for the C_9 keto carbonyl. Infrared studies indicate that upon one-electron oxidation the frequency of the C_9 keto carbonyl stretching mode of monomeric BChl *a* upshifts from 1684 to 1716 cm^{-1} (+32 cm^{-1}) in aprotic sovents (Mäntele et al., 1988). The magnitude of the 26- or 38-cm^{-1} upshift observed for P in the present spectra suggests that one BChl molecule in P carries nearly the full +1 charge. Indeed, if the unpaired electron were delocalized over both BChl molecules in P^+, a shift of only about half of that observed for monomeric BChl would be expected. It thus appears that the unpaired electron is localized over one BChl only, on the time scale of the RR effect (10^{-13} s); i.e., the unpaired electron does not share a common orbital of P_L and P_M. The 1641-cm^{-1} band in the P^+ spectrum can be assigned to the stretching mode of a H-bonded acetyl carbonyl of an oxidized BChl species having upshifted in frequency from 1620 cm^{-1}. An upshift (+11 cm^{-1}) of the acetyl carbonyl band can be observed in the FTIR spectra of BChl a^+ in THF (Mäntele et al., 1988). The probable 21-cm^{-1} upshift of the acetyl carbonyl stretching frequency upon P oxidation further supports hole localization on one BChl molecule.

From the crystal structure of the *Rb. sphaeroides* RC, only the acetyl carbonyl of P_L is likely to form a H-bond, that being with histidine L168 (Tiede et al., 1988). We may thus conclude that the acetyl carbonyl of P_L, which is H-bonded by His L168 in neutral P, is still H-bonded in the P^+ state, most likely with the same side chain. Thus the 1641- and 1717-cm^{-1} bands can be attributed to P_L, and no other band in the carbonyl stretching modes may be clearly assigned to P_M. As it is unlikely that the P_L and P_M bands are both degenerate, we conclude that P_M only weakly contributes in the P^+ spectrum.

From ENDOR/TRIPLE studies and spin-density calculations, it was concluded that the unpaired electron in P^+ of *Rb. sphaeroides* was delocalized but not equally shared by P_L and

P_M (Plato et al., 1990), thus deviating from C_2 symmetry of the dimer. Considering the results reported here, in a molecular orbital picture where P_L and P_M in the P^+ dimer would be strongly interacting (such an orbital picture has been proposed by Bocian and co-workers for strongly $\pi-\pi$ interacting porphyrin dimers in lanthanide–porphyrin sandwich complexes; Duchowski & Bocian, 1990a), it seems that the electron is removed primarily from a dimer molecular orbital comprised predominantly of a P_L molecular orbital. This could arise from the inequivalent protein environments and/or conformations of each of the P molecules (Deisenhofer & Michel, 1989), which could result in slightly different redox orbital energies of P_L and P_M. Such hole localization in asymmetric lanthanide–porphyrin sandwich complexes has been recently reported (Duchowski & Bocian, 1990b).

It is difficult to assess the relative magnitudes of the coefficients of the dimer redox orbital, but if one assumes that the observed magnitude of the shift of the keto carbonyl at 1717 cm^{-1} (+26 cm^{-1} in P^+ as compared to +32 cm^{-1} in vitro) is proportional to the hole density, it may be estimated that the orbital of P_L contributes ca. 80% to the dimer orbital. The absence or weakness of P_M^+ contributions may be explained by a difference in the RR scattering cross sections of the P_L^+ and P_M^+ species at 1064-nm resonance.

Conclusions

In this study, we have directly observed several ground-state vibrational modes of the primary donor in *Rb. sphaeroides* R26 in the 500–1800-cm^{-1} range with preresonance with its ^1P state, as well as of its cation radical in resonance with one or several bands of its 1000–1250-nm absorption region. The interaction pattern that we described for the carbonyl groups reconciles the Raman data with the X-ray crystallographic structures and sequence data, although more work is needed in order to understand the pattern of *Rb. sphaeroides* 2.4.1 (Robert & Lutz, 1986). Contrary to what is observed in the neutral state where both BChl molecules comprising P are enhanced to the same extent, only P_L seems to sizably contribute in the P^+ spectrum excited at 1064 nm. Moreover, the oxidation-induced shifts of both the H-bonded acetyl and the free keto carbonyl groups are consistent with a +1 charge preferentially (but not necessarily completely) localized on P_L. This might have relevance concerning the early intradimer charge-transfer mechanisms [see review by Friesner and Won (1989)]. Conceivably, application of the FT Raman methods to wild-type RCs from different bacterial species, as well as to mutant RCs exhibiting sizable charge-transfer mechanisms, should very soon allow a much better understanding of the physicochemical characteristics of the primary electron donors in bacterial RCs and, eventually, in higher plant systems.

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Cholate-Solubilized Erythrocyte Glucose Transporters Exist as a Mixture of Homodimers and Homotetramers†

Daniel N. Hebert* and Anthony Carruthers*

Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, Massachusetts 01605

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ABSTRACT: The molecular size of purified, human erythrocyte glucose transport protein (GLUT1) solubilized in cholic acid was determined by size-exclusion chromatography (SEC) and sucrose gradient ultracentrifugation. GLUT1 purified in the presence of dithiothreitol (GLUT1 + DTT) is resolved as a complex of average Stokes' radius 5.74 nm by SEC. This complex displays D-glucose-inhibitable cytochalasin B binding and, upon reconstitution into proteoliposomes, catalyzes cytochalasin B inhibitable D-glucose transport. GLUT1 purified in the absence of dithiothreitol (GLUT1 - DTT) is resolved by SEC as at least two particles of average Stokes' radii 5.74 (minor component) and 7.48 nm (major component). Solubilization of GLUT1 - DTT in the presence of dithiothreitol reduces the amount of 7.48-nm complex and increases the amount of 5.74-nm complex resolved by SEC. GLUT1 - DTT displays D-glucose-inhibitable cytochalasin B binding and, upon reconstitution into proteoliposomes, catalyzes cytochalasin B inhibitable D-glucose transport. Sucrose gradient ultracentrifugation of GLUT1 + DTT in cholate resolves GLUT1 into two components of 4.8 and 7.6 S. The 4.8S complex is the major component of GLUT1 + DTT. The reverse profile is observed upon sucrose gradient ultracentrifugation of GLUT1 - DTT. SEC of human erythrocyte membrane proteins resolves GLUT1 as a major broad peak of average Stokes' radius 7.48 nm and a minor component of 5.74 nm. Both components are characterized by D-glucose-inhibitable cytochalasin B binding. Purified GLUT1 is associated with approximately 26 tightly bound lipid molecules per monomer of transport protein. These data suggest that purified GLUT1 exists as a mixture of homodimers and homotetramers in cholate–lipid micelles and that the presence of reductant during solubilization favors dimer formation.

The facilitated diffusion of pentose and hexose monosaccharides across cell membranes is mediated by a family of integral membrane glycoproteins called glucose transporters. At least five isoforms of glucose transporter have been identified by using biochemical and recombinant DNA technologies (Mueckler et al., 1985; Birnbaum et al., 1986; Thorens et al., 1988; Kayano et al., 1988; Celenza et al., 1988; James et al., 1989). While advances in this area have been notable, a number of fundamental properties of glucose transport systems remain to be resolved. These include information regarding the secondary and higher order structures of the glucose transport proteins.

In this study we ask whether human erythrocyte glucose transporter (GLUT1)[1] exists as monomeric or as multimeric species in detergent extracts of membranes. Previous, target-size analyses suggest that the cytochalasin B binding and the sugar transport competent components of the human erythrocyte sugar transport system exist as 124- or 220-kDa

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* Author to whom correspondence should be addressed.

[1] Abbreviations: cmc, critical micellular concentration; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPC, egg phosphatidylcholine; GLUT1, human erythrocyte glucose transporter; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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