Symmetric Structural Features and Binding Site of the Primary Electron Donor in the Reaction Center of *Chlorobium*†

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Received March 8, 1995; Revised Manuscript Received May 22, 1995⊗

ABSTRACT: The protein binding interactions of the constituent bacteriochlorophyll *a* molecules of the primary electron donor, P_{840}, in isolated reaction centers from *Chlorobium limicola* f *thiosulphatophilum* and the electronic symmetry of the radical cation $P_{840}^{+\bullet}$ were determined using near-infrared Fourier transform (FT) Raman spectroscopy excited at 1064 nm. The FT Raman vibrational spectrum of P_{840} indicates that it is constituted of a single population of BChl *a* molecules which are spectrally indistinguishable. The BChl *a* molecules of P_{840} are pentacoordinated with only one axial ligand on the central Mg atom, and the π-conjugated C_2 acetyl and C_9 keto carbonyls are free of hydrogen-bonding interactions. The FT Raman spectrum of $P_{840}^{+\bullet}$ exhibits a 1707 cm^{-1} band attributable to a BChl *a* C_9 keto carbonyl group vibrational frequency that has upshifted 16 cm^{-1} upon oxidation of P_{840}; this upshift is exactly one-half of that expected for the one-electron oxidation of monomeric BChl *a in vitro*. The 16 cm^{-1} upshift, thus, indicates that the resulting +1 charge is equally shared between two BChl *a* molecules. This situation is markedly different from that of the oxidized primary donor of the purple bacterial reaction center of *Rhodobacter sphaeroides*, (i) which exhibits a 1717 cm^{-1} band that has upshifted 26 cm^{-1}, indicating an asymmetric distribution of the resulting +1 charge over the two constituent BChl *a* molecules, and (ii) whose H-bonding pattern with respect to the π-conjugated carbonyl groups is asymmetric. Therefore, according to the Raman data of P_{840} and $P_{840}^{+\bullet}$ presented here, the primary electron donor of *Chlorobium* is a highly symmetric dimer of BChl *a* molecules; this symmetry is with respect to the protein interactions of P_{840} and the positive charge distribution in $P_{840}^{+\bullet}$. The above results and conclusions are consistent with the proposal of a homodimeric structure of the *Chlorobium* reaction center.

In green sulfur bacteria, the primary photosynthetic events and transduction of solar light energy occur within a single photosystem, which shares many structural and functional features with photosystem I (PS I)[1] of higher plants and cyanobacteria. In particular, it was shown as early as 1969 that membrane fragments from these organisms were able to perform light-induced NAD^+ reduction (Buchanan & Evans, 1969; Knaff, 1978). This similarity with PS I was further substantiated by (i) the finding and characterization of iron−sulfur (FeS) centers as terminal electron acceptors in reaction centers (RCs) from *Chlorobium* (Jennings & Evans, 1977; Swarthoff *et al.*, 1981a; Nitschke *et al.*, 1990; Feiler *et al.*, 1992; Miller *et al.*, 1992; Kusumoto *et al.*, 1992; 1994; Oh-oka *et al.*, 1993), (ii) indirect evidence for the existence of a quinone-type electron acceptor analogous to A_1 in PS I (Nitschke *et al.*, 1987, 1990; Rigby *et al.*, 1994), and (iii) the nature of the primary electron acceptor, which was identified as a Chl *a* molecule, as A_0 in PS I (van de Meent *et al.*, 1992; Feiler *et al.*, 1994). On the other hand, RCs from green sulfur bacteria also share similarities with those from purple bacteria. As is the case for the purple bacteria, the primary electron donor of *Chlorobium*, P_{840}, appears to be a dimer of bacteriochlorophyll *a* (BChl *a*) molecules, probably of similar geometry (Olson *et al.*, 1976; Swarthoff *et al.*, 1981b; Vasmel *et al.*, 1984; Nitschke *et al.*, 1990). In an evolutionary model, Nitsche and Rutherford (1991) proposed a structure for *Chlorobium* RCs very similar to those of purple bacteria. A major difference, however, with respect to PS I and purple bacteria is that in *Chlorobium* only *one* gene coding for the RC could be found; this implies that the *Chlorobium* RC should consist of a homodimer of two large subunits each of ca. 68 kDa (Büttner *et al.*, 1992).

Despite the chemical and structural similarities between the primary electron donors of RCs from purple bacteria and from *Chlorobium*, both primary donors exhibit marked differences in their physicochemical properties. In particular, the oxidation midpoint potential of the $P_{840}/P_{840}^{+\bullet}$ couple is +240 mV (Fowler *et al.*, 1971; Prince & Olson, 1976); this value is at least 200 mV lower than those observed for other BChl *a*-type primary donors of purple bacteria (Prince & Dutton, 1978; Moss *et al.*, 1991; Williams *et al.*, 1992). Spectroscopic properties of P_{840} are also notably different from those of BChl *a*-containing purple bacteria (P_{870}). For example, as the name suggests, P_{870} exhibits its lowest Q_y transition at ca. 870 nm, as for *Rhodobacter sphaeroides*, whereas the primary donors from Chlorobiaceae exhibit this absorption maximum at 840 nm. As well, the width of this latter absorption band is much narrower (20 nm or 280 cm^{-1}, FWHM) than that of P_{870} (57 nm or 757 cm^{-1} FWHM).

† This work was supported by the EEC Science Program (to U.F.).

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⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: (B)Chl, (bacterio)chlorophyll; (B)Ph, (bacterio)-pheophytin; *C., Chlorobium*; ENDOR, electron nuclear double resonance; FeS centers F_A, F_B, and F_X, iron−sulfur centers F_A, F_B, and F_X; FT, Fourier transform; FWHM, full width at half-maximum; PS I/II, photosystem I/II; P_{840}, primary electron donor in green sulfur bacterial reaction centers; RC, reaction center; RR, resonance Raman; *Rb., Rhodobacter*; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/95/0434-11099$09.00/0

When P_{840} is oxidized, a near-infrared transition attributable to $P_{840}^{+\bullet}$ appears at ca. 1160 nm (Olson *et al.*, 1976) whereas for $P_{870}^{+\bullet}$ this band appears at ca. 1250 nm. Although significantly blue shifted as compared to the 1250 nm maximum of $P_{870}^{+\bullet}$, the 1160 nm absorption maximum of $P_{840}^{+\bullet}$ is still largely red shifted compared to that of monomeric BChl a^+ *in vitro* (ca. 900 nm; Fajer *et al.*, 1975), suggesting that $P_{840}^{+\bullet}$ keeps its dimeric character in its radical cation state.

These important physicochemical and spectroscopic differences between P_{840} and P_{870}, both of which are constituted of a BChl *a* dimer in both their neutral and radical cation states, have prompted us to characterize the structure and binding site interactions of P_{840}. In general, identification of protein interactions responsible for these differences provides an opportunity to understand the different structural factors which can modulate the properties of the primary electron donors in reaction centers, and of chlorophylls, in general.

In this paper we have characterized, using near-infrared Fourier transform Raman spectroscopy, the primary electron donor from *Chlorobium limicola* f *thiosulphatophilum*. The preparation of the reaction center complex used in this work is devoid of chlorosomal BChl *c* molecules and contains the iron−sulfur centers F_A and F_B (Feiler *et al.*, 1992). As well, it contains ca. 40 BChl *a* molecules per reaction center, some being bound to the remaining base-plate protein (the so-called FMO light-harvesting protein) (Feiler *et al.*, 1992; D. Albouy, U. Feiler, J. Sturgis, and B. Robert, unpublished results). Resonance Raman (RR) spectroscopy is a powerful technique to study chlorophyll pigments in various photosynthetic systems and yields direct information concerning the chemical nature and conformation of the respective pigments as well as their interactions with the protein (Lutz & Robert, 1988; Robert, 1995). Recently, RR spectroscopy has been successfully applied to the *Chlorobium* reaction center to determine the chemical nature of the primary acceptor, *in situ* (Feiler *et al.*, 1994). In studying the vibrational structure of the primary electron donors of BChl *a*-containing reaction centers, near-infrared Fourier transform (FT) Raman spectroscopy has been shown to be well suited (Mattioli *et al.*, 1991, 1992, 1994). This technique utilizes 1064 nm radiation to selectively, preresonantly enhance the vibrational Raman spectrum of P_{870} and resonantly enhance that of $P_{870}^{+\bullet}$, thus permitting the determination of the protein interactions assumed by P_{870} and deducing the electronic asymmetry of $P_{870}^{+\bullet}$. We have now applied this technique to the reaction center of *Chlorobium* to determine the intermolecular interactions assumed by each of the two Bchl *a* molecules of P_{840}, as well as the degree of delocalization of the + charge in $P_{840}^{+\bullet}$; these structural features are compared to those of P_{870} of the purple bacteria.

EXPERIMENTAL PROCEDURES

C. limicola f *thiosulphatophilum*, strain tassajara, was obtained from Norbert Pfennig (Konstanz, Germany). The cells were grown under strictly anaerobic conditions in a medium described by Biebl and Pfennig (1978). After 7 days of growth, cells were harvested by low-speed centrifugation and were stored at −20 °C until use.

The reaction center complex was purified as described by Feiler *et al.* (1992). Final RC preparations were in 0.1% dodecyl maltoside and 20 mM Tris-HCl, pH 8. An absorption spectrum of the isolated RC complex is shown in Figure 1. Samples were kept in 1 mM ascorbate and left to dark adapt for 30 min on ice before the absorption spectrum was recorded (reduced P_{840}) in the presence of 100 μM dithionite. To this same sample was added a ferricyanide stock solution to give a final concentration of 1.5 mM ferricyanide, and then a new absorption spectrum was recorded (oxidized P_{840}). For the Raman experiments, the RC preparations were first preconcentrated using Centricon 100 microconcentrators (Amicon) and then finally by ultracentrifugation at 350000*g* for 15 min in a Beckman TL 100 ultracentrifuge. The 10 μL samples were treated with either 1 mM dithionite or 1 mM ferricyanide in order to poise the RCs in their P_{840} or $P_{840}^{+\bullet}$ states, respectively; this was done by adding 1 μL of 10 mM stock solutions of dithionite and of ferricyanide to the 10 μL samples. It was verified that the addition of up to 5 mM ferricyanide did not alter the FT Raman spectra. In order to determine the relative contributions of the 827 nm absorbing species which is oxidized as well as $P_{840}^{+\bullet}$ in the presence of ferricyanide, we have also examined a similar *Chlorobium* RC preparation which is estimated to contain roughly one-half the number of the BChl *a* molecules per P_{840} as that of the preparation reported here (Albouy *et al.*, unpublished data). Under similar oxidizing conditions, the bleached 827 nm component is sizably diminished with respect to the bleached 845 nm band. The FT Raman spectrum of this latter preparation yields similar FT Raman spectra both in terms of bands which are bleached and in terms of those which appear upon oxidation of the sample, indicating that the contributions of the 845 nm absorbing species dominate the bleached components in the FT Raman spectrum and not those of the 827 nm absorbing species. Similarly, the appearance of the new bands in the FT Raman spectrum of the oxidized sample should arise from the species absorbing at 1148 nm, i.e., $P_{840}^{+\bullet}$.

Fourier transform (FT) Raman spectra were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous, diode-pumped Nd:YAG laser (Mattioli *et al.*, 1991). Spectra were recorded both at room temperature and at 15 K. For the low-temperature experiments, the sample was held in a gas-flow cryostat (SMC-TBT, France) in which cold helium gas was circulated. Typically, 200 mW of 1064 nm radiation was used to excite the Raman spectra at 4 cm^{-1} resolution.

Room temperature near-infrared absorption spectra (700−1300 nm) were recorded with the same Bruker IFS 66 interferometer operating in the absorption mode (Wachtveitl *et al.*, 1993). Samples were held in a 1 cm quartz cuvette.

RESULTS AND DISCUSSION

Figure 1 shows the room temperature near-infrared absorption spectra of the RC complex from *C. limicola* f *thiosulphatophilum* with P_{840} in its reduced and oxidized states. The absorption maximum at 816 nm arises from antenna BChl *a* pigments while the shoulder at 840 nm arises, at least partly, from neutral P_{840}. Upon the addition of ferricyanide, the 840 nm shoulder bleaches and a new band appears at 1158 nm (see inset). In the difference absorption, $P_{840}^{+\bullet}$-minus-P_{840}, two negative bands are observed at 845 and 827 nm. In the 800−900 nm region, the chemically

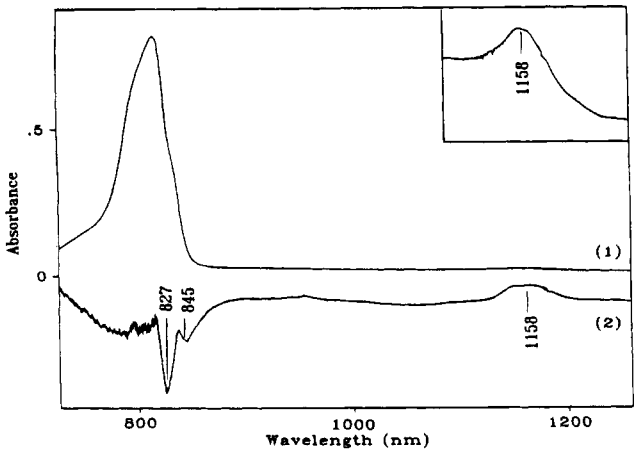

FIGURE 1: (1) Room temperature near-infrared electronic absorption spectrum of the *C. limicola* f *thiosulphatophilum* reaction center complex and (2) the $P_{840}^{+\bullet}$-minus-P_{840} difference spectrum (magnified 4 times). The RCs were poised in their $P_{840}^{+\bullet}$ and P_{840} states by the addition of ferricyanide and dithionite, respectively. The inset shows an enlarged view of the $P_{480}^{+\bullet}$ electronic absorption band.

induced $P_{840}^{+\bullet}$-minus-P_{840} difference spectrum shown in Figure 1 is similar to that in Feiler *et al.* (1992) and to the light-induced difference spectrum of RC-enriched *Chlorobium* membranes reported by Olson *et al.* (1976). In this same region, similar bands are seen in the low-temperature triplet-minus-singlet absorbance difference spectra of RC complexes from the green photosynthetic bacterium *Prosthecochloris aestuarii* (Vasmel *et al.*, 1984). Linear dichroism—absorption-detected magnetic resonance measurements on these RC complexes indicated that the ca. 840 and ca. 830 nm transitions were parallel (Vasmel *et al.*, 1984) and, therefore, they do not arise from an excitonically split transition.

We have observed that the negative 827 nm band in the difference spectrum can vary in intensity with respect to the 840 nm band, depending on the amount of BChl *a* molecules present in the reaction center preparation (unpublished observations). Thus, although the relative intensities of the 827 and 845 nm bleached bands show preparation-dependent variations, the relative intensities of the bleached 845 nm band and the positive 1158 nm band are essentially constant from preparation to preparation. This is consistent with the proposal that the bleached 845 nm band arises from the *Chlorobium* primary donor while the 827 nm component is most likely associated with some BChl *a* antenna pigment.

On the basis of its absorption spectrum (Figure 1), the FT Raman spectrum of the preparation used in this work excited at 1064 nm should result in (i) the preresonant enhancement of P_{840} contributions when the latter is in its neutral state, (ii) the bleaching of these P_{840} contributions when P_{840} is oxidized, and (iii) the appearance of resonantly enhanced Raman contributions of the $P_{840}^{+\bullet}$ species which absorbs at 1158 nm. We also expect to see relatively smaller changes in the FT Raman spectrum due to the species absorbing at 827 nm (see below).

Fourier Transform Raman Spectra of the Reduced and Oxidized RCs. FT Raman spectra of BChl *a*-containing reaction centers such as those from *Rb. sphaeroides*, excited with 1064 nm radiation, involve a selective preresonant enhancement of the vibrational spectrum of neutral P_{870} via

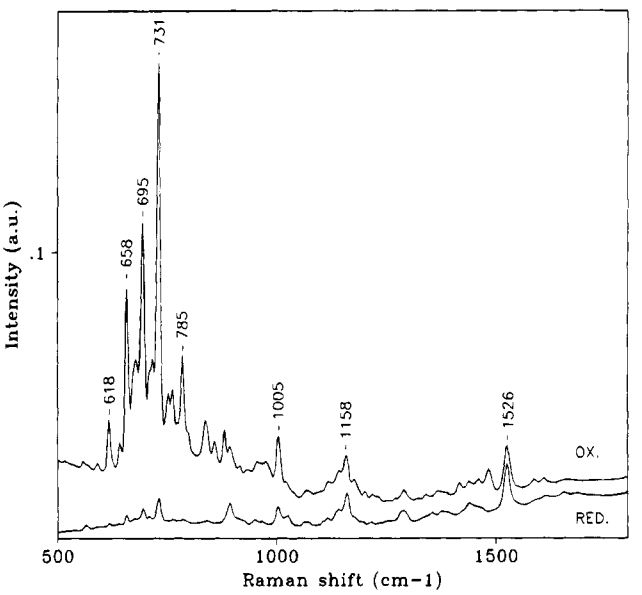

FIGURE 2: Low-temperature Fourier transform Raman spectra of the isolated RC complex from *C. limicola* f *thiosulphatophilum* in its reduced P_{840} state in the presence of dithionite (RED) and in its $P_{840}^{+\bullet}$ state in the presence of ferricyanide (OX). Excitation was at 1064 nm, 200 mW laser power, spectral resolution was 4 cm^{-1}, coaddition of 8000 scans.

its ca. 870 nm electronic transition (Mattioli *et al.*, 1991, 1993). When P_{870} is oxidized, 1064 nm radiation results in a genuine resonance condition with the 1250 nm transition of $P_{870}^{+\bullet}$ so that its Raman contributions are enhanced; since 1064 nm is ca. 1400 cm^{-1} higher in energy than 1250 nm, 1064 nm radiation is in resonance within a vibronic satellite region of the 1250 nm absorption band.

Figure 2 shows the 15 K FT Raman spectra of the *Chlorobium* RC preparation in its P_{840} and $P_{840}^{+\bullet}$ states, in the presence of dithionite and ferricyanide, respectively. The most pronounced difference between these two spectra is the large enhancement of a cluster of bands at ca. 700 cm^{-1} for the ferricyanide-treated preparation. In several experiments involving separate individual preparations, both at room and low temperature, the appearance of this intense cluster of bands strictly correlates with the presence of the 1158 nm absorption band attributed to $P_{840}^{+\bullet}$. Assuming that 1158 nm is, or is near, the electronic origin of this absorbing species, then one could expect the resonance enhancement of modes of vibrational frequencies at ca. 700 cm^{-1} since the excitation wavelength of 1064 nm is ca. 700 cm^{-1} higher in energy than 1158 nm. This resonance enhancement phenomena in a 0−1 vibronic satellite has been described in chlorophylls (Mattioli *et al.*, 1990) and in particular for the $P_{870}^{+\bullet}$ species in *Rb. sphaeroides* (Mattioli *et al.*, 1991). It is expected that a similar phenomenon occurs for P_{840} and $P_{840}^{+\bullet}$ and that modes at ca. 700 cm^{-1} could be significantly enhanced (see above).

Figure 3 shows an expanded view of Figure 2 in the high-frequency region (1550−1750 cm^{-1}). These spectra have been normalized with respect to (i) the complex Raman contributions at ca. 2900 cm^{-1} arising primarily from the C−H stretching modes of the protein and detergent and (ii) the 1526 cm^{-1} band of the carotenoid (not shown). These two bands are not expected to change between dithionite and ferricyanide treatment (Mattioli *et al.*, 1991, 1993). For RCs containing the neutral P_{840} species, the 1612 cm^{-1} band arises

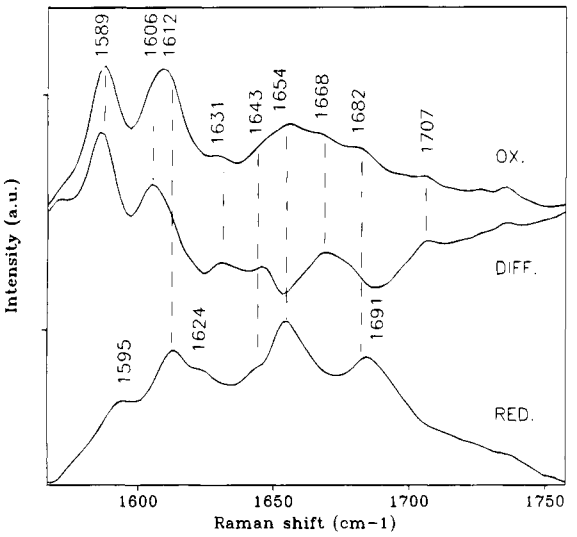

FIGURE 3: Expansion of the carbonyl stretching region of Figure 2 for the FT Raman spectra of oxidized (OX) and reduced (RED) RCs of *C. limicola* f *Thiosulphatophilum*. Also shown is an oxidized-minus-reduced RC difference spectrum (DIFF) calculated by normalizing the respective spectra on the complex C−H band at ca. 2900 cm^{-1} on the 1526 cm^{-1} band of the carotenoid molecule.

from the C_aC_m methine bridge stretching modes of the BChl *a* molecules present in the sample; its observed high frequency indicates that the central Mg atoms of these molecules possess one axial ligand only (Cotton & Van Duyne, 1981). In the region of 1620−1705 cm^{-1}, the stretching mode vibrations of the C_2 acetyl and C_9 keto carbonyls contribute to the Raman spectra (Lutz, 1984; Feiler *et al.*, 1994). The specific vibrational frequencies of these modes reflect the strength of intermolecular interactions in which they are engaged. Excitation of the Raman spectrum using 1064 nm radiation favors contributions from pigments which absorb nearest this wavelength, i.e., P_{840} in dithionite-treated preparations. The P_{840} contributions are identified by noting which bands are bleached from the Raman spectrum upon $P_{840}^{+\bullet}$ formation, where the 840 nm absorption band is also bleached. This is accomplished by comparing the FT Raman spectra of *Chlorobium* RC preparations treated with dithionite to those treated with ferricyanide. New bands which appear in the FT Raman spectrum upon $P_{840}^{+\bullet}$ formation should arise from $P_{840}^{+\bullet}$ species, resonantly enhanced via its 1158 nm absorption transition. We note that the $P_{840}^{+\bullet}$-minus-P_{840} absorption difference spectrum (Figure 1) also exhibited a negative band at 827 nm in addition to the bleaching of the 840 nm band. Thus, we expect some contributions of the 827 nm absorbing species when comparing the FT Raman spectra of the *Chlorobium* RC sample in its reduced and chemically oxidized states.

Inspection of the two spectra in Figure 3, as well as the computed difference spectrum, leads to the conclusion that two carbonyl bands are predominantly bleached upon ferricyanide treatment at 1654 and 1691 cm^{-1}; these two bands are, thus, attributable to P_{840} or to the species giving rise to the bleached absorption band at 827 nm (see Figure 1). As well in Figure 3, there is a downshift of some spectral intensity under the 1612 cm^{-1} band in the reduced spectrum, toward 1606 cm^{-1} in the oxidized spectrum. This same behavior is observed for the 1607 cm^{-1} band in the FT Raman spectra of reduced purple bacterial reaction centers which shifts to 1600 cm^{-1} upon oxidation of P_{870} (Mattioli *et al.*, 1991). In order to investigate the possibility that the bands bleached at 1654 and 1691 cm^{-1} arise from the bleaching of P_{840}, we also recorded FT Raman spectra on *Chlorobium* RC preparations which exhibited significantly less bleaching of the 827 nm component (see Experimental Procedures section). For these preparations, similar spectral characteristics as mentioned above were observed; the 1654 and 1691 cm^{-1} bands dominated the difference spectrum, and the same downshift of the 1612 cm^{-1} band was observed (data not shown). From these observations we conclude that the 1612, 1654, and 1691 cm^{-1} bands should not arise from the 827 nm absorbing species but rather from P_{840}. The preresonance enhancement of the P_{840} species is greater than that of the 827 nm species, which may be expected since 840 nm is closer in energy to 1064 nm excitation than is 827 nm.

The 1691 cm^{-1} band that is bleached appears to be too broad (20 cm^{-1} FWHM) compared to what is expected for a single BChl *a* Raman band representing a homogeneous population (ca. 14 cm^{-1} FWHM) (Mattioli *et al.*, 1993). As well, it is asymmetric on the high-frequency side, suggesting that there is another band of weaker intensity at ca. 1695 cm^{-1} contributing to the difference spectrum. Also from Figure 3, a weak component at 1624 cm^{-1} also appears to bleach; however, its intensity is less than one-third of those of the 1654 and 1691 cm^{-1} bands. The 1624 and ca. 1695 cm^{-1} Raman bands most likely arise from the BChl *a* molecule(s) responsible for this 827 nm component, which, incidently, is expected to exhibit lower preresonance Raman enhancement at 1064 nm than the P_{840} species. As well, the intensity of the 1624 and 1695 cm^{-1} Raman bands are diminished in preparations where the intensity of the negative 827 nm absorption band in Figure 1 is also diminished (data not shown). For the case of RCs from purple bacteria, the presence of a + charge on P_{870} does not result in the significant bleaching of Raman contributions from species other than P_{870} (Mattioli *et al.*, 1991, 1993) which dominates. Indeed, for these RCs $P_{870}^{+\bullet}$ formation results in a small (ca. 1 nm) electrochromic shift of the accessory BChl *a* transition at ca. 800 nm. However, for the case of RCs reported here, $P_{840}^{+\bullet}$ formation by treatment with ferricyanide seems to result in more of a bleaching of an electronic transition at 827 nm rather than an electrochromic shift in the spectral region. This, together with the difference in wavelength of the concerned transitions (i.e., ca. 800 nm for the purple bacterial RCs as compared to ca. 830 nm for *Chlorobium* RCs), could explain why additional bleachings are observed in the FT Raman spectra of oxidized *Chlorobium* RCs.

FT Raman Spectra of P_{840}. From the above discussion, obvious FT Raman bands in the high-frequency region attributable to neutral P_{840} are 1612, 1654, and 1691 cm^{-1}. The band at 1612 cm^{-1} arises from the C_aC_m methine bridge stretching modes of the BChl *a* molecules. Its observed frequency and narrow bandwidth (14 cm^{-1} FWHM) at 15 K (Mattioli *et al.*, 1993) indicate that both BChl *a* molecules constituting P_{840} possess one axial ligand each as is the case for P_{870} in *Rb. sphaeroides* (Mattioli *et al.*, 1991). The 1654 and 1691 cm^{-1} bands arise from carbonyl group stretching modes. *In vitro* Raman studies have shown that for BChl *a* the frequency of the C_2 acetyl carbonyl vibrational mode is observed between 1665 (free) and 1620 cm^{-1} (hydrogen bonded), whereas that of the C_9 keto carbonyl may be observed in the 1710−1650 cm^{-1} range (Lutz, 1984); the

extent of these downshifts reflects the strength of the H-bond.

The assignments of the FT Raman bands corresponding to these conjugated carbonyl groups of P_{870} from *Rb. sphaeroides* have been thoroughly discussed (Mattioli *et al.*, 1994). The 1691 cm^{-1} band of P_{840} is consistent only with a C_9 keto carbonyl which is free, not engaged in a H-bond. The 1654 cm^{-1} can arise from either a strongly H-bonded C_9 keto carbonyl group or from a free C_2 acetyl carbonyl group of BChl *a*. Since Raman spectra of BChl *a* molecules should exhibit contributions from both the C_2 acetyl and C_9 keto carbonyl groups, the 1654 cm^{-1} band must correspond to a C_2 acetyl carbonyl whose vibrational frequency reflects that it is not engaged in a hydrogen bond. Thus, for P_{840}, it appears that no conjugated carbonyl of its constituent BChl *a* molecules is involved in H-bonding. Furthermore, the observation of only two distinct Raman bands, each one attributable to a C_2 and C_9 carbonyl group of the P_{840} species, is consistent with a single population of BChl *a* molecules, all sharing the same or similar protein interactions and environments.

FT Raman Spectra of $P_{840}^{+\bullet}$. As mentioned above, bands appearing in the FT Raman spectra upon P_{840} oxidation should arise from P_{840} in its radical cation state. In the FT Raman spectrum of *Chlorobium* RCs treated with ferricyanide, and thus in the $P_{840}^{+\bullet}$ state, there are several new bands which appear in the high-frequency region, most obviously at 1589, 1606, and 1707 cm^{-1}. Because the 1707 cm^{-1} band is in a region which is spectrally uncongested, its appearance in the FT Raman spectrum of the sample upon oxidation is obvious and unambiguous. Similar to what is observed for the case of $P_{870}^{+\bullet}$ in *Rb. sphaeroides* at low temperature (Mattioli *et al.*, 1992, 1994), the 1612 cm^{-1} band (C_aC_m stretching mode) of P_{840} downshifts upon oxidation to 1606 cm^{-1}, although to a lesser extent. The 1707 cm^{-1} is observed in a spectral region where the C_9 keto carbonyl stretching mode of a BChl a^+ species is expected to contribute. *In vitro* infrared (Mäntele *et al.*, 1988) and RR studies (Heald & Cotton, 1990) on monomeric (B)Chl *a* in nonprotic solvents have shown that there is a ca. 32 cm^{-1} upshift of the C_9 keto carbonyl group vibrational frequency upon one-electron oxidation. For the case of $P_{870}^{+\bullet}$ in *Rb. sphaeroides* a band at 1717 cm^{-1} was observed in the oxidized RC spectra and represented a 26 cm^{-1} upshift in frequency of the C_9 keto carbonyl of the BChl *a* molecule of P_{870} bound to the L subunit (P_L) (Mattioli *et al.*, 1991, 1993). The 26 cm^{-1} upshift observed for $P_{870}^{+\bullet}$ compared to the 32 cm^{-1} observed for monomeric BChl *a* indicates that a resulting + charge in the oxidized, radical cation $P_{870}^{+\bullet}$ dimer is not equally shared but primarily resides on one of the BChl *a* molecules constituting P_{870}. Using the simple assumption that the observed upshift is proportional to the + charge localization and that a +32 cm^{-1} upshift represents 100% localization, we have estimated the +26 cm^{-1} upshift of $P_{870}^{+\bullet}$ to represent ca. 80% localization of the + charge on one of the two BChl *a* molecules, namely, P_L (Mattioli *et al.*, 1991, 1993). This simple analysis is in general agreement with ENDOR and electron spin modulation measurements on $P_{870}^{+\bullet}$ (Lendzian *et al.*, 1993; Davis *et al.*, 1993; Mattioli *et al.*, 1994).

The 1707 cm^{-1} band of a C_9 keto carbonyl of $P_{840}^{+\bullet}$ is unusually low in frequency as compared to other oxidized BChl *a* primary donor dimers of purple bacteria (Mattioli *et al.*, 1992). Its frequency relative to that of 1691 cm^{-1} observed in the P_{840} neutral form represents an upshift of only +16 cm^{-1}. This value is exactly one-half of the 32 cm^{-1} and represents the value expected for complete delocalization of the + charge over two BChl *a* molecules.

Deduced Structure of P_{840}. (A) Protein Interactions. The FT Raman data reported here indicate that the two BChl *a* molecules constituting P_{840} experience similar if not identical protein environments. Furthermore, these environments impose no hydrogen-bonding interactions with the conjugated C_2 acetyl and C_9 keto carbonyls of P_{840}. This highly symmetric situation is in contrast with that of the primary donors from purple bacteria where asymmetric H-bonding patterns impose asymmetric protein environments with respect to the two BChl molecules constituting P. For the case of *Rb. sphaeroides*, the C_2 acetyl carbonyl of P_L is engaged in a H-bond with His L168 while the other C_2 acetyl carbonyl and remaining C_9 keto carbonyls are free (Yeates *et al.*, 1988; El-Kabbani *et al.*, 1991; Chirino *et al.*, 1994; Ermler *et al.*, 1994; Mattioli *et al.*, 1991), and for the case of *Rhodopseudomonas viridis*, only the C_9 keto carbonyl of P_L is free while the other C_9 keto and the two C_2 acetyl carbonyl groups are H-bonded (Michel *et al.*, 1986a; Deisenhofer & Michel, 1989; Zhou *et al.*, 1989).

(B) Electronic Structure of $P_{840}^{+\bullet}$. In addition to the symmetric protein interactions experienced by P_{840}, the FT Raman data of the radical cation $P_{840}^{+\bullet}$ indicate that the + charge is equally shared between the BChl *a* molecules. Recently, on the basis of ENDOR and Special TRIPLE measurements, Rigby *et al.* (1994) have also concluded that the electron spin density in $P_{840}^{+\bullet}$ is highly symmetrically distributed between the two BChl *a* molecules. Again, this situation is in contrast to what is observed for the purple bacteria where the unpaired spin density is asymmetrically distributed (Lendzian *et al.*, 1993; Davis *et al.*, 1993; Mattioli *et al.*, 1991). For the case of P_{700}^+ in PS I, the unpaired electron spin density also appears highly asymmetric if not totally localized [for a review, see Lubitz (1991); Davis *et al.*, 1993].

Homodimeric Nature of P_{840}. Recently, the gene for the reaction center of *C. limicola* f *thiosulphatophilum* has been sequenced, and only *one* gene coding for the RC core protein could be determined (Büttner *et al.*, 1992). This situation, which is identical to that observed for heliobacteria (Liebl *et al.*, 1993), is in contrast to the heterodimeric structures of the purple bacterial, PS I and PS II reaction centers (Michel *et al.*, 1986b; Golbeck & Bryant, 1991; Trebst, 1986) which consist of two different core polypeptides.

The Raman data reported here indicate that the *Chlorobium* primary donor is a highly symmetric dimer, with respect to both BChl−protein interactions and electronic structure. This is consistent with the proposal that the *Chlorobium* reaction center is a protein homodimer imparting a symmetric environment to the two BChls of P_{840}.

Recently in mutant reaction centers from *Rb. sphaeroides*, it has been shown that the addition of histidine-donated H-bonds to C_2 or C_9 keto carbonyl groups of P_{870}, in general, raises the redox midpoint potential of the P/P^+ couple (Lin *et al.*, 1994). Similarly, the replacement of His L168 with a phenylalanine residue, thus rupturing the H-bond to the C_2 acetyl carbonyl of P_L, resulted in a decrease of 95 mV to the oxidation potential of P_{870} (Lin *et al.*, 1994). A similar effect could at least in part explain the particularly low redox potential of P_{840}, which is 240 mV compared to ca. 450−500 mV for P_{870} from *Rb. sphaeroides* (Prince & Dutton,

1978; Moss *et al.*, 1991; Williams *et al.*, 1992; Lin *et al.*, 1994). For *Chlorobium* RCs, we have found that the C_2 and C_9 carbonyl groups of P_{840} are not involved in H-bonding with the protein, and thus a H-bond to the C_2 acetyl carbonyl of P_{840} via a histidine residue is not present. Comparing these two situations, one might expect P_{840} to be ca. 100 mV lower in redox potential than P_{870}. However, the redox potential of the BChl *a* dimer P_{840} is ca. 200−250 mV lower than that of P_{870}, and clearly other factors are influencing the redox potential.

One factor could be the highly symmetric nature of P_{840} in both protein interactions and electronic structure as described above. A more symmetric, "dimeric" primary donor could result in a lower redox potential compared to a similar BChl *a* dimer which is more asymmetric and therefore retaining some "monomeric" character. However, two observations would counter this argument: (i) the presumable lower exciton absorption maximum of the P_{840} BChl *a* dimer is ca. 400 cm^{-1} higher in energy than that of P_{870}, and (ii) the bandwidth of the 840 nm band appears significantly narrower than that of P_{870}. These observations could reflect weaker exciton interaction and/or less charge-transfer character for P_{840} as compared to P_{870}. This would imply that the protein environment in the vicinity of, and/or the geometry of, the primary donor is somewhat different between P_{840} and P_{870}.

The PsaA and PsaB core proteins of the PS I RC are predicted to possess 11 transmembrane helices (Fish *et al.*, 1985). The histidine residues suggested to act as the binding ligands to the primary donor P_{700} were proposed to be located in helix 8 of PsaA and PsaB (Robert & Möenne-Loccoz, 1990). Conserved histidine residues in helix 8 of the sequence of the *C. limicola* RC protein (Büttner *et al.*, 1992) and of the *Heliobacillus mobilis* RC protein (Liebl *et al.*, 1993) have also been proposed as the binding ligands of their respective primary donors. Recently, however, the location of the putative P_{700} histidine ligand in helix 8 has been challenged on the basis of evolutionary arguments (Vermaas, 1994; Rutherford & Nitschke, 1995; Nitschke *et al.*, 1995) and by site-directed mutagenesis studies on *Chlamydomonas reinhardtii* in which the purported histidine ligand in the predicted helix 8 of PsaB as well as two other histidine residues in the same helix has been genetically altered (Cui *et al.*, 1995). These evolutionary models and experimental results have led to the proposal that the P_{700} binding ligands could be found in helix 10 where indeed conserved histidine residues are found for PsaA and PsaB of maize as well as for the RC proteins of *C. limicola* (Büttner *et al.*, 1992) and *H. mobilis* RC protein (Liebl *et al.*, 1993).

Assuming structural analogies of the primary donor of the purple bacteria extend to P_{840} in *Chlorobium*, the absence of H-bonding interaction at the level of the acetyl carbonyl group of each BChl *a* molecule constituting P_{840}, as determined by the Raman data presented in this work, is consistent with the histidine residues of either the predicted helix 8 or 10 as being the binding ligands of P_{840}. Both C_2 acetyl carbonyl groups of the primary donor of *Rps. viridis* are engaged in H-bonds with His L168 and Tyr M195 (Deisenhofer & Michel, 1989; Zhou *et al.* 1989); both of these histidine residues are five positions away from the binding ligand of each BChl molecule constituting P (His L173 and His M200). The conserved His L168 residue also forms a H-bond with the C_2 acetyl carbonyl group of *Rb. sphaeroides* as seen in the X-ray crystal structure (El-Kabbani *et al.*, 1991; Chirino *et al.* 1994; Ermler *et al.*, 1994) and spectroscopically (Mattioli *et al.*, 1991, 1994). A histidine residue, such as His L168, being five positions away from the BChl-binding histidine, is not conserved in either helix 8 or helix 10 in *Chlorobium* and PS I.

ACKNOWLEDGMENT

We thank Drs. W. Nitschke and A. W. Rutherford for stimulating discussions and Dr. A. N. Webber for communicating results prior to publication.

REFERENCES

Biebl, H., & Pfennig, N. (1978) *Arch. Microbiol. 117*, 9−16.
Buchanan, B. B., & Evans, M. C. W. (1969) *Biochim. Biophys. Acta 180*, 123−129.
Büttner, M., Xie, D.-L., Nelson, H., Pinther, W., Hauska, G., & Nelson, N. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8135−8139.
Chirino, A. J., Lous, E. J., Huber, M., Allen, J. P., Schenck, C. C., Paddock, M. L., Feher, G., & Rees, D. C. (1994) *Biochemistry 33*, 4584−4593.
Cotton, T. M., & Van Duyne, R. P. (1981) *J. Am. Chem. Soc. 103*, 6020.
Cui, L., Bingham, S. E., Kuhn, M., Käss, Lubitz, W., & Webber, A. N. (1995) *Biochemistry 34*, 1549−1558.
Davis, I. H., Heathcote, P., MacLachlan, D. J., & Evans, M. C. W. (1993) *Biochim. Biophys. Acta 1143*, 183−189.
Deisenhofer, J., & Michel, H. (1989) *EMBO J. 8*, 47−54.
El-Kabbani, O., Chang, C.-H., Tiede, D., Norris, J., & Schiffer, M. (1991) *Biochemistry 30*, 5361−5369.
Ermler, U., Fritzsch, G., Buchanan, S. K., & Michel, H. (1994) *Structure 2*, 925−936.
Fajer, J., Brune, D. C., Davis, M. S., Forman, A., & Spaulding, L. D. (1975) *Proc. Natl. Acad. Sci. U.S.A 72*, 4956−4960.
Feiler, U., Nitschke, W., & Michel, H. (1992) *Biochemistry 31*, 2608−2614.
Feiler, U., Albouy, D., Pourcet, C., Mattioli, T. A., Lutz, M., & Robert, B. (1994) *Biochemistry 33*, 7594−7599.
Fish, L. E., Kück, U., & Bogorad, L. (1985) in *Molecular Biology of the Photosynthetic Apparatus* (Steinback, K. E., Bonitz, B., Arntzen, C. J., & Bogorad, L., Eds.) pp 111−120, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Fowler, C. F., Nugent, N. A., & Fuller, R. C. (1971) *Proc. Natl. Acad. Sci. U.S.A 68*, 2278−2282.
Golbeck, J. H., & Bryant, D. A. (1991) *Curr. Top. Bioenerg. 16*, 83−177.
Heald, R. L., & Cotton, T. M. (1990) *J. Phys. Chem. 94*, 3968−3975.
Jennings, J. V., & Evans, M. C. W. (1977) *FEBS Lett. 75*, 33−36.
Knaff, D. B. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 629−640, Plenum, New York.
Komiya, H., Yeates, T. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012−9016.
Kusumoto, N., Inoue, K., Nasu, H., Takano, H., & Sakurai, H. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. I, pp 389−392, Kluwer Academic, Dordrecht, The Netherlands.
Kusumoto, N., Inoue, K., Nasu, H., Takano, H., & Sakurai, H. (1994) *Plant Cell Physiol. 35*, 17−25.
Lendzian, F., Huber, M., Isaacson, R. A., Endeward, B., Plato, M., Bönigk, B., Möbius, K., Lubitz, W., & Feher, G. (1993) *Biochim. Biophys. Acta 1183*, 139−160.
Liebl, U., Mockensturm-Wilson, M., Trost, J. T., Brune, D. C., Blankenship, R. E., & Vermaas, W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7124−7128.
Lin, X., Murchison, H. A., Nagarajan, V., Parson, W. W., Allen, J. P., & Williams, J. C. (1994) *Proc. Nalt. Acad. Sci. U.S.A. 91*, 10265−10269.
Lubitz, W. (1991) in *Chlorophylls* (Scheer, H., Ed.) pp 903−944, CRC Press, Boca Raton, FL.
Lutz, M. (1984) in *Advances in Infrared and Raman Spectroscopy* (Clark, R. J. H., & Hester, R. E., Eds.) Vol. 11, pp 211−300, Wiley, New York.

Lutz, M., & Robert, B. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, pp 347−411, Wiley-Interscience, New York.
Mäntele, W. G., Wollenweber, A. M., Nabedryk, E., & Breton, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8468−8472.
Mattioli, T. A., Haley, L. V., & Koningstein, J. A. (1990) *Chem. Phys. 140*, 317−329.
Mattioli, T. A., Hoffmann, A., Robert, B., Schrader, B., & Lutz, M. (1991) *Biochemistry 30*, 4648−4654.
Mattioli, T. A., Robert, B., & Lutz, M. (1992) in *The Photosynthetic Bacterial Reaction Center: Structure, Spectroscopy, and Dynamics* (Breton, J., & Vermeglio, A., Eds.) pp 127−132, Plenum, New York.
Mattioli, T. A., Hoffmann, A., Sockalingum, D. G., Schrader, B., Robert, B., & Lutz, M. (1993) *Spectrochim. Acta 49A*, 785−799.
Mattioli, T. A., Williams, J. C., Allen, J. P., & Robert, B. (1994) *Biochemistry 33*, 1636−1643.
Michel, H., Epp, O., & Deisenhofer, J. (1986a) *EMBO J. 5*, 2445−2451.
Michel, H., Weyer, K. A., Gruenberg, H., Dunger, I., Oesterhelt, D., & Lottspeich, F. (1986b) *EMBO J. 5*, 1149−1158.
Miller, M., Liu, X., Snyder, S., Thurnauer, M. C., & Biggins, J. (1992) *Biochemistry 31*, 4354−4363.
Moss, D. A., Leonhard, M., Bauscher, M., & Mäntele, W. (1991) *FEBS Lett. 283*, 33−36.
Murchison, H. A., Alden, R. G., Allen, J. P., Peloquin, J. M., Taguchi, A. K. W., Woodbury, N. W., & Williams, J. C. (1993) *Biochemistry 32*, 3498−3505.
Nitschke, W., & Rutherford, A. W. (1991) *Trends Biochem. Sci. 16*, 241−245.
Nitschke, W., Feiler, U., Lockau, W., & Hauska, G. (1987) *FEBS Lett. 218*, 283−286.
Nitschke, W., Feiler, U., & Rutherford, A. W. (1990) *Biochemistry 29*, 3834−3842.
Nitschke, W., Mattioli, T. A., & Rutherford, A. W. (1995) in *Origin and Evolution of Biological Energy Conversion* (Baltscheffsky, H., Ed.) VCH, New York (in press).
Oh-oka, H., Kakutani, S., Matsubara, H., Malkin, R., & Itoh, S. (1993) *Plant Cell Physiol. 34*, 93−101.
Olson, J. M., Prince, R. C., & Brune, D. C. (1976) *Brookhaven Symp. Biol. 28*, 238−246.
Prince, R. C., & Olson, J. M. (1976) *Biochim. Biophys. Acta 423*, 357−362.
Prince, R. C., & Dutton, P. L. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 439−453, Plenum, New York.
Rigby, S. E. J., Thapar, R., Evans, M. C. W., & Heathcote, P. (1994) *FEBS Lett. 350*, 24−28.
Robert, B. (1995) in *Advances in Photosynthesis* (Hoff, A. J., & Amesz, Eds.) Vol. XX, Chapter 10, Kluwer, Dordrecht (in press).
Robert, B., & Möenne-Loccoz, P. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 65−68, Kluwer, Dordrecht.
Rutherford, A. W., & Nitschke, W. (1995) in *Origin and Evolution of Biological Energy Conversion* (Baltscheffsky, H., Ed.) VCH, New York (in press).
Swarthoff, T., Gast, P., Hoff, A. J., & Amesz, J. (1981a) *FEBS Lett. 130*, 93−98.
Swarthoff, T., Gast, P., & Hoff, A. J. (1981b) *FEBS Lett. 127*, 83−86.
Trebst, A. (1986) *Z. Naturforsch. 41C*, 240−245.
van de Meent, E. J., Kobayashi, M., Erkelens, C., van Veelan, P. A., Otte, S. C. M., Inoue, K., Watanabe, T., & Amesz, J. (1992) *Biochim. Biophys. Acta 1102*, 371−378.
Vasmel, H., den Blanken, H. J., Dijkman, J. T., Hoff, A. J., & Amesz, J. (1984) *Biochim. Biophys. Acta 767*, 200−208.
Vermaas, W. F. J. (1994) *Photosynth. Res. 41*, 285−294.
Wachtveitl, J., Farchaus, J. W., Das, R., Lutz, M., Robert, B., & Mattioli, T. A. (1993) *Biochemistry 32*, 12875−12886.
Williams, J. C., Alden, R. G., Murchison, H. A., Peloquin, J. M., Woodbury, N. W., & Allen, J. P. (1992) *Biochemistry 31*, 11029−11037.
Yeates, Y. O., Komiya, H., Chirino, A., Rees, D. C., Allen, J., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7993−7997.
Zhou, Q., Robert, B., & Lutz, M. (1989) *Biochim. Biophys. Acta 977*, 10−18.

BI950538V