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Structure of the Primary Donor of *Rhodopseudomonas sphaeroides*: Difference Resonance Raman Spectroscopy of Reaction Centers

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ABSTRACT: Resonance Raman spectra of the primary donor in its ground, neutral state were obtained from reaction centers of *Rhodopseudomonas sphaeroides*, wild type, by using difference methods. Three distinct methods yielded essentially identical difference spectra, arising from two unequivalent bacteriochlorophyll *a* molecules. These spectra permitted the molecular interaction states of the magnesium atoms and of the keto and acetyl carbonyls of the primary donor bacteriochlorophylls to be described. From these data, a molecular model of the primary donor of *Rps. sphaeroides*, wild type, is proposed. This model involves no mutual binding interactions between the two bacteriochlorophylls, in the neutral, ground state, through their magnesium atoms and their acetyl carbonyls.

The first steps of bacterial photosynthesis, involving the creation of a donor–acceptor ion pair from trapped energy of incoming photons, occur in specialized membrane-bound pigment–protein complexes named reaction centers (RC).[1] These proteic complexes have been isolated in highly pure yet functional states from several species of bacteria (Gingras, 1978) and their primary photochemistry is now reasonably well understood (Parson, 1982).

The reaction centers of purple photosynthetic bacteria generally appear to contain four bacteriochlorophylls (BChl), two bacteriopheophytins (BPheo), two quinones, a non-heme iron or manganese atom, and a carotenoid. The primary charge separation that initiates the electron transfer involves a particular structure, named the primary donor P.

The primary donor of many purple photosynthetic bacteria appears to consist of a pair (Norris et al., 1971) of bacteriochlorophyll molecules, which assume strong electronic coupling. Much interest has been devoted to the structure of this assembly: polarized electronic absorption spectroscopies and, particularly, paramagnetic resonance methods (Hoff, 1982) of the reaction center and of molecular models have led to various pictures. Among others, the following structural predictions were made from spectroscopic data: the two molecules should be related by a C_2 axis and their dihydrophorbin planes should be closely parallel, with a plane to plane distance of about 3.5 Å; the conjugated parts of the two macrocycles should overlap partially only (Fong, 1975; Boxer & Closs, 1976; Shipman et al., 1976; Wasielewski et al., 1977; Maggiora, 1979; Lubitz et al., 1984), and the Q_Y transition moments of the monomeric molecules should be noncollinear (Paillotin et al., 1979). However, this picture remained incomplete and controversial. In particular, no direct information was available on the intermolecular interactions that stabilize the above structure. Recent X-ray crystallographic studies brought decisive information about the structure of the primary donor in *Rhodopseudomonas viridis* (Deisenhofer et al., 1984). Spectroscopic data indicate that this structure is most probably largely transposable to those present in reaction centers of many other purple bacteria, e.g., *Rhodopseudomonas sphaeroides* (Breton, 1985; Lubitz et al., 1985). Additional knowledge provided by X-ray studies concerns the detailed relative arrangement of the two BChl *a* molecules, which have been shown to overlap at the levels of their rings I and to be related one to each other by a C_2 symmetry (Deisenhofer et al., 1984). The spatial resolution of these data, although still limited by the lack of the complete sequences of the polypeptide chains, permitted some conclusions to be drawn about the intermolecular bonding of the two BChls.

[1] Abbreviations: BChl, bacteriochlorophyll *a*; BPheo, bacteriopheophytin *a*; LDAO, lauryldimethylammonium oxide; RC, reaction center(s); *Rps.*, *Rhodopseudomonas*; *Rsp.*, *Rhodospirillum*; RR, resonance Raman; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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These conclusions essentially concerned the coordination states of the magnesium atoms, which clearly appeared to be liganded to amino acid side chains of α-helices of the L and of the M polypeptides (Deisenhofer et al., 1984). Close distances between the acetyl or methyl groups to magnesium of the adjacent molecule were taken as indicating that the acetyl groups should occupy the second liganding positions of the Mg atoms (Deisenhofer et al., 1984, 1985a).

Resonance Raman spectroscopy of the bacteriochlorin *a* pigments of reaction centers of *Rps. sphaeroides* and of *Rhodospirillum rubrum* (Lutz, 1979, 1981, 1984; Lutz et al., 1976) yielded direct information about the interaction states of these pigments in the ground state. One of these conclusions was that the four BChl molecules (i.e., the two constituting the primary donor and the two accessory molecules) should have five-coordinated Mg atoms, i.e., each binding a single external ligand (Lutz, 1981). The present study was aimed at selectively producing resonance Raman spectra of the primary donor pair alone. Previous observations were less selective, being limited to unspecified contributions in spectra also containing contributions either from the two accessory BChls (Lutz, 1979, 1984) or from the BPheo and accessory BChl molecules (Lutz, 1981). Preliminary accounts of this study have been given (Lutz & Robert, 1985; Robert & Lutz, 1985a).

Experimental Procedures

Materials. Cells of *Rps. sphaeroides* 2.4.1 (wild type) were grown anaerobically in a modified Hutner medium at 30 °C. Chromatophores were prepared as previously described (Robert & Lutz, 1985b). Crude reaction centers were obtained by treating chromatophores with 0.3% LDAO and then centrifuging them for 1 h at 55000*g*. The supernatant was fractionated by precipitation with ammonium sulfate (0.2 g/mL) and dialyzed overnight against Tris-HCl, 25 mM (pH 8) and 0.1% LDAO. Reaction centers were purified on DEAE-cellulose (DE-52, Whatman). After a new dialysis against the Tris–LDAO buffer, LDAO was exchanged against Triton X-100 on a DEAE-cellulose column. For Raman experiments, RC were concentrated to ca. 100 μM with a Centricon (Amincon) system. Reduction of the quinone electron acceptor was performed by adding excess sodium dithionite or dithiothreitol.

Resonance Raman experiments were conducted on a Jobin Yvon spectrometer (Ramanor HG2S-UV) with a 363.8-nm excitation wavelength from an argon laser (Spectra Physics Model 171). During the RR experiments, the sample temperature was kept at 20 or 80 K by a flow of cold gaseous helium and was measured near to the sample. Grazing incidence of the excitation laser beam was used in order to prevent reabsorption of the Raman photons by the sample. In these conditions, and with laser radiant powers not higher than 20 mW, less than 2 mW penetrated the sample. Typical spectral resolution at 1000 cm^{-1} was 8 cm^{-1}. The signal to noise ratios were improved by summation of individual spectra in a multichannel analyzer (Tracor Northern 1710).

Methods. Previous work on reaction centers from *Rps. sphaeroides* strains and on *Rsp. rubrum* (Lutz, 1979) has shown that RR spectra of the bacteriochlorin pigments can be obtained in both Q_X and Soret resonance conditions. At Q_X resonance, the bacteriochlorophylls and the bacteriopheophytins can be observed independently, with 528–550- and 580–620-nm excitations, respectively. Yet, the information content of these spectra appears somewhat limited, especially because of the absence of sizable contributions from the stretching modes of the conjugated carbonyls (Lutz et al., 1976). These groups are known to play key roles in molecular interactions of the chlorophylls (Katz et al., 1978; Lutz, 1984). Stretching modes of the conjugated carbonyls are active in Raman spectra obtained at Soret resonance conditions (Lutz, 1981). In these conditions, however, due to almost perfect overlap of the Soret bands of the BChl and BPheo in reaction centers, all six bacteriochlorin pigments are expected to contribute in RR spectra excited at the top of the Soret band. Indeed, up to 10 components have been observed in the carbonyl-stretching region (1620–1705 cm^{-1}), in which 12 unequivalent, carbonyl vibrators are expected to contribute, i.e., one stretching mode from the 9-keto group and one from the 2-acetyl carbonyl from each individual molecule (Lutz, 1981).

Selectively observing the primary donor contributions can thus be achieved only by difference spectroscopy. The laser beam used in producing RR spectra has an actinic effect on the reaction center preparations, inasmuch as the resonance effect is obtained when the excitation wavelength matches a vibronic transition of the investigated molecules, here the bacteriochlorins, i.e., when a high proportion of the incoming photons is actually absorbed by the sample. We recently showed (Robert et al., 1985) that sizable amounts (up to 20%) of the spheroidene molecules may assume the lower triplet excited state (5-μs lifetime) in chemically reduced RC of *Rps. sphaeroides* 2.4.1, by continuous wave excitation at the weakly absorbed 545-nm wavelength.

It thus appeared possible, by acting on the illumination level due to the Raman probing laser beam, to modify the amount of centers with their primary donors in a neutral, ground state being present in the sample. In untreated reaction centers a steady-state illumination results in the presence of variable amounts of the radical cation of the primary donor, P^+. In the low-temperature conditions of the RR experiments, generally conducted around 20 K, the lifetime of P^+ is 100 ms (Mc Elroy et al., 1974), and near to 100% of the RC probed may be expected to be oxidized in a typical RR experiment. Similarly, in chemically reduced centers, in which the forward electron transfer is blocked, high amounts of a triplet state of P870 designated P^R, the lifetime of which is 100 μs, may build up in the sample if the transfer of this triplet to the carotenoid is impossible or limited (Parson & Monger, 1976).

Both the radical cation and triplet states of the primary donor have extinction coefficients that are smaller than those of the neutral species at the top of the Soret band of the latter (Parson & Monger, 1976). Their contributions to the RR spectra excited at 364 nm should accordingly be smaller than those of the neutral pigments. Hence, differences between RR spectra obtained at low and at high illumination levels from either untreated or chemically reduced RC, at Soret resonance, should primarily arise from the primary donor in its neutral, ground state.

A third difference RR experiment was devised, making use of the marked temperature dependence of the triplet–triplet transfer efficiency from state P^R to the carotenoid, in reaction centers from wild types of certain species such as *Rps. sphaeroides* (Parson & Monger, 1976; Schenck et al., 1984). Because of a decreased transfer efficiency from P^R to spheroidene below 60 K, chemically reduced centers, probed at constant illumination levels, should contain smaller amounts of P^R and hence higher amounts of P at 80–100 K than at 20 K.

Results

Resonance Raman spectra of reaction centers from *Rps. sphaeroides* 2.4.1 excited at 363.8 nm contain three strong bands in the 1500–1750-cm^{-1} range (Figure 1). A 1540-cm^{-1}

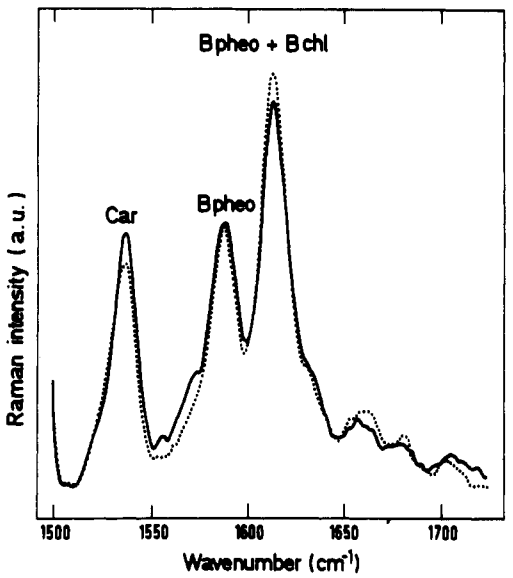

FIGURE 1: Resonance Raman spectra (1500–1740-cm^{-1} regions) of dithionite-treated reaction centers from *Rps. sphaeroides* 2.4.1 at 20 (solid line) and 80 K (dotted line): excitation, 363.8 nm; spectral resolution, 8 cm^{-1}. The spectra were normalized on the bacteriopheophytin band at 1590 cm^{-1}. See text.

band arises from the stretching modes of C=C bonds of spheroidene (Lutz et al., 1976). A 1615-cm^{-1} band, arising from both BChl and BPheo, has been assigned to a mode primarily involving stretching of the methine bridges. A third band at 1590 cm^{-1} essentially arises from an unspecified mode of BPheo involving stretching of both C=C and C=N bonds. This latter band masks a weak ca. 1580-cm^{-1} mode of the BChl molecules. Weaker bands in the 1630–1710-cm^{-1} region arise from the stretching modes of conjugated carbonyls of most if not all of the six bacteriochlorin pigments in various states of interactions (Lutz, 1979, 1981, 1984).

The two spectra of Figure 1 were obtained from dithionite-treated centers, at constant illumination. The solid- and dotted-line spectra were obtained at 20 and 80 K, respectively, and were normalized on the 1590-cm^{-1} band of Bpheo. As predicted (cf. Methods), the carotenoid band at 1540 cm^{-1} is stronger at 20 K than at 80 K, due to a more efficient triplet–triplet transfer from P^R to spheroidene at the latter temperature. This results in a smaller amount of spheroidene in the ground state being present in the sample at 80 K than at 20 K. Conversely, the 1615-cm^{-1} band intensity is higher at 80 K than at 20 K, due to a lower amount of P^R and hence a higher amount of P870 in the ground state at 80 K than at 20 K.

Two such spectra, each the sum of 35 individual scans, are shown in Figure 2, together with their difference. The latter was obtained by using a relative weighing that ensured an almost complete cancellation of the 1590-cm^{-1} band, corresponding to the relative contributions at 1580 and 1615 cm^{-1} expected for a BChl species. The difference spectrum consists of positive contributions only, located at 1612, 1637, 1660, and 1684 cm^{-1}. The absence of negative contributions indicates that, as expected, none of the intermediate states of the bacteriochlorin pigments that are present in higher amounts in the sample at lower temperature is significantly contributing in the difference Raman spectrum. The observed difference spectrum quite likely arises from BChl molecules in the ground, neutral state, as estimated from the presence of the 1612-cm^{-1} band and from its intensity relative to the 1637-, 1660-, and 1684-cm^{-1} bands. The latter bands have frequencies quite consistent with those expected for stretching of acetyl and keto

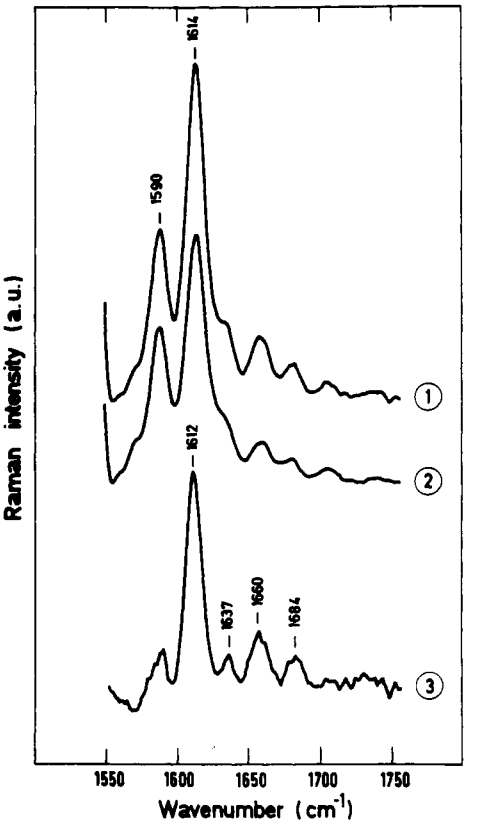

FIGURE 2: Resonance Raman spectra (1550–1750-cm^{-1} regions) of dithionite-treated reaction centers from *Rps. sphaeroides* 2.4.1 at low temperature: excitation, 363.8 nm; spectral resolution, 8 cm^{-1}. (1) Sum of 35 scans on the sample set at 80 K; (2) sum of 35 scans on the same sample set at 20 K; (3) difference between spectra 1 and 2. For normalization, see text.

carbonyls of BChl (Lutz, 1984). The fact that there are three bands in the 1620–1700-cm^{-1} region indicates that two BChl molecules are contributing, as would be expected for a contribution from the primary donor alone.

The spectra of Figure 3 were obtained from dithionite-treated reaction centers, at a fixed 20 K temperature. The upper spectrum was recorded at a lower illumination level than the middle spectrum. The difference spectrum (bottom) was obtained with near cancellation of the BPheo band at 1590 cm^{-1}. In the 1600–1750-cm^{-1} region, this difference spectrum is extremely similar to that of Figure 2, exhibiting no negative bands, and the same number of positive bands, at the same frequencies, and with remarkably similar relative intensities.

The same observations hold for the difference spectrum of Figure 4, which was obtained from RR spectra of untreated reaction centers, recorded at a fixed 20 K temperature and at high and low illumination levels, respectively. A slight increase in relative intensity of the 1684-cm^{-1} band, as compared to that observed in reduced RC spectra, may, however, be significant. It may be noted, in addition, that normalizing the two spectra on the 1540-cm^{-1} carotenoid band resulted in a satisfactory, near cancellation of the BPheo 1590-cm^{-1} band. This shows that, as expected, neither the carotenoid nor the BPheo had its ground-state concentration altered by the illumination change. Identical difference spectra were also obtained when subtracting low-illumination RR spectra of untreated centers from spectra of ferricyanide-treated reaction centers (not shown). These experiments also showed that under high illumination levels the weakening of the 1615-cm^{-1} band relative to that of the 1590-cm^{-1} band was ca. 80% of that observed with ferricyanide-treated centers, thus confirming that high proportions of the centers may be kept in their P^+

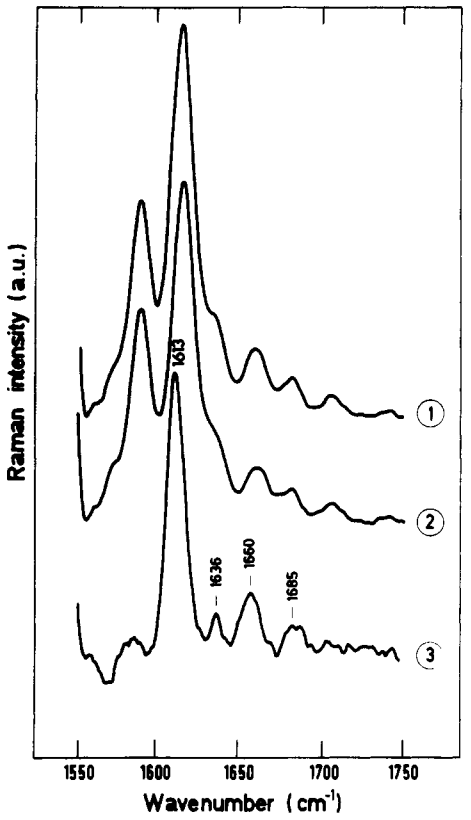

FIGURE 3: Resonance Raman spectra (1550–1750-cm^{-1} regions) of dithionite-treated reaction centers from *Rps. sphaeroides* 2.4.1 at 20 K: excitation, 363.8 nm; spectral resolution, 8 cm^{-1}. (1) Sum of 18 scans under low illumination conditions; (2) sum of 18 scans under high illumination conditions; (3) difference between spectra 1 and 2. For normalization, see text.

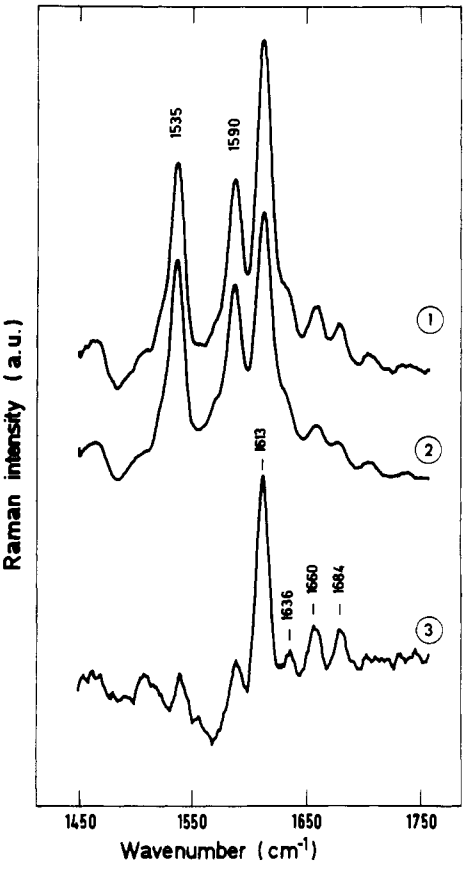

FIGURE 4: Resonance Raman spectra (1450–1750-cm^{-1} regions) of untreated reaction centers from *Rps. sphaeroides* 2.4.1 at 20 K: excitation, 363.8 nm; spectral resolution, 8 cm^{-1}. (1) Sum of 13 scans under low illumination conditions; (2) sum of 13 scans under high illumination conditions; (3) difference between spectra 1 and 2. For normalization, see text.

state at high illumination levels in our experimental conditions. Control experiments, finally, showed that RR spectra of ferricyanide-treated centers were insensitive to changes in illumination levels (not shown).

Discussion

The difference resonance Raman spectra obtained, in the 1550–1750-cm^{-1} region, from reaction centers of *Rps. sphaeroides* 2.4.1, can be ascribed to BChl *a* molecules in their neutral states, as estimated from the band frequencies and relative intensities (Lutz, 1984). From the observed number of carbonyl bands, three, the number of BChl molecules contributing is most likely to be two. We identify these two molecules with those constituting the primary donor P. This assignment is very strongly supported by the remarkable similarity, in both band frequencies and relative intensities, of the difference spectra obtained by the three different methods described above. Indeed, neutral P is the only molecular species that is common to the three types of experiments and the concentration of which is expected to vary in each of them. In addition, the observed similarity between the three types of difference spectra probably eliminates any possibility of artifactual origins for these spectra. For example, changes in Raman scattering cross sections of any pigment of the reaction center conceivably might result from differences in sample temperature at high and low illumination levels. Such an origin for the difference spectra can safely be ruled out by considering the identical results of the two sets of experiments on reduced reaction centers, run at constant and at variable illumination levels, respectively. Indeed, in the latter experimental conditions, the possible temperature difference between the two states of the reaction center and hence its possible spurious spectral effects should have been opposite to the temperature difference imposed between the corresponding two states in the constant illumination experiment.

Changes in Raman scattering cross sections of any pigment of the reaction center may also result indirectly from changes in redox or electronic states of other molecules, e.g., via conformational changes in the protein. In particular, the experiment at variable illumination levels on untreated RC at 20 K involves a different redox state of the quinone in reaction centers in the P or P^+ states. This difference, however, does not provide any spurious contribution to the difference spectrum, inasmuch as this spectrum is almost identical with those obtained from chemically reduced centers, which, in these low-temperature experiments, involve the Q^- state only (Dutton et al., 1978). Recent calculations indicated that excited-state mixing should occur between P and the accessory bacteriochlorophylls (Parson et al., 1985). Oxidation of P, as well as its excitation to the P^R state, should modify these interactions and hence the Raman cross sections of the accessory bacteriochlorophylls, which might then contribute in the present difference spectra. However, two BChl molecules only are participating, to first order, in these spectra, as well as in those obtained with 351.1-nm excitation. This indicates that such contributions must be small.

Ground-State Interactions on the Primary Donor Molecules. The 363.8-nm RR spectrum of the neutral, ground-state primary donor of *Rps. sphaeroides* 2.4.1 provides the following information on the ground-state interactions assumed by the two BChl *a* molecules.

(*A*) *Magnesium Liganding.* The ca. 1600-cm^{-1} methine bridge stretching mode is observed at 1612 cm^{-1} for P (Figures 2–4). The half-width of the RR band is 13 cm^{-1} only, indicating that both BChl molecules vibrate at the same frequency and hence that both have their magnesiums each binding a single external ligand. Indeed, comparing RR spectra of BChl *a* mono- and disolvated by pyridine, Cotton and Van Duyne (1981) proposed that the ca. 1600-cm^{-1} mode should occur 15 cm^{-1} higher when the Mg atom is five-coordinated than when it is six-coordinated. We confirmed this proposal by comparing RR spectra of BChl *a* disolvated by tetrahydrofuran, pyridine, or 1-methylimidazole, in all of which this mode occurs near to 1605 cm^{-1}, and RR spectra of BChl *a* monosolvated by 2-methylpyridine or 2-methylimidazole (Robert & Lutz, 1985b). The frequency of the methine mode is indirectly sensitive to the liganding of Mg, probably through the conformation of the $MgN_4L(L')$ grouping. The higher, ca. 1615-cm^{-1} frequency of this mode, as observed for P, most likely reflects (Lutz et al., 1982) the 0.4–0.54-Å out of plane position that the Mg atom is known to adopt when bound to a single strong ligand such as histidine, a peptidic carbonyl (Tronrud et al., 1986), or water (Tronrud et al., 1986; Serlin et al., 1975; Chow et al., 1975). The lower, ca. 1605-cm^{-1} frequency should correspond to an almost in-plane position of the Mg atom, when binding two equivalent external ligands.

This direct observation of five-coordinated states of the magnesiums of the primary donor of *Rps. sphaeroides* confirms previous conclusions drawn from studies of the low-frequency regions of RR spectra of the six bacteriochlorin pigments altogether (Lutz, 1981). These spectra contain a ca. 300-cm^{-1} band arising from a BChl mode involving Mg–N stretching, and the frequency of which is sensitive to the number of ligands on the magnesium (Lutz et al., 1982; Lutz, 1984). The 295-cm^{-1} frequency of this band, with no 300-cm^{-1} component, indicated that all four BChls of *Rps. sphaeroides* RC have single liganded magnesiums (Lutz, 1981). However, the relative contribution of neutral P in these spectra was not determined and may have been small.

(*B*) *Carbonyl-Stretching Modes.* Four distinct carbonyl-stretching vibrations should contribute in the 1620–1710-cm^{-1} range of RR spectra of ground-state P. Three bands only are observed. This and the overlap of the possible frequency ranges of the keto and of the acetyl carbonyl stretching modes (ca. 1640–1710 and 1620–1665 cm^{-1}, respectively) mean that more than one set of assignments can be considered for those bands.

One of these sets, however, has to be preferred over the others. Free acetyl carbonyls of BChl *a* vibrate at 1655–1665 cm^{-1} (Lutz, 1984). Hence, the 1637- and 1660-cm^{-1} bands can be assigned to the two acetyls of P. The latter frequency indicates a group free from intermolecular bonding and the former, an interacting group. The 1684-cm^{-1} band necessarily arises from a moderately interacting keto carbonyl. The second keto vibrator may be accidentally degenerate either with the other one at 1684 cm^{-1} or with the 1660-cm^{-1} acetyl frequency. A persistent shoulder observed at ca. 1662 cm^{-1} in many spectra (Lutz & Robert, 1985) may actually arise from this second keto group.

Other interpretations of the νC=O region of RR spectra of P appear much less likely. Assigning the ca. 1660-cm^{-1} band to both acetyl carbonyl modes, then nearly degenerate, would compel us to assign the 1636-cm^{-1} band to a keto carbonyl. A 1636-cm^{-1} frequency would be unusually low for a keto carbonyl, particularly in a biological environment. Indeed, the keto carbonyls of chlorophyll *a*, the RR spectra of which contain this contribution only in the discussed range, did not yield any stretching frequency lower than 1651 cm^{-1} in eukaryote protein–pigment complexes (Lutz, 1977, 1984; Lutz et al., 1979). Frequencies close to 1640 cm^{-1} actually were observed for this mode in RR spectra of hydrated polymers only (Lutz, 1977, 1984).

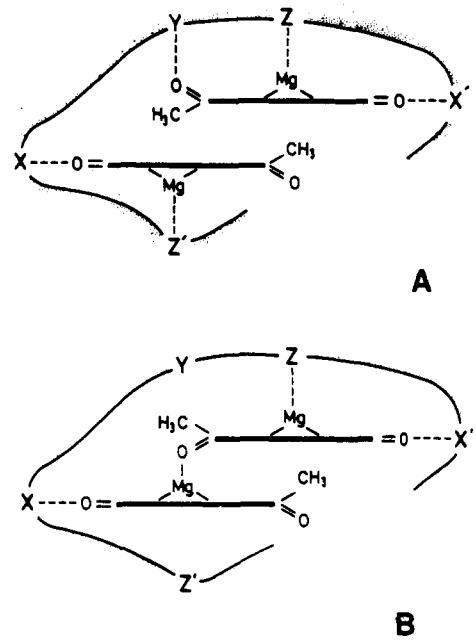

FIGURE 5: Molecular models for the ground-state binding interactions assumed by the magnesiums and the keto and acetyl carbonyls of the primary donor bacteriochlorophylls of *Rps. sphaeroides*, wild type. These models are based on the present Raman data, assuming that, as for *Rps. viridis* (Deisenhofer et al., 1984), the two macrocycles overlap at the level of rings I. Binding sites X, X', Y, Z, and Z' are all likely to be proteic, although this is not necessarily so. Z and Z' are most likely to be histidine side chains. Model A is strongly preferred over model B (see text).

Finally, the possibility that a ketone carbonyl vibrator might be inactive in the RR spectrum of P cannot be excluded at this stage. However, as far as the two BChls constituting P necessarily contribute in the RR spectrum, the keto carbonyl of one of them could be drawn out of resonance only because of a marked out of plane conformation (Lutz et al., 1982). Such a possibility could probably occur only through a strong interaction of this group with an external site, a state which is not qualitatively different from that indicated by the more likely interpretation of the RR spectrum of P presented above.

Hence, the more probable set of assignments for the carbonyl-stretching region of P involves one acetyl carbonyl free from any binding interaction, a second acetyl carbonyl intermolecularly bonded, and two intermolecularly bonded keto carbonyls.

Preliminary results obtained by the same methods for the primary donor of *Rsp. rubrum* (Zhou, 1985; Zhou Qing, B. Robert, and M. Lutz, unpublished results) fully confirm this set of assignments. Indeed, difference RR spectra of the primary donor of this species yielded three carbonyl bands at the same frequencies as those observed for *Rps. sphaeroides*, together with an additional 1700-cm^{-1} band, necessarily arising from a free keto carbonyl. Hence, the assignments of the 1636- and 1660-cm^{-1} bands to acetyl-stretching modes are confirmed. Moreover, a relative weakening of the ca. 1660-cm^{-1} band of *Rps. rubrum*, compared to that of *Rps. sphaeroides*, strongly suggested that, in the latter species, the second keto carbonyl contributes at 1660 cm^{-1}.

Structure of the Primary Donor of Rps. sphaeroides. The present Raman data allow two alternative models for the

structure of the primary donor of *Rps. sphaeroides* to be proposed (Figure 5). These models are based on the very likely assumption (Breton, 1985; Lubitz et al., 1985) that the relative arrangements of the two BChl molecules are very much the same in *Rps. sphaeroides* and in *Rps. viridis* and that, in both species, they overlap at the levels of their rings I. Hence, the binding interaction observed on the acetyl carbonyl of one of the two BChls of the primary donor of *Rps. sphaeroides* may be either with the Mg atom of the second BChl (lower scheme) or with an external site (upper scheme). The 1637-cm^{-1} frequency of the bound carbonyl does not discriminate between these two possibilities. Indeed, nearly identical frequencies have been observed for the stretching modes of acetyl carbonyls, both in (BChl *a*)$_n$ oligomers (Lutz, 1979, 1984) and in the soluble BChl *a*–protein complex from *Prosthecochloris aestuarii* (Lutz, 1981; Lutz et al., 1982), in which no such oligomers are present (Matthews et al., 1979).

On the other hand, X-ray crystallographic data on reaction centers from *Rps. viridis* clearly show that the magnesiums of both primary donor BChls interact with the protein (Deisenhofer et al., 1984), at the level of side chains of histidine residues 173 (L subunit) and 200 (M subunit) (Deisenhofer et al., 1985b). These two residues are also present at the same positions in the L and M chains of *Rps. sphaeroides* reaction centers (Williams et al., 1984). Moreover, histidine side chains are known to constitute privileged ligands for the BChl magnesiums in antenna complexes from green (Matthews et al., 1979) and purple bacteria (Robert & Lutz, 1985b). In addition, in vitro RR experiments have shown that imidazole derivatives indeed constitute strong ligands for the magnesiums of BChl *a* and readily prevent formation of (BChl *a*)$_n$ oligomers, i.e., the formation of any keto or acetyl to magnesium bonds (Robert, 1983).

Taken together, these data strongly suggest that the single ligand bound to the magnesium of the primary donor BChl that has its acetyl C=O free from binding (the lower molecule in the schemes of Figure 5) should be an histidine side chain rather than the acetyl group of the adjacent molecule. Hence, the upper scheme of Figure 5, which involves no direct BChl–BChl bond in the ground state, appears much more likely than the lower scheme.

On the basis of the same arguments, the binding site (Z in Figure 5) of the magnesium of the second BChl molecule is also most likely to be an histidine side chain.

The ligands interacting with the two keto carbonyls (X and X′ in Figure 5) and with one of the acetyl groups (Y in Figure 5A) are also likely to be proteic, although this is not necessarily so. There is a high probability that ligands X and X′ of the two keto groups are chemically distinct, on the basis of the 25-cm^{-1} frequency difference observed between their stretching frequencies. Indeed, when bound to a single type of site (e.g., in oligomers, the Mg atom of another chlorophyll), the frequency of the stretching mode of keto group appears to span a narrower frequency range (probably less than 12 cm^{-1}, whatever the chlorophyll and the structure of the oligomer; Lutz, 1979, 1984).

Comparison of Structures of the Primary Donors of Rps. sphaeroides and Rps. viridis. The Raman-based model for the primary donor of *Rps. sphaeroides* (Figure 5A) differs from the model proposed for that of *Rps. viridis* on the basis of crystallographic data (Deisenhofer et al., 1984, 1985a) essentially by the fact that the latter postulates two Mg to acetyl C=O bonds, in addition to the two observed Mg to protein bonds (Deisenhofer et al., 1984), thus involving six-coordinated magnesiums. Although the model for *Rps. viridis* may be reconsidered upon further refinement of the X-ray data (J. Deisenhofer, personal communication), this difference between the two models may reflect interspecific variability in the ground-state structure of the primary donor of purple bacteria, as suggested also by the above-mentioned Raman data on the primary donor of *Rsp. rubrum* (Zhou, 1985). However, the present resonance Raman data clearly show that, in the primary donor of *Rps. sphaeroides*, one of the magnesiums cannot be bound to the adjacent acetyl C=O, even though paramagnetic resonance data (Lubitz et al., 1985) indicated that these groups may well assume as short a distance in the latter species as in *Rps. viridis*. Several geometrical and energetic parameters may be involved in actually preventing any sizable C=O···Mg interaction in the sixth position, which may be relevant as well to the case of the primary donor of *Rps. viridis*. Current resonance Raman studies on reaction centers of *Rps. viridis* may help to solve this problem in the near future.

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Articles

Specific Overproduction and Purification of the Cytochrome b_{558} Component of the Cytochrome *d* Complex from *Escherichia coli*†

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ABSTRACT: In *Escherichia coli* strain GR84N[pNG10], the cloned gene for subunit I of the membrane-bound cytochrome *d* complex resulted in the overproduction of cytochrome b_{558} and facilitated purification of this cytochrome. Extracting membranes with 1% Triton X-100 followed by two chromatographic steps yielded a single band on sodium dodecyl sulfate–polyacrylamide gels corresponding to subunit I (M_r 57 000). Purified cytochrome b_{558} was in its native state as determined by difference absorption spectroscopy and by potentiometric analysis. Both the membranes of strain GR84N[pNG10] and the purified subunit I lacked the other two spectroscopically defined cytochromes, b_{595} (previously "a_1") and *d*, of the cytochrome *d* complex. Reconstitution of cytochrome b_{558} in phospholipid vesicles demonstrated that cytochrome b_{558} can be reduced by ubiquinol but that it does not reduce molecular oxygen. Heme extraction of cytochrome b_{558} yielded an extinction coefficient of 22 000 M^{-1} cm^{-1} for the wavelength pair of 560 and 580 nm in the reduced-minus-oxidized spectrum. The mutation on pNG10 that eliminates subunit II was mapped to a 250 base pair DNA fragment.

The cytochrome *d* complex is a two-subunit terminal oxidase found in the inner membrane of *Escherichia coli*. The purified complex contains three distinct cytochromes, cytochromes b_{558} and *d* and the cytochrome previously described as cytochrome a_1 (Miller & Gennis, 1983). Although cytochrome a_1 absorbs at 595 nm in the reduced-minus-oxidized spectrum, it does not contain an *a*-type heme (Miller & Gennis, 1983) but apparently contains protoheme IX (Lorence et al., 1986). Therefore, cytochrome a_1 will be referred to as cytochrome b_{595} in this report.

Cytochrome b_{595} has a second absorbance peak at 560 nm that overlaps with the absorbance of cytochrome b_{558} (Koland et al., 1984), an overlap that has made the quantitation of cytochrome b_{558} difficult. Subunit I has been previously shown (Green et al., 1984b) to be the cytochrome b_{558} component

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