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Intergeneric structural variability of the primary donor of photosynthetic bacteria: resonance Raman spectroscopy of reaction centers from two *Rhodospirillum* and *Rhodobacter* species

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Several structural properties of the primary donor of *Rhodobacter sphaeroides*, wild type, have been obtained recently from difference resonance Raman spectra (Robert, B. and Lutz, M. (1986) Biochemistry 5, 2303–2309). In order to test the interspecific variability of the primary donor structure, we extended those observations to reaction centers of *Rhodospirillum rubrum*, wild type, using the same difference methods. Difference resonance Raman spectra of the primary donor are extremely close to those obtained for *Rhb. sphaeroides*. Identical conclusions can be drawn for the two species about the molecular interaction states of the magnesium atoms and of the acetyl carbonyl groups of both bacteriochlorophyll *a* molecules constituting their primary donors. However, the keto carbonyl of one of the bacteriochlorophylls, which is intermolecularly bound in *Rhb. sphaeroides*, is free from bonding in *Rsp. rubrum*. It thus appears that there is a limited interspecific variability of the structure of the primary donor in Rhodospirillales. These results also demonstrate an interspecifically variable, environmental asymmetry around the two primary donor molecules, which should variably affect charge distributions over these two molecules, in transient intermediates states of P, according to the species considered.

Introduction

The first steps of the primary events of photosynthesis take place in specialized pigment-protein complexes called reaction centers (RC). All reaction centers that have been isolated up to now from Rhodospirillales contain four molecules of bacteriochlorophyll (BChl), two molecules of bacteriophaeophytin (Bph) and two quinone molecules, one of which is generally lost during extraction. Absorption of a photon by isolated RCs induces a one-electron oxidation of a specific structure called the primary donor (P), followed by a sequence of electron transfers and by the reduction of a quinone molecule. This sequence of light-induced reactions appears, as far as it has been studied, very conservative for all Rhodospirillales studied so far. Much knowledge has been recently gained about the structures in which these reactions occur. In particular, the reaction centers extracted from the BChl *b*-containing bacteria *Rhodopseudomonas viridis* have been crystallized and much information about their general features could be extracted from a 3 Å

Abbreviations: BChl, bacteriochlorophyll *a*; Bph, bacteriopheophytin *a*; DEAE, diethylaminoethyl; LDAO, lauryldimethyl ammonium oxide; RC, reaction centers; *Rhb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; *Rsp*, *Rhodospirillum*.

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resolution electron density map obtained from X-ray studies [1]. It was shown that the six bacteriochlorin pigments contained in RCs are symmetrically arranged by pair along a C_2 axis. This study also confirmed the fact that the primary donor P is constituted by two strongly electronically interacting BChls, as had been proposed before [2].

However, until very recently, our knowledge has been very sparse concerning many details of these structures, such as the bonding interactions assumed by the different pigments present in the RCs. Yet, most of these interactions must play determinant roles in ensuring the very high efficiency with which the separation and stabilization of charges occurs, not only because they determine the relative distances and orientations of the pigments, but also because they most probably largely influence their electronic structures and redox properties. In particular, the precise structure of P, as well as the ground state interactions involving its two constitutive BChl molecules, are of particular current interest, because of their obvious relevance to the understanding of the mechanisms of the primary charge separation.

Many of these structural parameters can be determined by resonance Raman spectrometry. Indeed, it has been shown that information can be obtained from this method about the conformations and intermolecular binding states of the dihydrophorbin rings of BChl and of Bph and their conjugated substituents, as well as about the local permittivities around them [3–6]. The highest information content of the resonance Raman spectra of bacteriochlorin pigments is obtained when resonance is with the Soret electronic transition and includes data on the intermolecular binding states of their conjugated 2-acetyl and 9-ketone carbonyls. The main problem in studying RCs under these conditions of excitation is that limited selectivity is obtained amid the Raman contributions of the six pigments of the RC. However, we recently developed a new difference method permitting us to obtain selectively the resonance Raman spectra of the primary donor in RCs extracted from *Rhb. sphaeroides* [7,8]. This method involves controls of the dynamic equilibrium states assumed by the samples during resonance Raman experiments through control of the illumination, redox potential and temperature parameters. From such resonance Raman spectra, several conclusions could be drawn about the structure of the primary donor. In particular, we concluded that, in *Rhb. sphaeroides* RCs, the Mg atoms of the two BChl were each singly liganded to a molecular site which was not the partner BChl in P [7–9]. In addition, from these data we could propose tentative assignments for the whole set of carbonyl bands arising from the six bacteriochlorin pigments [8]. It appears of interest to compare local structures around the primary donor and the other pigments in reaction centers of different bacterial species or genera of Rhodospirillales. Such comparisons should permit to determine which among these molecular interactions are essential in ensuring the efficiency of the primary photochemistry of the RC. A recent comparison indeed suggested possible differences between the structures of the primary donors of *Rhb. sphaeroides,* as observed by resonance Raman spectroscopy, and of *Rps. viridis*, as observed by X-ray crystallography [1,8–10].

In this paper, we present results concerning reaction centers from *Rhodospirillum (Rsp.) rubrum,* and we compare them to our previous data on *Rhb. sphaeroides.* Preliminary accounts of this work have been presented in Refs. 11 and 12.

Material and Methods

Cells of *Rsp. rubrum,* strain S1 (wild type) were grown anaerobically in a Hutner modified medium. Chromatophores were prepared as in Ref. 6. LDAO was used to prepare reaction centers. A key step in preparing RC from these chromatophores resides in the mild solubilization of the intracytoplasmic membrane [13,32], which permits, after ultracentrifugation at $180\,000 \times g$ for 1 h, to obtain a crude extract enriched in RC (RC : B880 = 1). The best results were obtained by treating freshly prepared chromatophores ($A_{880} = 50$) with 0.25% LDAO and incubating them for 25 min at room temperature. If the chromatophores have been congelated, the optimal incubation time with LDAO is longer (50 min). However, the maximum enrichment obtained in this case is always lower than with fresh chromatophores. Crude RC fractions were then purified by HPLC chro-

matography, using a DEAE-cellulose column with a 0 to 1 M NaCl gradient (eluting buffer, Tris-HCl (pH 8.0)/25 mM, Triton 0.05%). A typical absorption spectrum of RCs obtained by this method is presented in Fig. 1. On this basis, these preparations can be favorably compared, in terms of their pigments content, with previously published ones [13,32]. A typical A_{280} : A_{800} absorbance ratio is 2.5. These preparations could be frozen and kept at −80°C for periods longer than 1 month without any apparent modification of their absorption spectra.

Resonance Raman spectra were recorded at about 30 K, using a 363.8 nm excitation wavelength, which insures resonance at the top of the Soret bands of the bacteriochlorin pigments. The experimental set-up and recording procedure have been described in Refs. 4 and 9. In order to obtain resonance Raman spectra of the primary donor, two different types of difference experiment were conducted. Both methods made use of the actinic effect of the probe laser beam on the reaction centers, resulting in the primary charge separation. Depending on this steady-state illumination level, variable amounts of excited radical states of the primary donor may build up in the samples. In a first type of experiment, resonance Raman spectra were recorded from untreated RCs at low and high irradiances, respectively. We showed [9] that, in Raman experiments conducted in the higher irradiance conditions (corresponding to about 2 mW of 364 nm light penetrating the sample) near to 100% of the RCs probed might be expected to be oxidized. The second type of experiment involved chemically reduced RCs, in which high amounts of a triplet state of P-870 (named P^R) may build up upon steady-state illumination, provided that transfer from this state to the carotenoid is prevented. Poising the centers at a temperature of about 20 K ensured this latter condition [9]. Both the radical cation and triplet states of P do not participate much to the resonance Raman spectra of RCs excited at 3.63.8 nm. Hence, differences between spectra obtained at low and high illumination levels from either untreated or chemically reduced RCs should primarily arise from the primary donor in its neutral ground state. In this work, we will present results only concerning the higher frequency regions of the RR spectra (1450–1750 cm^{-1}). Indeed, in this frequency range are present bands from which much information can be extracted. A methine bridge stretching mode of the BChls occurs around 1615 cm^{-1}. The frequency of this band is sensitive to the coordination number of the central Mg of the molecule, being located near 1600 cm^{-1} when 6-coordinated and around 1615 cm^{-1} when 5-coordinated [6,14]. The stretching modes of the two conjugated 2-acetyl and 9-keto carbonyl groups of bacteriochlorin pigments also occur in this range. These groups have been shown to play key roles in the intermolecular interactions assumed by the bacteriochlorin pigments, both in vivo and in vitro [15–17].

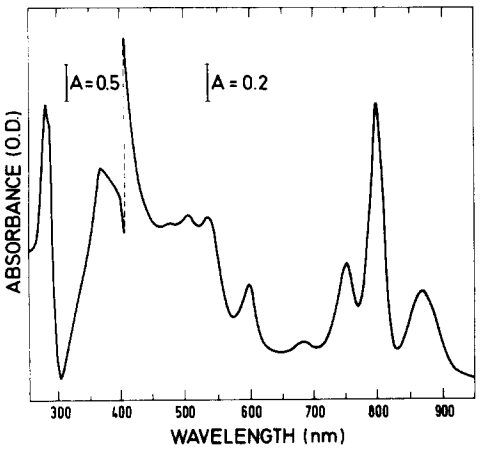

Fig. 1. Room-temperature absorption spectrum of reaction centers isolated from *Rsp. rubrum* strain S1.

Results and Discussion

Fig. 2 compares the 1450–1750 cm^{-1} regions of resonance Raman spectra obtained from untreated RCs of *Rsp. rubrum* and of *Rhb. sphaeroides* with low (Fig. 2a) and high (Fig. 2b) radiant powers of the Raman analysis laser beam, respectively. In this region, the 1590 cm^{-1} band arises predominantly from Bph and the 1614 cm^{-1} one from both BChl and Bph. The weak bands between 1620 and 1720 cm^{-1} arise from the stretching modes of the conjugated 2-acetyl and 9-keto carbonyls of the bacteriochlorin pigments. Differences occur between spectra of *Rsp. rubrum*

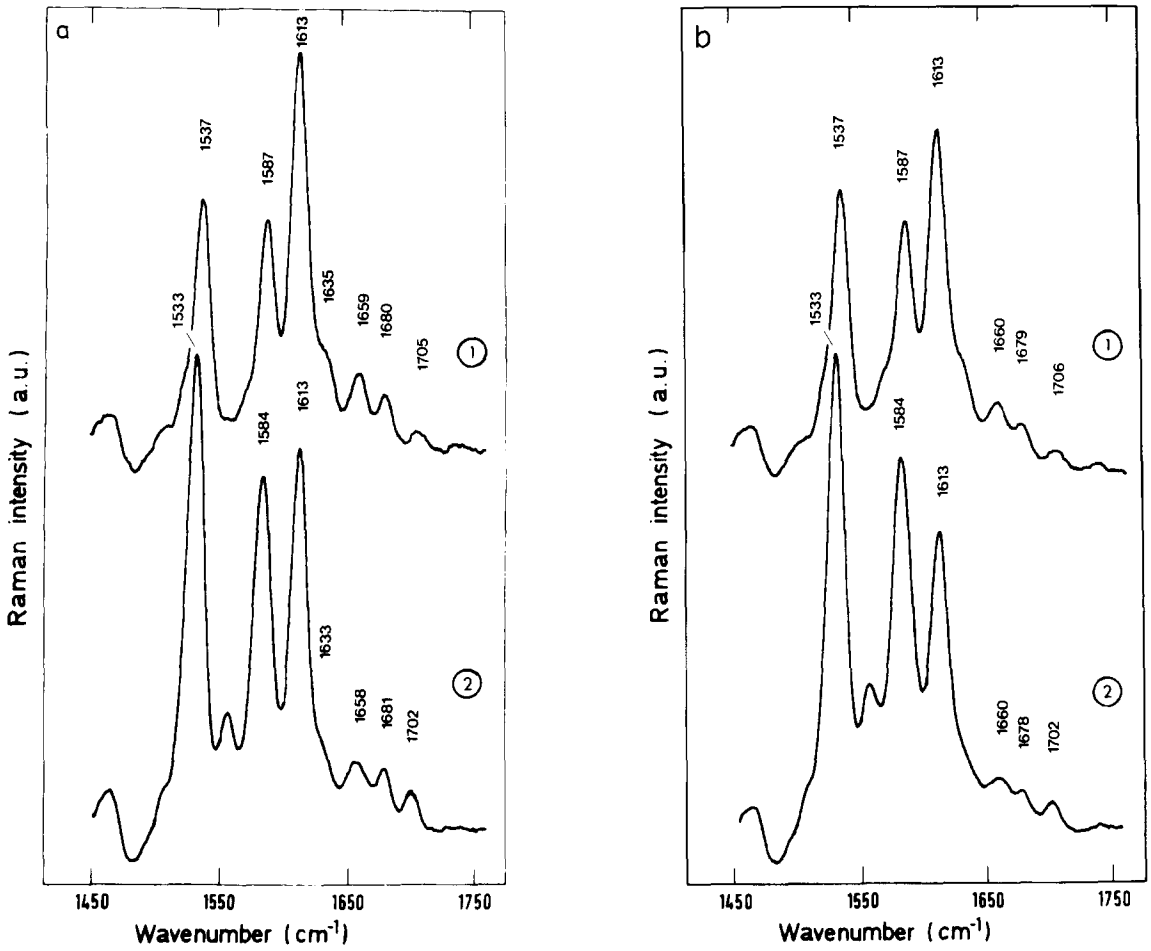

Fig. 2. (a) Resonance Raman spectra (1450–1750 cm^{-1} region) of untreated reaction centers, under low irradiance conditions. (1) From *Rbh. sphaeroides* 2.4.1; (2) from *Rsp. rubrum* S1. $T = 20$ K; excitation wavelength, 363.8 nm. (b) Resonance Raman spectra (1450–1750 cm^{-1} region) of untreated reaction centers, under high irradiance conditions. (1) From *Rbh. sphaeroides* 2.4.1; (2) from *Rsp. rubrum* S1. $T = 20$ K ;excitation wavelength, 363.8 nm.

and Rhb. sphaeroides, both at low and high irradiance. These differences primarily concern the relative intensities with which the vibrational modes of the individual pigments participate in the spectra of the whole RC. In *Rsp. rubrum* spectra, the 1533 cm^{-1} band arising from the ν_1 mode of spirilloxanthin is much stronger than the 1537 cm^{-1} band arising from spheroidene in *Rhb. sphaeroides* RCs: this is most likely due to the differences occurring between the electronic absorption spectra of the two molecules; on the other hand, the 1590 cm^{-1} band, arising from the two Bph molecules of the center, is also much stronger in the spectra of *Rsp. rubrum* RCs than in those from *Rhb. sphaeroides,* taking the 1614 cm^{-1} band as a reference. This relative enhancement concerns all of the observable BPh bands in the 50–1750 cm^{-1} region (data not shown). This effect is most likely due to differences in the relative positions and oscillator strengths of the Soret absorption bands of individual pigments for the two types of reaction center. Indeed, in the Soret region, bands arising from BChl and Bph are almost perfectly superimposed, and although a very small shift, e.g., 1 nm, of certain of these bands relative to the others and to the Raman excitation wavelength can be very difficult to detect, such a shift may induce sizable changes in the

relative contributions of the different pigments to the spectrum of the whole RC (Robert, B., unpublished results).

Small differences only are observed between the 1620–1720 cm^{-1} spectral regions of the RCs from *Rsp. rubrum* and *Rhb. sphaeroides* (Fig. 2). Most of them are relative intensity differences, which may be accounted for by the above-mentioned differences in relative contributions from the Bph and BChl pigments. Such are the stronger relative intensities of the 1700 cm^{-1} and 1660 cm^{-1} bands, relatively to the 1680 cm^{-1} band intensity, in spectra of *Rsp. rubrum* than in those of *Rhb. sphaeroides.*

The difference Raman spectrum of Fig. 4(1) was obtained by subtracting resonance Raman spectra of untreated RCs of *Rsp. rubrum* recorded under low and high irradiance values, respectively (cf. Fig. 2a and 2b). The difference spectrum of Fig. 4(2) was obtained in the same conditions from experiments on dithionite-treated RCs from *Rsp. rubrum* (Fig. 3). In both calculations, the relative weighings of the terms of the differences were choosen for optimal cancellation of the 1533 cm^{-1} band of spirilloxanthin. Except for the features at 1570–1590 cm^{-1}, both spectra of Fig. 4(1) and 4(2) probably contain positive contributions only. The presence of a strong band at 1613 cm^{-1}, and of a weaker one at 1590 cm^{-1} indicate that these spectra arise from BChl. The presence of four bands in the 1620–1720 cm^{-1} region, which can be assigned to carbonyl stretching modes, indicate that two unequivalent BChl molecules are contributing. Fig. 4 also demonstrates a close similarity between difference spectra in Fig. 4(1) and 4(2) obtained from *Rsp. rubrum* RCs using distinct experimental conditions. Taken together, these observations lead us to conclude, as we previously did for *Rhb. sphaeroides* 2.4.1 [9], that the spectra in Refs. 4(1) and 4(2) essentially arise from contributions of the primary donor P in its neutral ground state. As far as the low irradiance conditions corresponded to less than one photon absorbed per RC per second, it appears most likely, in addition, that these contributions are from the primary donors in conformationally relaxed RCs. The features observed in the 1450–1580 cm^{-1} region of difference spectra of Figs. 4(1) and 4(2) should not be considered as

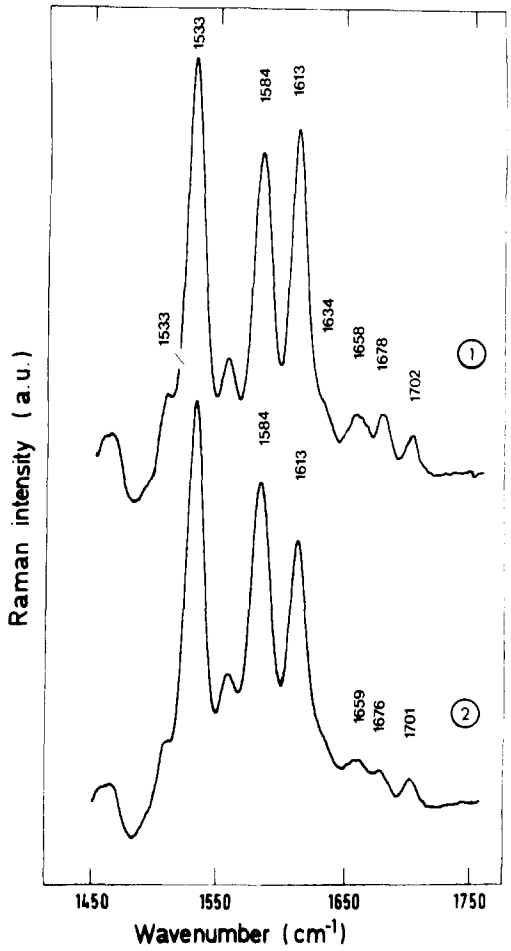

Fig. 3. Resonance Raman spectra (1450–1750 cm^{-1} region) of dithionite-treated reaction centers from *Rsp. rubrum*, strain S1. (1). Low irradiance conditions; (2) high irradiance conditions. $T = 20$ K; excitation wavelength, 363.8 nm.

perfectly reliable, due to the presence of the strong carotenoid and phaeophytin bands in the parent spectra. However, persistent positive bands near to 1505 and 1535 cm^{-1} might arise from primary donor BChl [18]. Possible negative bands near to 1575 cm^{-1} conceivably might originate from contributions of radical cation (Fig. 4(1)) and of triplet state (Fig. 4(2)) of primary donor BChl. Removal of a bonding pi-electron either by ionization or by promotion into a non-bonding orbital should indeed result in downshifts of many skeletal modes [19,20].

Resonance Raman spectra of the primary donors of *Rsp. rubrum* and of *Rhb. sphaeroides*

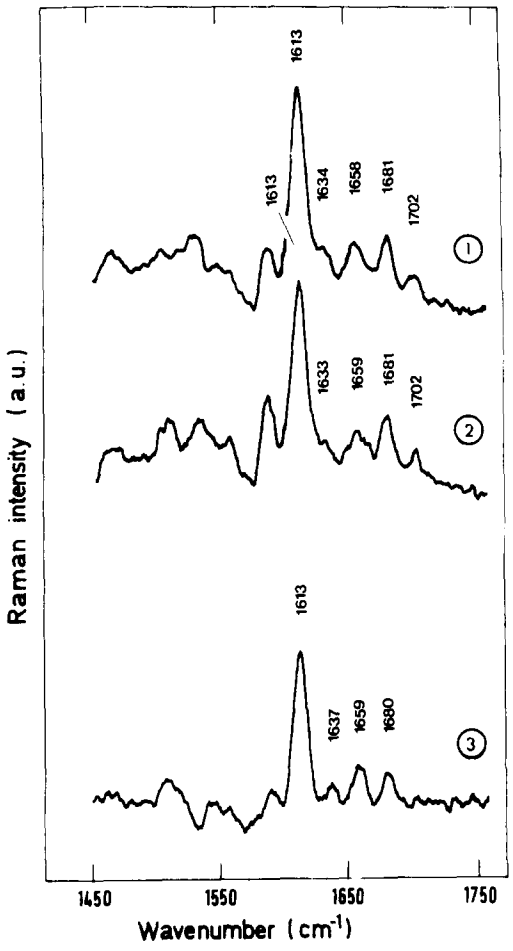

Fig. 4. Difference resonance Raman spectra (1450–1750 cm^{-1} region). (1) Difference between spectra of untreated RCs from *Rsp. rubrum* S1 recorded under low and high irradiance conditions, respectively. Normalization: see text. (2) Difference between spectra of dithionite-treated RCs from *Rsp. rubrum* S1 recorded under low and high irradiance conditions, respectively. Normalization: see text. (3) Difference between spectra of untreated RCs from *Rhb. sphaeroides* 2.4.1 recorded under low and high irradiance conditions, respectively (see Ref. 11).

exhibit close similarities and clear differences (compare Fig. 4(1), (2) and (3): indeed, the positions of the three lower frequency carbonyl bands are the same in *Rhb. sphaeroides* and in *Rsp. rubrum* spectra, but an additional band is present at 1700 cm^{-1} in spectra of *Rsp. rubrum* reaction centers. Moreover, an intensity difference relative to that of the 1680 cm^{-1} band is observed between the 1660 cm^{-1} bands of the resonance Raman spectra of the two species. An unequivocal assignment of the four C=O stretching bands present in resonance Raman spectra of *Rsp. rubrum* is possible, based on the frequency ranges observed, both in vitro and in vivo, for the acetyl and for the keto carbonyl stretching modes, i.e., about 1620–1665 and 1640–1710 cm^{-1}, respectively [5,18]. In as far as the 2-acetyl stretching frequency has never been observed to occur above 1665 cm^{-1}, the 1680 and 1700 cm^{-1} bands necessarily arise from ketonic C=O groups, which should be weakly bonded and free from intermolecular bonding, respectively. Hence, the 1637 cm^{-1} and 1660 cm^{-1} bands must arise from acetyl carbonyl groups of the primary donor respectively bound and free from intermolecular bonding, respectively.

As was the case previously for *Rhb. sphaeroides* [9], from these results we are able to propose the molecular model of Fig. 5A. This model makes use of two items of information yielded by X-ray crystallographic data on reaction centers from *Rps. viridis* [1]. The first one is the overlap on the BChls at the level of their pyrrole rings I. It appears legitimate to transpose this arrangement from *Rps. viridis* to *Rsp. rubrum* on the basis of recent ENDOR studies [21] which indicated very close structures for the primary donor of both species. The second item of information transposed from the X-ray data analysis is the fact that the Mg atoms of both BChls of the primary donor were observed to be bound to the protein [1] and more precisely to histidine side-chains [22]. Because no complete sequences of the L and M polypeptides are yet available for *Rsp. rubrum*, it is still not known whether histidine residues 173 (L subunit) and 200 (M subunit) which are present both in RC apoprotein of *Rps. viridis* and of *Rhb. sphaeroides* are conserved in that of *Rsp. rubrum*. However, the primary donors of these three species share such close structural and functional properties that it appears very unlikely that such a key structural parameter as the Mg liganding might qualitatively differ in the *Rsp. rubrum* primary donor and might involve non proteic sites. Hence, as far as the present resonance Raman data demonstrate that the Mg of both primary donor molecules each bind a single ligand, it appears reasonable to assume that these ligands are proteic in nature, and are not the partner primary donor

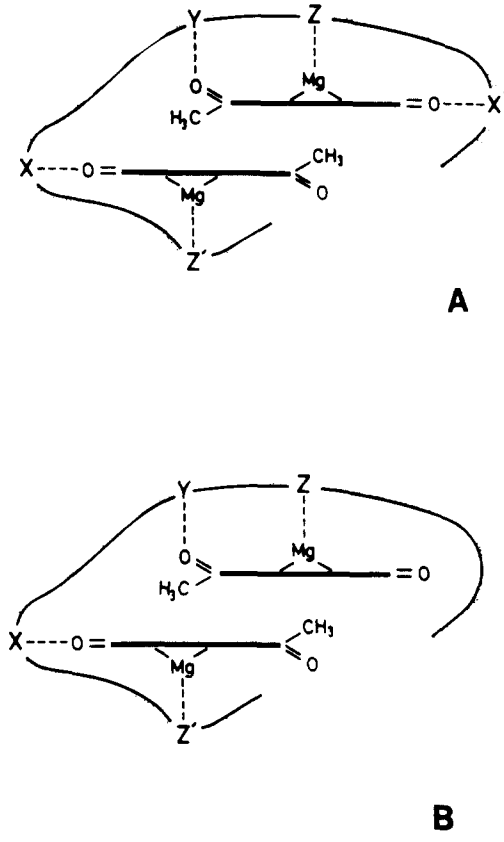

Fig. 5. Molecular models for the ground-state binding interactions assumed by the magnesiums and by the keto and acetyl carbonyls of the primary donor bacteriochlorophylls of *Rhb. sphaeroides* wild type (A) and of *Rsp. rubrum*, wild type (B).

BChl, or a foreign molecule such as water. As noted previously [9], the present resonance Raman spectra of the primary donor of *Rsp. rubrum* also bring full confirmation of the interpretation that we have proposed earlier for those of the primary donor of *Rhb. sphaeroides*. Indeed, the nearly identical frequencies observed for the approx. 1635 and 1660 cm^{-1} bands in spectra of both species (Fig. 4) strongly support their common assignment to stretching of the acetyl groups, for *Rhb. sphaeroides* as well as for *Rsp. rubrum*. Even the relative weakening of the 1660 cm^{-1} band of *Rsp. rubrum*, compared to that present in *Rhb. sphaeroides* spectra (Fig. 4) strengthens the earlier proposal that the keto carbonyl which vibrates at 1700 cm^{-1} in the primary donor of *Rsp. rubrum*, actually vibrates near 1660 cm^{-1} in that of *Rhb. sphaeroides* [9].

Differences in the resonance Raman spectra of the primary donors of *Rhb. sphaeroides* and *Rsp. rubrum* may thus be interpreted by a single structural difference, which concerns the intermolecular binding of the keto carbonyl of one of the two BChl constituting P (cf. Fig. 4). This group may belong either to molecule P_A, located on the side of the high-probability pathway for electrons, or to molecule P_B. In any case, its interaction state does not appear to be essential in determining either the molecular and electronic structures of P, or its main functional properties, in view of the close similarities observed between RCs of *Rsp. rubrum* and of *Rhb. sphaeroides* [23]. However, this interaction state may well influence certain of these properties significantly. For example, the P^+ states of *Rhb. sphaeroides* R 26 and of *Rsp. rubrum* G 9 yielded distinct triple electron resonance spectra, a difference that Lubitz et al. ascribed to unspecified environment-induced differences in dimer geometries [21]. These environmental differences may largely consist of the difference in interaction states of the keto groups of the P_A (or P_B) molecules which is observed in the present experiment.

The present results demonstrate that, as for the primary donor of *Rhb. sphaeroides*, environmental interactions assumed by each of the two primary donor molecules of *Rsp. rubrum* are not identical, hence breaking the overall C_2 symmetry which most probably [21] relates P_A and P_B, as in the case of the primary donor of *Rps. viridis* [1]. More precisely, the lack of any intermolecular bonding of the keto group of one of the primary donor molecule of *Rsp. rubrum* results in a still higher environmental asymmetry around P for the latter species than for *Rhb. sphaeroides*. At this point, it should be noted that in drawing the model of Fig. 5B for the primary donor of *Rsp. rubrum*, the free keto carbonyl group has been arbitrarily ascribed to the molecule which has a bonded acetyl group and not to that in which this latter group is free. The alternate possibility, in which one of the bacteriochlorophylls has both its conjugated carbonyls free, and the other one has both of them intermolecularly bonded, would correspond to a still higher asymmetry of the primary donor.

Because this environmental asymmetry concerns substituents which are largely conjugated with the pi-electron systems of P_A and P_B [5], it is likely to influence the structure of the intermediate functional states of P. Firstly, it may result in a sizeable asymmetry in the time-averaged, unpaired charge densities which occur on P in the P^+ or P^R states, irrespective of a possible hopping of unpaired charges between P_A and P_B on optical timescales, in both the P^+ [19] and P^R states [24,25]. Such an average asymmetry has indeed been deduced from EPR measurements of the P^R state of single crystals of *Rps. viridis* reaction centers [26], and has been proposed to occur in the P^+ state of the same species, in order to explain values of the EPR linewidths which were intermediate between those expected for a monomer BChl and for a symmetric $(BChl)_2$ dimer [26,27].

Secondly, such an environmental asymmetry, as mediated by the conjugated carbonyls, might break the C_2 symmetry of P in the lowest singlet excited state, hence permitting charge separation within the primary donor. Possible occurrence of such an early P^{+-} charge transfer state of P indeed has been recently postulated from hole-burning [28–30] and subpicosecond absorption experiments [31].

Moreover, resonance Raman data on the primary donor of *Rsp. rubrum* and of *Rhb. sphaeroides* indicate that, if actually significantly dependent on the interaction states of the conjugated carbonyls, which appears most likely, the amounts of asymmetry of the P^+, P^R, and perhaps P^{+-} states should depend on the bacterial species considered. Testing of this prediction must, however, await a larger body of data concerning charge repartition over P_A and P_B for different bacterial species, as well as information on other possibly effective sources of environmental asymmetry for P, i.e., on asymmetrically distributed local charges and/or pi-conjugated systems close to the dihydrophorbin rings of P_A and P_B.

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