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Protein–prosthetic group interactions in bacterial reaction centers: resonance Raman spectroscopy of the reaction center of *Rhodopseudomonas viridis*

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Resonance Raman spectra have been obtained, at low temperature, for the carotenoid, bacteriopheophytin *b* and primary donor bacteriochlorophyll *b* molecules within reaction centers of *Rhodopseudomonas viridis*. The carotenoid molecule was shown to assume the same stereoisomerism as other reaction center-bound carotenoids, consisting of a 15-15′ *cis* configuration twisted into a non-planar conformation. RR spectra of each of the two bacteriopheophytins were obtained independently. Consistently with X-ray data (Michel et al. (1986) EMBO J. 5, 2445–2451), the keto carbonyl group of the acceptor molecule appeared to be H-bonded, while that of the second molecule was free. On the other hand, the acetyl groups of both molecules were shown to be both engaged in intermolecular bonding. Difference resonance Raman spectra of the primary donor molecules in the ground state permitted a precise description of the interaction states of their magnesium atoms and conjugated carbonyl groups. Comparisons of protein sequences and of resonance Raman data between the two species *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* further permitted unambiguous determination of the vibrational frequencies of each of the four conjugated carbonyl groups of the primary donor.

Introduction

The primary photochemical events of bacterial photosynthesis occur within an integral membrane protein named the reaction center (RC). Reaction centers isolated from several species of purple bacteria (*Rhodospirillales*) generally contain ten prosthetic groups, bound to a three-polypeptide apoprotein. These prosthetic groups consist of four bacteriochlorophyll (BChl) *a* or *b* molecules, two bacteriopheophytins (BPheo) *a* or *b*, two quinones (one of which is often lost during isolation), one transition metal ion (generally Fe^{2+}), and one carotenoid molecule. An RC-bound cytochrome may also be found in some species [1]. Two of the BChl molecules are generally believed to constitute the primary electron donor, P. One of the two BPheo molecules constitutes an intermediate electron acceptor, as do the quinone molecule(s) [2]. Several approaches have yielded much structural knowledge about reaction centers. Recently, X-ray crystallographic studies have given decisive information about the structures of RCs from *Rhodopseudomonas (Rps.) viridis* [3–5] and from *Rhodobacter (Rhb.) sphaeroides* [6,7]. The approx. 3 Å resolution, three-dimensional models built from these data, in addition to the information they themselves provide, should permit incorporation of many other structural data, mainly of spectroscopic origin. Such are structural data yielded by vibrational spectroscopy, and particularly resonance Raman spectroscopy, which provides detailed information on prosthetic group conformations and interactions with the protein, with spatial resolutions which can be much higher than those of X-ray models [8,9]. Conversely, most spectroscopic methods, not being tied to a crystalline state of the sample, are applicable to reaction centers of any bacterial species, and hence also are essential in transposing structural knowledge gained from crystallographic studies to RC samples which have not been crystallized. Such samples may consist of RCs from other bacterial origin than those which have been

Abbreviations: RC, reaction center; BPheo, bacteriopheophytin; *Rps., Rhodopseudomonas; Rhb., Rhodobacter; Rsp., Rhodospirillum;* LDAO, lauryldimethylammonium oxide.

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crystallized, but also of crystallizable RCs which have been structurally modified and/or poised in functional states which have not yet been obtained as crystals. Recent examples of such transpositions are a resonance Raman study of the primary donor in RCs from *Rhodospirillum (Rsp.) rubrum* [10], and a resonance Raman study of the effect of P^+ formation on the local environments of the accessory BChls, in the R26 carotenoidless mutant of *Rhb. sphaeroides* [11].

Several resonance Raman studies have been conducted on RCs from *Rhb. sphaeroides,* concerning the carotenoid conformation in both the ground singlet and lowest excited triplet states [12–14], the compared interaction states of the BPheo molecules [12,15], the structure of the primary donor [16] and, recently, the interactions assumed by the accessory BChls [11]. No such set of resonance Raman data is yet available on RCs from *Rps. viridis*. This is due essentially to their bacteriochlorin groups consisting of the *b* rather than of the *a* derivatives. Indeed, BChl *b* is very photooxidizable, particularly in solution, and the in vitro resonance Raman studies needed to interpret in situ spectra have long suffered from this drawback. Only recently has the improvement of isolation and purification methods of the *b*-type bacteriochlorins [17] permitted such studies [18]. In the following, we report a resonance Raman study of the states of the primary donor, bacteriopheophytins and carotenoid in reaction centers from *Rps. viridis*. We discuss the results with respect to those recently yielded by X-ray crystallography, as well as with respect to those currently available about RCs from other bacterial species. Preliminary accounts of certain of these results have been given in Refs. 18–20.

Material and Methods

Preparation of reaction centers

Reaction centers were isolated by LDAO treatment (5%) from chromatophores (absorbance at 1015 nm: 50 *A*) of *Rps. viridis* following the procedure of Clayton and Clayton [21], modified as follows. The ammonium sulfate precipitation was performed prior to chromatography on hydroxyapatite; LDAO was exchanged for cholate by overnight dialysis at 4°C against a pH 8 buffer containing 0.1% cholate and 10 mM Tris-HCl. For the Raman experiments, the reaction centers were brought to a final concentration ensuring a 20 *A* absorbance at 830 nm. All these steps were conducted in subdued light and at 4°C. An electronic absorption spectrum of such a preparation at room temperature is illustrated in Fig. 1.

Resonance Raman spectroscopy

Resonance Raman spectra were recorded from samples kept at about 30 K by a flow of cold gaseous helium directly leaching the illuminated site. Several

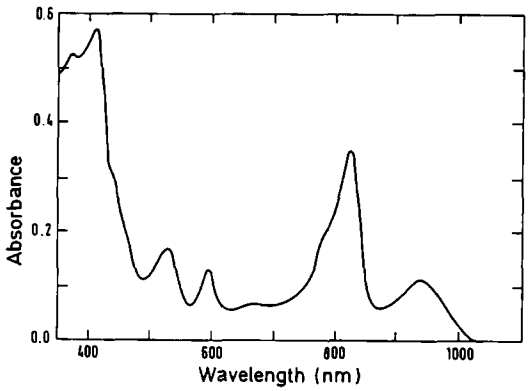

Fig. 1. Room-temperature electronic absorption spectrum of reaction centers (untreated) isolated from *Rps. viridis*.

excitation wavelengths were used in order to enhance contributions from the different prosthetic groups selectively. The 363.8 and 496.5 nm excitations were obtained by an argon ion laser (Spectra Physics model 171.03). Excitations in the range 530–550 nm were generated from the laser dye Coumarin 6. Resonance Raman spectra excited at 364 nm were detected using a Jobin-Yvon HG2S – UV spectrometer, while those excited at longer wavelengths were detected using a CODERG PHO spectrometer. Both were equipped with monochannel detection systems. Signal-to-noise ratios were improved by summation of individual spectra using a Tracor Northern multichannel analyzer, which also processed these spectra.

Difference resonance Raman methods

The highest information content of Raman spectra of bacteriochlorophylls excited in the near-ultraviolet-visible range is available at Soret resonance. All six bacteriochlorins present in bacterial reaction centers have almost coincident Soret bands, and hence are more-or-less all contributing in resonance Raman spectra excited in this region. In order to obtain specific vibrational information on the primary donor only, we recently developed difference methods permitting us to obtain selectively resonance Raman spectra of the primary donor [16]. These methods take advantage of the actinic effect of the laser beam used in the resonance Raman experiment, which can populate the reaction center preparation, in dynamic equilibrium conditions, with variable amounts of either a radical cation state or a triplet state of the primary donor depending on experimental conditions. From our past experience on other RCs [16,10], both of these states are poor Raman scatterers when excitation is in the Soret bands of the neutral, ground-state pigments. This phenomenon is probably due, in part, to the smaller absorption coefficients of both of these species in this range [22–124]. In addition, there are indications that many porphyrin radicals are weaker scatterers than the neu-

tral species under equivalent excitation conditions (see, for example, Refs. 25–27). Hence, difference resonance Raman spectra obtained in this way should essentially arise from the primary donor in its neutral, ground state.

In reaction centers prepared from *Rps. viridis*, the cytochromes can rapidly rereduce the oxidized primary donor, preventing any sizeable build-up of state P^+. This effect was overcome by oxidizing the cytochromes, prior to illumination, by 200 μM $K_3Fe(CN)_6$. In order to populate the P^R state in chemically reduced centers, we avoided formation and build-up of the I^- state by working at 30 K and by changing the illuminated site every 5 min.

Normalization of the resonance Raman spectra obtained under low and high irradiance of the sample proved somewhat difficult for the *Rps. viridis* species. This difficulty arises from the extreme weakness of the C = C stretching band of the carotenoid under 363.8 nm excitation, which generally constituted an excellent internal intensity standard for other bacterial species [10,16]. We thus used the approx. 1585 cm^{-1} bacteriochlorin band for this purpose. This band predominantly arises from ground state, neutral BPheo *b*, the amount of which is not expected to vary in these experiments. However, it also contains a sizeable contribution from BChl *b* [18], and the difference spectra presented in Fig. 5 are the results of normalizations based on the 1585 cm^{-1} band intensities, with an ad hoc, approx. 5% additional correction. This correction ensures a residual, positive contribution at 1585 cm^{-1} in the difference spectrum, corresponding to the relative intensities of the 1585 and 1610 cm^{-1} bands expected for BChl *b*. We checked that modifying the weighting of the terms of this difference of spectra by $\pm 10\%$ from that given by normalization on the 1585 cm^{-1} bands did not qualitatively alter the appearance of the 1620–1710 cm^{-1} region of the difference spectrum.

Resonance Raman spectra and interaction states of bacteriochlorophyll and bacteriopheophytin b

In vitro studies have shown that the higher-frequency regions (1550–1750 cm^{-1}) of Raman spectra of BChl *b* and BPheo *b* resonant in the Soret transitions contain much of the same modes as the *a*-type derivatives and hence yield the same information [18]. Like those of *a*-type derivatives, these spectra are dominated by an approx. 1610 cm^{-1} band, which most likely arises from a methine bridge stretching mode [28–31]. In BChl *b*, as in BChl *a* [29,30], the frequency of this mode is sensitive to the coordination number of the magnesium atom, being approx. 1600 cm^{-1} when this atom is six-coordinated, and approx. 1615 cm^{-1} when it is five-coordinated [18]. The stretching modes of the keto and acetyl carbonyl groups of BChl *b* and BPheo *b* occur near 1700 and 1668 cm^{-1}, respectively, when these groups are free from any intermolecular interactions [18]. These frequencies are similar to those found for the *a*-type derivatives [8,32], indicating that they are not sensitive to the presence of the ethylidene group. Hence, they should assume the same downshifts as those of the *a*-type derivatives when engaged in a given molecular interaction [8,32]. They are also expected to be found in the same frequency range, i.e., 1660–1710 cm^{-1} (keto) and 1620–1665 cm^{-1} (acetyl).

Results and Discussion

Excitation in the 545–535 nm range: the bacteriopheophytins at Q_x resonance

The Raman scattering of either of the two BPheo molecules present in the RCs from *Rps. viridis* can be selectively enhanced by excitation in their respective Q_x transitions. Fig. 2 shows the higher-frequency regions of resonance Raman spectra of untreated RCs from *Rps. viridis* at 30 K, excited at 534.1 and at 545.8 nm. These two spectra both have strong bands at 1611 cm^{-1} and shoulders at 1593–1595 cm^{-1}, but they qualitatively differ at higher frequencies. The 534-nm-excited spectrum indeed contains a strong shoulder at 1627 cm^{-1}, while the 546-nm-excited one contains a band at 1635 cm^{-1}. Also, the latter spectrum exhibits a very weak 1678 cm^{-1} band, while the former has a complex feature around 1700 cm^{-1}. It is thus very likely that each of these two spectra arises with good mutual selectivity from each of the BPheo molecules of the center. According to differential absorption measurements of the I^- state [33], the 546-nm-excited spectrum corresponds to the acceptor $BPheo_L$ and the 535-nm-excited

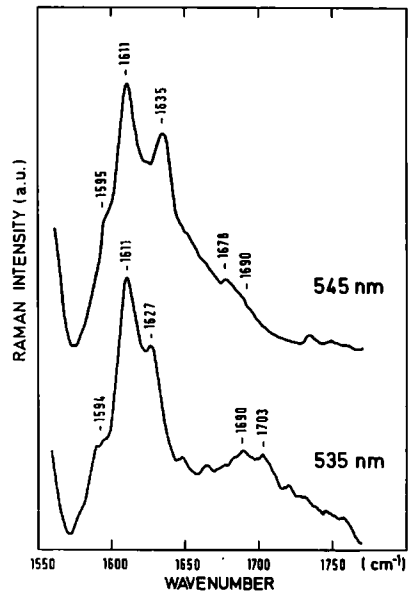

Fig. 2. Resonance Raman spectra (1550–1750 cm^{-1} regions) of BPheo at 30 K in untreated reaction centers from *Rps. viridis*. Excitations 534.1 and 545.8 nm. Spectral resolution, 6 cm^{-1}.

one to molecule $BPheo_M$. Comparisons of these resonance Raman spectra in the whole 200–1700 cm^{-1} range, with those of isolated BPheo *b* excited under the same conditions, confirmed that the features observed in the higher-frequency region from the reaction center all arise from BPheo, and not from the carotenoid or cytochrome moieties. In particular, no change could be observed in this spectral region when the redox state of the bound cytochrome was changed (data not shown).

Along the lines of our previous assignments for BPheo *b* [18], the 1635 and 1627 cm^{-1} bands observed for *Rps. viridis* most probably arise from the stretching modes of the acetyl carbonyl groups of $BPheo_L$ and of $BPheo_M$, respectively (see Material and Methods). The 25 and 33 cm^{-1} downshifts assumed by these vibrators from the wavenumber of the interaction-free state (approx. 1660 cm^{-1}) indicate that they are both intermolecularly bound.

The keto carbonyl group of $BPheo_L$ can be assigned the stronger, 1678 cm^{-1} component observed at higher frequency in the 545-nm-excited spectrum. This assignment is more ambiguous for $BPheo_M$, the resonance Raman spectrum of which exhibits a broader band with two components at 1690 and 1703 cm^{-1}. We tentatively assign the latter component to $BPheo_M$ and the former to a spurious contribution from, for example, additional BPheo *b* or *a* present in the preparation as an impurity. Indeed, the 545-nm-excited spectrum also contains a weak shoulder at 1690 cm^{-1}, suggesting that this additional molecular species might have a Q_x band broader than those of $BPheo_L$ and $BPheo_M$, as expected for a contaminant species in a non-native, ill-defined environment. On this basis, the 1678 and 1700 cm^{-1} wavenumber values can be assigned to the stretching modes of the keto carbonyl groups of $BPheo_L$ and $BPheo_M$, respectively. These values indicate that the former group is intermolecularly bound, while the latter is free from interaction. It may be noted that these conclusions would not be qualitatively different if considering that the stretching mode of the 9-keto group of $Bpheo_M$ should be assigned the 1690 cm^{-1} component rather than the 1700 cm^{-1} one, because both indicate free rather than H-bonded states.

Hence, the present spectra demonstrate that $BPheo_L$, the normal primary acceptor, has both its conjugated carbonyls intermolecularly bound, while $BPheo_M$ retains a free keto carbonyl. The frequencies of these four vibrators are almost identical to those observed for the homologous molecules in RCs from *Rhb. sphaeroides* [15]. This indicates that the interaction states of the homologous carbonyls are identical in the two species. In particular, X-ray crystallography of RCs from *Rps. viridis* has indicated that the keto carbonyl group of the acceptor $BPheo_L$ molecule is most probably H-bonded to the (protonated) carboxyl function of the side-chain of Glu L104 [5]. This residue is conserved in *Rhb. sphaeroides* [34] and thus also probably constitutes the donor site of the H-bond which is shown by resonance Raman spectroscopy to involve the keto group of $BPheo_L$. On the other hand, the M chains of the RC proteins of *Rps. viridis* and of *Rhb. sphaeroides* do not have glutamic acid, but rather valine and threonine residues, respectively, at the corresponding positions. In *Rps. viridis*, the valine M131 side-chain indeed has no suitable H bond donor group, in perfect agreement with our present resonance Raman data. In *Rhb. sphaeroides,* the secondary alcohol function of threonine M133 might bind the keto group of $BPheo_M$ [35]. Hence, resonance Raman data further indicate that this grouping actually should assume a position unfavorable to such a binding.

The 25 cm^{-1} difference observed between the stretching modes of the keto groups of the BPheo L and M molecules, in both *Rps. viridis* and *Rhb. sphaeroides* RCs, corresponds to a H-bond energy of about 3.5 kcal/mol, according to Badger-type rules proposed by Zadorozhnyi and Ishchenko [36]. This difference in H-bonding between the acceptor and accessory BPheo molecules has been proposed to contribute in the functional asymmetry of the bacterial reaction center [5,37]. Indeed, substitution of Glu L104 by a leucine residue in RCs of *Rhb. capsulatus* induced a deceleration of the electron transfer between P and $BPheo_L$ by a factor of 1.5 [38]. Furthermore, the recent observation of a very similar structure in the PS II reaction center also suggested a definite functional role for the H-bonding of the keto carbonyl of the acceptor (bacterio)pheophytin molecule [39].

Thus, the structural pictures given by RR spectroscopy about the interaction states of the keto carbonyls of the BPheo L and M molecules, in both *Rps. viridis* and *Rhb. sphaeroides* RCs is perfectly consistent with those given by X-ray crystallography and protein sequences, as well as with the recent infrared data of Mäntele et al. [40].

The agreement between resonance Raman data and the current structural models derived from X-ray crystallography is not as satisfactory when the interaction states of the acetyl groups of the Bpheo molecules, in RCs of the *Rps. viridis* and *Rhb. sphaeroides* species, are considered. Because their stretching frequencies are downshifted by 33 and 41 cm^{-1} from that of the free vibrator (about 1668 cm^{-1} [18]), these four carbonyls are seen by resonance Raman spectroscopy as sizably intermolecularly bound (e.g., by H bonds of 5–6 kcal/mol), and yet no suitable ligands are found in the X-ray models [5] except, perhaps, for $BPheo_L$ in *Rhb. sphaeroides* [35]. It might be argued that these downshifts actually could originate either from high local permittivity or from strong perturbation of the conjugated π system of the molecules. These possibilities do not appear very likely. Indeed, even a dielectric constant as high as 50 is not expected to induce a shift

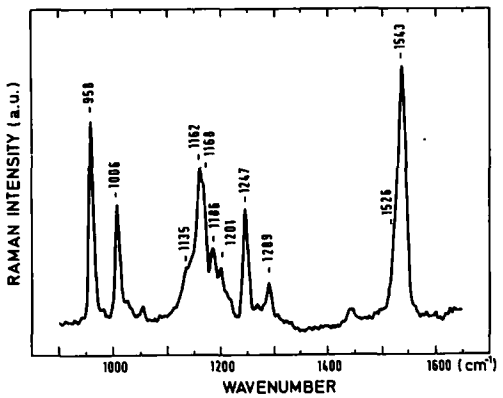

Fig. 3. Resonance Raman spectrum (900–1750 cm^{-1} region) of carotenoids in untreated reaction centers of *Rps. viridis* at 30 K. Excitation 496.5 nm. Spectral resolution, 6 cm^{-1}.

larger than about 20 cm^{-1}, according to measurements performed by Koyama and his co-workers [41] on the keto stretching frequency of chlorophyll *a*. On the other hand, variations in electron densities on the dihydrophorbin ring which would be large enough to shift the stretching mode of one of the conjugated carbonyls by as much as 30 cm^{-1} would certainly result in conspicuous frequency shifts of other resonance Raman modes of the molecule, particularly skeletal ones, as well as in large relative intensity changes of the resonance Raman bands. This is not observed for the RC BPheos, and the hypothesis of about 5 kcal/mol intermolecular bonding of each of their acetyl carbonyls is by far the most likely one.

In any case, resonance Raman spectroscopy indicates that very conservative local proteic environments accommodate the bacteriopheophytin molecules in RCs from *Rps. viridis* and from *Rhb. sphaeroides*.

Excitation at 496.5 nm: resonance of carotenoid modes

Excitation of RCs from *Rps. viridis* in the 410–500 nm range results in resonance Raman spectra which contain variable contributions from the carotenoid, bacteriochlorins and cytochromes, depending on the redox state of the latter and on the excitation wavelength within this range (data not shown). Resonance Raman spectra excited at 496.5 nm contain contributions from the carotenoid alone (Fig. 3). As compared to those of the bulk carotenoid of the chromatophore, these spectra share common specificities with those of other RC-bound carotenoids such as spheroidene in *Rhb. sphaeroides* [12,42]. These specificities, already observed in crude preparations of RCs from *Rps. viridis* [42], include an upshift of the C = C stretching band from 1535 to 1542 cm^{-1}, and the occurrence of an additional band at 1248 cm^{-1}. As discussed previously, these features are characteristic of a 15-15′ *cis* configuration [14,43]. Another specificity of resonance Raman spectra of the RC-bound carotenoid in *Rps. viridis* consists of a marked enhancement of a complex band at 959 cm^{-1} (Fig. 3). This indicates that the carotenoid molecule, in addition to its *cis* configuration, assumes an out of plane deformation [14]. These resonance Raman data in particular exclude that the isomer present in RCs from *Rps. viridis* might be 13-14 *cis*. Indeed, 13-14 *cis* isomers of C_{40} carotenoids do not yield the approx. 1240 cm^{-1} band which is common to 15-15′ *cis* forms in vitro and to RC-bound carotenoids [43,44]. On the other hand, the 13-14 *cis* isomers generally give rise to a conspicuous resonance Raman band near to 1135 cm^{-1}, which is not observed in resonance Raman spectra of the RC-bound carotenoids [13,43]. However, mixtures of the 15-*cis* and all-*trans* conformations may sometimes be found in some samples, probably due to differences in the isolation procedures (e.g., nature and concentration of the detergent) or to the storage conditions (Zhou and Robert, unpublished work). A striking difference between the functions of the carotenoid in bacterial RCs of *Rhb. sphaeroides* and in those of *Rps. viridis* is that, in the former, this molecule may trap the triplet state of the primary donor, at room temperature, with a high efficiency [45], whereas this triplet-triplet transfer has been shown not to occur in *Rps. viridis* [45]. On the basis of resonance Raman data, it may be concluded that these different behaviours cannot result from differences in the configurations of the conjugated, isoprenic chains, which are both 15-15′ *cis*, or, most probably, in their conformations, which are both out of plane. Similarly, in *Rhb. sphaeroides* RCs, the yield of the triplet-triplet transfer between P and the carotenoid drastically decreases between 100 and 30 K [45], while no modification of the carotenoid configuration or conformation is detected in resonance Raman spectra in this range of temperature [42]. Hence, the differences in triplet-triplet transfer efficiency observed between the two species cannot be ascribed to these parameters, and have to result from other ones, such as the chemical nature of the carotenoid, its distance from bacteriochlorophyll B_M and the properties of the protein around.

Excitation at 363.8 nm: the bacteriochlorins at Soret resonance

Excitation of untreated reaction centers from *Rps. viridis* at 363.8 nm, close to the top of the Soret band common to all six bacteriochlorin pigments, results in the resonance Raman spectrum of Fig. 4. As expected from these resonance conditions, this spectrum contains only very weak contributions from the carotenoid (band at 1542 cm^{-1}), and none from the cytochrome hemes. In order to ascertain this latter point, control resonance Raman experiments were conducted on isolated horse-heart cytochrome *c* excited at this wavelength. In the reduced as well as in the oxidized state, this molecule yields its strongest resonance Raman band at about 1370 cm^{-1}. On the other hand, changing the redox state

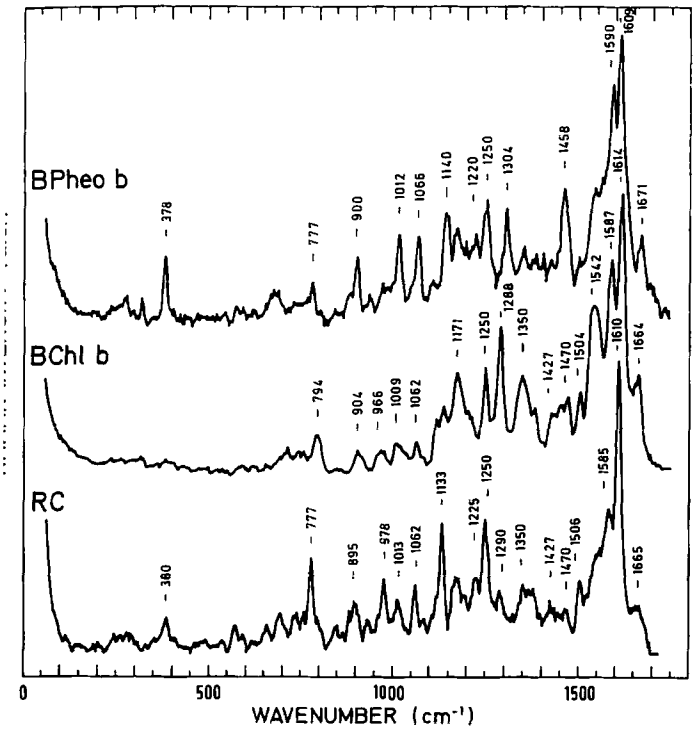

Fig. 4. Resonance Raman spectra (50–1750 cm^{-1} regions) of BChl *b* and BPheo *b* in hexane, and of untreated reaction centers from *Rps. viridis* at 30 K. Excitation 363.8 nm. Spectral resolution, 8 cm^{-1}.

of the bound cytochrome in *Rps. viridis* RCs does not induce any variations in this frequency range of their resonance Raman spectra excited at 363.8 nm. On this basis, it may be concluded that the four hemes of the bound cytochrome *c* are not sizably contributing in these spectra.

A comparison of a resonance Raman spectrum from *Rps. viridis* RCs excited at 363.8 nm with those of isolated BChl *b* and BPheo *b* obtained under the same conditions (Fig. 4.) indicates predominant contributions, in the lower frequency region, from the BPheo molecules, as indicated by bands at, for example, 380, 777, 1013, 1066 and 1225 cm^{-1}. The BPheo contributions are comparatively weaker in the higher-frequency region. Resonance Raman bands specific to BChl *b* are also observed throughout the spectrum, e.g., at 794, 1290, 1350, 1427 and 1470 cm^{-1}. Participation of both BChl and BPheo molecules in the 363.8-nm-excited spectrum is confirmed by inspection of the carbonyl stretch region, which contains components at 1655 and 1665 cm^{-1}, which cannot arise from the BPheo molecules (see above), and which has a comparatively weak band near to 1705 cm^{-1}, where the stretching mode of the 9-keto group of $BPheo_M$ is known to occur.

Primary donor

Following the difference methods presented in Material and Methods, it has been possible to extract contributions from the primary donor in the 363.8-nm-excited spectra (Fig. 5). These contributions are considerably smaller than those previously extracted from resonance Raman spectra of RCs from *Rhb. sphaeroides* and *Rsp. rubrum*, excited under the same conditions [10,16]. This phenomenon may have, at least in part, the same origin as the very low contribution from the primary donor in resonance Raman spectra of the R26, carotenoidless mutant from *Rhb. sphaeroides* (see discussion in Ref. 11).

Fig. 5 displays difference spectra obtained from ferricyanide-treated and dithionite-treated centers from *Rps. viridis*. The ($P\text{-}P^R$) and ($P\text{-}P^+$) spectra from *Rps. viridis* are very similar: they both involve a major band at 1610–1613 cm^{-1}, two bands at 1666–1668 and 1686–1688 cm^{-1}, two weak shoulders near to 1628 and 1635 cm^{-1}, and a possible band at 1652 cm^{-1}. This indicates that these difference spectra contain no sizeable contributions from the P^+ or P^R species, respectively, which might have contributed specific negative bands, and that they should essentially arise from the primary donor in its neutral, ground state.

Given that resonance Raman spectra of the *a*- and *b*-type bacteriochlorins are very similar in the region considered [18], the above proposal is also supported by the fact that the present difference spectra also bear very close similarity to resonance Raman spectra of the primary donors of BChl-*a*-containing species obtained in the same conditions. More precisely (Table I), in addition to a dominant band at 1611–1613 cm^{-1}, these

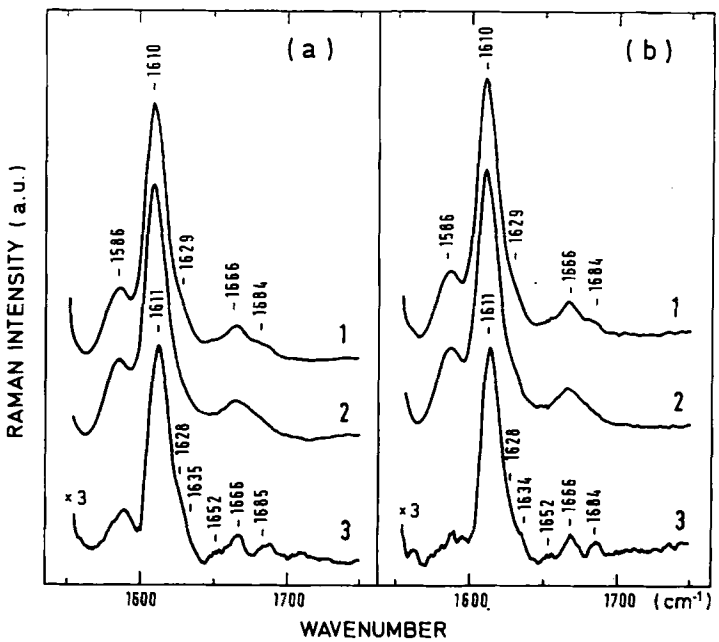

Fig. 5. Resonance Raman spectra (1150–1750 cm^{-1} regions) of reaction centers from *Rps. viridis* treated with 200 μM potassium ferricyanide (A) or with 20 mM sodium ascorbate (B) at 30 K. Excitation 363.8 nm. Spectral resolution, 8 cm^{-1}. (1) Low irradiance conditions. (2) High irradiance conditions. (3) Difference between spectra 1 and 2. Normalization: see Material and Methods.

TABLE I

Assignment of the stretching modes of carbonyl vibrators active in (P − P$^+$) and (P − P^R) difference resonance Raman spectra (1620–1720 cm^{-1} region) of bacterial reaction centers in three genera of Rhodospirillales

P_L, P_M, primary donor bacteriochlorophylls; $BChl_L$, accessory bacteriochlorophyll; Ac, acetyl carbonyl group; K, keto carbonyl group; *vir, Rps. viridis; sph, Rhb. sphaeroides; rub, Rsp. rubrum;* Residue number is that of *Rps. viridis* (Ref. 5).

Wavenumbers (cm^{-1})			Assignment
Rps. viridis [a]	*Rhb. sphaeroides* [b]	*Rsp. rubrum* [c]	
1628			P_MAc...Tyr (M195)
1635	1637	1634	P_LAc...His (L168)
1652			$BChl_L$Ac...?
1665	1660	1658	P_LK...Thre (L248) (*vir*) P_MAc free (*sph, rub*) P_LK...? (*sph*)
1685	1684	1681	P_MK...loosely bound? (Ile (M282))
		1702	P_LK free

[a] This work.
[b] From Ref. 16.
[c] From Ref. 10.

spectra have three bands in common in the carbonyl stretching region, namely at 1635–1637, 1659–1665 and 1680–1685 cm^{-1}. The difference resonance Raman spectra of *Rps. viridis* have additional, specific features at 1628 and 1652 cm^{-1}. At Soret resonance, each bacteriochlorin *b* molecule may contribute two carbonyl stretching bands in the 1620–1710 cm^{-1} range, arising from its 9-keto and 2-acetyl, conjugated groups, respectively. Hence, a maximum number of four distinct frequencies can be contributed by the primary donor alone in the 1620–1710 cm^{-1} range, and an additional contribution has to be considered in order to explain the present difference RR spectra from *Rps. viridis* RCs. Leaving this problem aside for a moment, we note that the two bands at 1628 and 1635 cm^{-1} can arise only from acetyl C = O groups, that the two bands at 1652 and 1665 cm^{-1} may arise from acetyl or keto carbonyls, while the 1685 cm^{-1} band can arise only from keto carbonyls (see Material and Methods, and Refs. 8, 16). By analogy with our previous assignments for the primary donors of *Rhb. sphaeroides* [16] and of *Rsp. rubrum* [10], we propose that the acetyl groups of the primary donor BChls vibrate at 1628 and 1635 cm^{-1}, and that their keto carbonyls vibrate at 1665 and 1685 cm^{-1}.

The remaining, very weak band at 1652 cm^{-1} in difference resonance Raman spectra of *Rps. viridis* is ascribed to the acetyl carbonyl of the accessory molecule $BChl_L$ on the following basis. We have shown previously [11] that, in resonance Raman spectra of RCs of the R26 mutant of *Rhb. sphaeroides* excited at 363.8 nm, the contribution from the neutral primary donor is negligibly small in the 1550–1710 cm^{-1} region. Because of this effect, it can be observed that the primary charge separation is accompanied by a downshift of an approx. 1689 cm^{-1} band and by a weakening of a 1660 cm^{-1} band. These features were ascribed to the keto and acetyl carbonyls, respectively, of $BChl_L$. This phenomenon is thought to play a functional role in the primary charge separation and should take place in other reaction centers; its Raman signature should, however, be generally masked, in difference resonance Raman spectra, by the stronger contributions from the neutral primary donor. However, it has been observed in difference resonance Raman spectra from RCs of *Rhb. capsulatus* (Robert, B., Zhou, Welte and Lutz, unpublished work). Inasmuch as the primary donor contribution in difference resonance Raman spectra from RCS of *Rps. viridis* excited in the same conditions is significantly lower than for *Rhb. sphaeroides* (see above), it is very likely that the 1652 cm^{-1} feature is homologous to the 1660 cm^{-1} one observed for R-26 RCs and corresponds to a weakening of the stretching mode of the acetyl carbonyl of $BChl_L$ induced by the primary charge separation.

In this interpretation, both the P_L and P_M molecules of the primary donor of *Rps. viridis* have their acetyl groups strongly bound (1628 and 1635 cm^{-1} bands), most likely to the protein [16]. One of these molecules also has its keto carbonyl strongly interacting with a probable proteic partner (1665 cm^{-1} band). The second primary donor BChl has a loosely bound, or even free keto carbonyl (1685 cm^{-1}). Indeed, Koyama and coworkers recently observed that, for chlorophyll *a* in polar solvents, the stretching mode of the free keto carbonyl may occur in the 1702–1680 cm^{-1} range, depending on the dielectric constant of the solvents [34]. The approx. 15 cm^{-1} downshift observed for this mode of the primary donor of *Rps. viridis* may correspond either to a weak H-bonding (about 2.2 kcal · mol^{-1}), or to a high local permittivity [46].

This picture of the ground-state interactions assumed by the conjugated carbonyls of the primary donor BChls of *Rps. viridis* is entirely consistent with the structural model of this RC derived from X-ray crystallography [5]. Indeed, this model indicates that the acetyl group of molecule P_L can be bound to the side-chain of His L168, and that of molecule P_M to the side-chain of Tyr M195. Both of the NH and OH groups of these amino acids may establish the rather strong H bonds observed in resonance Raman spectra of P. The keto group of P_L may be H-bonded to the hydroxyl group of the side-chain of Thr L248, while that of P_M, being in the vicinity of Ile M282, has no suitable electron acceptor nearby to establish a localized bond. According to the above remark, the latter group may vibrate at 1685

cm^{-1}, while the H-bonded keto group of P_L may vibrate at 1665 cm^{-1} (Table I).

Further assignment of each of the two 1628 and 1635 cm^{-1} frequencies to the proper primary donor molecule can be made by comparing resonance Raman and sequence data for the two species *Rps. viridis* and *Rhb. sphaeroides*. In both species, homologous His residues (L168 in *Rps. viridis*) can bind the acetyl carbonyl of the P_L molecules. Hence, the 1635 cm^{-1} frequency being almost identical to the 1636 cm^{-1} value observed for one of the acetyl groups of the primary donor of *Rhb. sphaeroides* [16], both the corresponding modes must be assigned to the histidine-bound P_L molecules in both species, at odds with a recent proposal [35].

Consequently, the tyrosine-bound acetyl group of the P_M molecule of *Rps. viridis* should vibrate at 1628 cm^{-1}. This latter group is clearly free from any interaction in *Rhb. sphaeroides*, vibrating at 1660 cm^{-1} [16]. Consistent with this observation, Tyr M195 is replaced by a phenylalanine residue in the M chain of *Rhb. sphaeroides*, which is unable to provide H-bonding to the acetyl of P_M. This observation is also at odds with the proposal of Allen and Yeates et al. [6,35] that, in *Rhb. sphaeroides*, the side-chain of Tyr M210 provide the acetyl of P_M with an H-bond equivalent to that provided by Tyr M195 in *Rps. viridis*.

As discussed previously for *Rhb. sphaeroides* [16], the difference spectra of Fig. 5 permit us to determine the coordination numbers of the magnesiums of the P_L and P_M molecules, on the basis of the frequency and width of the Ca-Cm stretching band located around 1611 cm^{-1} (see Material and Methods). In vitro studies have shown that, as for BChl *a* [29,30], the wavenumber of this band is around 1600 cm^{-1} when the magnesium atom of BChl *b* bears two external ligands, i.e., is hexa-coordinated, and above 1610 cm^{-1} when it is penta-coordinated [18]. The 1611–1613 cm^{-1} wavenumbers of the corresponding bands in the difference spectra of Fig. 5 indicate that the two primary donor BChls of *Rps. viridis* have five-coordinated magnesiums. The 16 cm^{-1} half-bandwidths, which are not higher than those observed for BChl *b* in solution, indicate that the two molecules should vibrate at closely the same frequency, and hence should share very similar geometries around their magnesiums [47,48]. The likely contribution of a carbonyl group of molecule $BChl_L$ in these difference spectra is not expected to be accompanied by any other sizable contributions, in particular at 1611 cm^{-1} [11]. The refined model of the RC of *Rps. viridis* derived from X-ray data by Deisenhofer, Michel et al. [5] also indicated that the magnesium atoms of the primary donor BChls should be both five-coordinated.

Hence, perfectly consistent pictures of the interaction states of the magnesium atoms and conjugated carbonyls of the primary donor molecules are given by resonance Raman spectra and by the refined X-ray model of the reaction center of *Rps. viridis*. This gives a satisfactory basis for interpreting the resonance Raman spectra of the primary donors of other species in structural terms.

Interspecific variability of the primary donor structure

Table I confirms [10] that a genetic variability does exist in the interactions of the conjugated carbonyls of the primary donor molecules with the protein. Comparison of the stretching frequencies of these carbonyls in *Rps. viridis, Rhb. sphaeroides* and *Rsp. rubrum* indeed shows that the acetyl group of molecule P_M and the keto group of molecule P_L may be either free or intermolecularly interacting. The Thr (L248) residue with which the keto group of P_L most likely interacts (Ref. 5 and this work) is replaced by a Met residue in *Rhb. sphaeroides*, the side-chain of which cannot provide any strong H-bonding. Hence, even though this keto group shares the same interaction energy as that of *Rps. viridis*, this interaction should be with another molecular site. On the other hand, the acetyl group of molecule P_L and the keto group of molecule P_M vibrate at the same frequencies, and hence should each assume the same interaction states within the three above bacterial species.

The recent sequencing of the L and M polypeptides of the reaction center of *Rsp. rubrum* showed that His L168 and Ile M282 were conserved in *Rsp. rubrum*, but that Thr L248 and Tyr M195 were replaced by a methionine and a phenylalanine residue, respectively [49]. These results are in complete agreement with resonance Raman spectra of the primary donors in both species, and confirm our predictions about the nature of the residues potentially interacting with the primary donor of *Rsp. rubrum* [10,20].

In a preceding paper [10] we noted that the unequivalence of the sets of molecular interactions assumed by the conjugated carbonyls of the P_L and P_M molecules in the bacterial primary donor should play a role in the unequal repartition of the time-averaged unpaired charge densities over P_L and P_M in the P^+ and P^R states. We noted further that this asymmetry should accordingly be species-dependent, on the basis of the interspecific variability of these molecular interactions shown by resonance Raman. Together with recent results on charge repartition [50], the present results on *Rps. viridis* give additional data useful for future testing of this hypothesis.

It may be worth noting, finally, that the structural variations affecting the interaction states of the acetyl group of molecule P_M and the keto group of molecule P_L do not appear to affect, to first order, the primary events in the reaction center, whereas the interaction states of the acetyl group of P_L, H-bonded with a His residue, and of the keto group of P_M, probably free but located in a rather polar micro-environment, might constitute important parameters in the structure and func-

tion of the bacterial primary donor.

Further interspecific comparisons of the structures of the primary donor in non-sulfur as well as in sulfur purple bacteria currently are underway and will be presented elsewhere (Zhou, Q., Robert, B., Vermeglio, A. and Lutz, M., unpublished data).

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