Proteic Events following Charge Separation in the Bacterial Reaction Center: Resonance Raman Spectroscopy

Bruno Robert* and Marc Lutz

Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France

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ABSTRACT: Raman spectra of bacteriochlorin pigments of reaction centers from the R 26 carotenoidless strain of *Rhodobacter sphaeroides* have been studied at low temperature and at Soret resonance. These spectra show that the primary charge separation is accompanied by changes in the liganding of the 9-keto carbonyl group and, most probably, in the environment of the 2-acetyl carbonyl of the accessory bacteriochlorophyll molecule B_L located along the normal electron pathway (L branch) of the reaction centers. These environmental changes around B_L are observed when the primary donor (P) is chemically or photochemically oxidized. They are triggered by the ionization of P rather than by the electron transfer. They persist when P is brought in its triplet state P^R. These changes in liganding of the 9-keto group may involve the side chain of tyrosine M-208 or bound water possibly located in the vicinity of B_L. It appears likely that the protein conformational change occurring around B_L might play a functional role in the asymmetric functioning of the reaction centers and/or in the limiting of backreactions during the first electron-transfer steps.

The light reactions of bacterial photosynthesis take place in specialized protein–pigment complexes localized in the intracytoplasmic membranes of photosynthetic bacteria named reaction centers (RC).[1] These complexes contain six bacteriochlorin pigments, namely, two bacteriohaeophytins (Bpheo) and four bacteriochlorophylls (BChl), as well as two quinones and a non-heme iron. Recently, RCs from *Rhodopseudomonas* (*Rps.*) *viridis* were crystallized, and X-ray diffraction studies of these crystals allowed an electron density map to be obtained with a 2.9-Å resolution. These results permitted a precise description of the relative topology of the different pigments of the RC (Deisenhofer et al., 1984) and of the arrangement and conformations of the three polypeptides (L, M, and H) composing the apoprotein of the reaction centers (Deisenhofer et al., 1985). Spectroscopic investigations have demonstrated the existence of extremely rapid electron-transfer steps following the charge separation. The electron moves from the primary donor, consisting of two electronically interacting BChls, to a Bpheo in approximately 3 ps and then to a quinone in approximately 200 ps (Woodbury et al., 1985; Martin et al., 1986). The role in the electron transfer of the "accessory" BChl molecule located along the normal electron pathway is not yet completely clear. If these charge-transfer steps are now rather well characterized, very little information is still available on the possible roles of the proteic core during these steps, as well as on the protein conformational changes which are likely to be induced by charge separation. Nevertheless, several hypotheses have already been proposed concerning a possible involvement of protein conformational changes in the functioning of the reaction center: in order to explain changes in linear dichroism spectra, Vermeglio and Paillotin (1982) invoked changes in the relative orientations of the pigments following charge separation in *Rps. viridis*. Kleinfeld et al. (1984) suggested that a change in protein conformation should occur after charge separation, in order to explain illumination-dependent differences in the recombination behavior of the RCs during freezing. By providing information about intermolecular interactions assumed by the different pigments within reaction centers, resonance Raman spectroscopy potentially gives direct information about protein conformational changes in the local environments of these pigments, i.e., about these conformational changes that may directly influence charge separation and electron transfer.

In this paper, we present the first evidence of such a light-induced protein conformational change, and we discuss the possible mechanisms that may induce this change in the protein.

Experimental Procedures

Samples. Cells of *Rhodobacter* (*Rhb.*) *sphaeroides* strain R 26 were grown anaerobically in a modified Hutner medium. Chromatophores were prepared as in Robert and Lutz (1985), and RCs were purified from these chromatophores, with a DEAE HPLC column (Berger et al., 1984). Samples were kept for the experiments in a Tris (25 mM)–Triton (0.1%) buffer. Absorption spectra of RCs purified by this method are presented in Figure 1(1). A typical absorbance A_{280}:A_{800} ratio is 1.4 for such preparations. Selective bleaching of one of the "accessory" BChl was performed by borohydride treatment, following the procedure of Marotti et al. (1985). Absorption spectra of borohydride-treated RCs are presented in Figure 1(2).

Methods. Resonance Raman (RR) spectroscopy has been shown to be a particularly sensitive method for studying intermolecular interactions as well as conformations of the photosynthetic pigments in bacterial protein–pigment complexes, constituting the antenna (Robert & Lutz, 1985; Robert et al., 1984) as well as reaction centers (Robert & Lutz, 1986). The high-frequency region of RR spectra of the bacteriochlorin pigments excited near the top of their Soret absorption tran-

* Author to whom correspondence should be addressed.

[1] Abbreviations: Bchl, bacteriochlorophyll; Bpheo, bacteriophaeophytin; RC, reaction centers; RR, resonance Raman; *Rps.*, *Rhodopseudomonas*; *Rhb.*, *Rhodobacter*; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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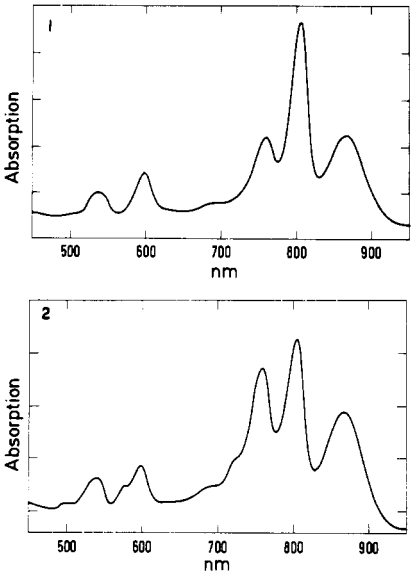

FIGURE 1: Room temperature electronic absorption spectra of reaction centers from *Rhb. sphaeroides* R 26 strain: (1) untreated; (2) borohydride treated.

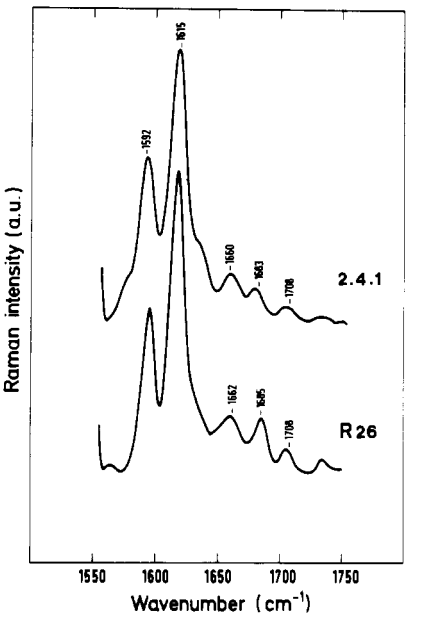

FIGURE 2: Resonance Raman spectra (1550–1750-cm^{-1} region) of reaction centers from (1) *Rhb. sphaeroides* 2.4.1; (2) *Rhb. sphaeroides* R 26. Experimental conditions: T = 30 K; excitation wavelength = 363.8 nm.

sition, by use of the 363.8-nm line of the Argon laser, contains bands arising from stretching of the conjugated carbonyls of several if not all of these pigments (2-acetyl and 9-keto groups). Both of these C=O groups have been shown to play important roles in anchoring the pigments in the proteic matrix of the complexes [for a review, see Lutz and Robert (1988)]. Recent results also suggest that the conjugated carbonyls of BChl *a* may actually predominate over the methyl ester carbonyl in ensuring this function (Egan & Fenna, 1988). Resonance Raman spectra thus provide indirect information about the local environments of both BChl and Bpheo in the bacterial RC. We recently developed methods selectively yielding RR spectra of the primary donor alone (Robert & Lutz, 1986). These methods make use of the actinic effect of the probe laser beam on the reaction centers and involve control of the dynamic equilibrium states assumed by the samples during RR experiments through control of the irradiance, redox potential, and temperature parameters.

Resonance Raman spectra were recorded at 30 K, with a 363.8-nm excitation wavelength. The experimental setup and recording procedure have been described in Robert and Lutz (1986), and an estimation of the irradiance values used to accumulate P^+ as well as P^R has been given in the same reference. Typically, less than 2 mW penetrated the sample during "high-irradiance" Raman experiments. By playing both on the laser power and on the focusing of the beam on the frozen samples, it was possible to decrese irradiance at the sample by an estimated factor of 500 in "low-irradiance" experiments, while keeping the Raman signal at acceptable levels. RR spectra used for computing difference spectra are each sums of 20–50 individual scans.

RESULTS

Figure 2 presents the higher frequency region (1550–1750 cm^{-1}) of RR spectra of RCs from the R 26 carotenoidless mutant of *Rhb. sphaeroides* and of RCs extracted from the wild-type strain (2.4.1). The following features are present in both spectra: (i) a band at 1592 cm^{-1} mainly arising from Bpheo, (ii) a stretching mode of the methine bridges of the BChls and Bpheos around 1615 cm^{-1} [this mode has been shown to be sensitive to the coordination number of the central Mg of Bchl, being located near 1600 cm^{-1} when this atom is 6-coordinated and around 1615 cm^{-1} when it is 5-coordinated (Cotton & Van Duyne, 1982; Robert & Lutz, 1985)], and (iii) the stretching modes of the 2-acetyl and 9-keto carbonyl groups, the frequencies of which are sensitive to environmental interactions, such as van der Waals and H-bonding.

Differences in the relative intensities of the different bands arising from the C=O stretching modes clearly appear between spectra of the two strains. Interestingly, all the carbonyl stretching bands that appear modified in RR spectra of RCs extracted from the R 26 strain correspond to modes that have been previously attributed to the two BChls constituting the primary donor, i.e., the 1637-, 1660-, 1683-cm^{-1} bands (Robert & Lutz, 1986); the 1637-cm^{-1} band (which appears in Figure 2 as a little shoulder of the 1615-cm^{-1} band) is weaker in R 26 RR spectra than in 2.4.1 ones; the 1660-cm^{-1} band is also weaker in R 26 RR spectra, and very slightly upshifted (2 cm^{-1}), while the 1683-cm^{-1} band is stronger and slightly upshifted to 1685 cm^{-1}. Intensity differences between C=O stretching bands of both wild-type and R 26 strains of *Rhb. sphaeroides* are measured by taking the whole C=O stretching area as reference.

Curves 1 and 2 of Figure 3 present RR spectra of untreated R 26 RCs obtained at 363.8 nm with high and low irradiances from the excitation laser beam, respectively. As might be expected from the weak contribution from the primary donor in RR spectra of R 26 RCs, no sizable difference in the intensity ratio of the two main bands at 1592 and 1615 cm^{-1} appears in these two spectra. This behavior differs from those of RR spectra of RCs from both *Rhb. sphaeroides* 2.4.1. and *Rsp. rubrum* S1 reaction centers, in which the primary donor significantly contributes (Robert & Lutz, 1986; Zhou et al., 1987). In RR spectra of both of these RCs, the buildup of sizable amounts of radical excited states of P indeed results primarily in changes of the intensity ratios of the 1592- and 1615-cm^{-1} bands, due to a weakening of the contributions from neutral P.

However, illumination-dependent differences can be observed in the carbonyl stretching regions of RR spectra of R 26 RCs. For instance, the 1685-cm^{-1} feature, which appears as a single, well-resolved band in RR spectra recorded under low irradiance, appears as a shoulder of the 1662-cm^{-1} band

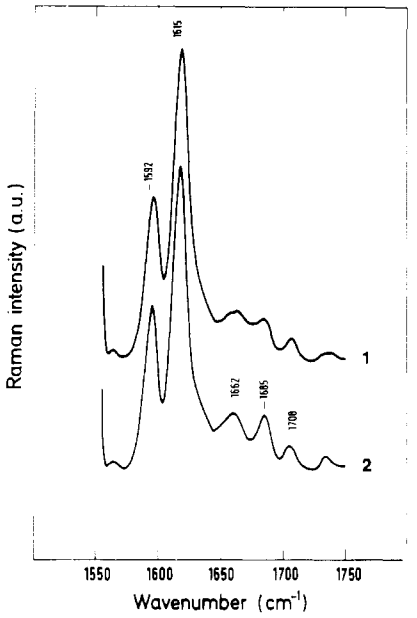

FIGURE 3: Resonance Raman spectra (1550–1750-cm^{-1} region) of reaction centers from *Rhb. sphaeroides* R 26: (1) high irradiance; (2) low irradiance. Experimental conditions: T = 30 K; excitation wavelength = 363.8 nm.

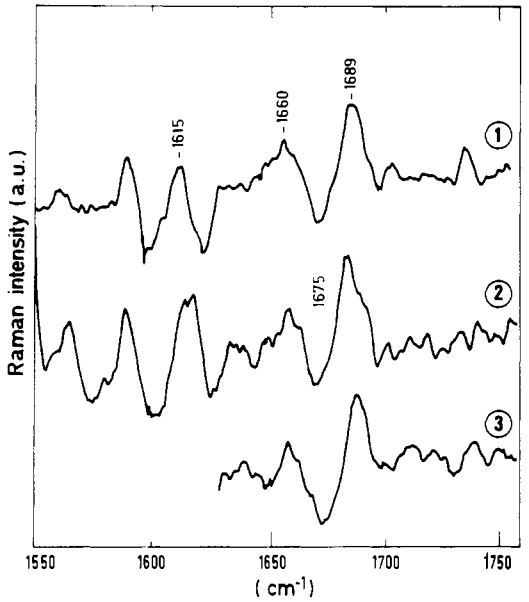

FIGURE 4: Difference resonance Raman spectra (1550–1750-cm^{-1} region) obtained from reaction centers of *Rhb. sphaeroides* R 26: (1) nontreated, low irradiance minus high irradiance; (2) dithionite-treated, low irradiance minus high irradiance; (3) nontreated reaction centers minus ferricyanide-treated reaction centers. Experimental conditions: T = 30 K; excitation wavelength = 363.8 nm. Normalization procedures, see text.

at higher irradiance [Figure 3(1,2)]. A difference between spectra of Figure 3 is presented in Figure 4(1). For computation of this difference, any of the bands arising from skeletal modes of BChl or Bpheo may be taken as an internal standard, because no measurable difference is induced in the 900–1615-cm^{-1} region by the changes in irradiance at the sample. The same difference spectra were also obtained from samples with $(NH_4)_2SO_4$ added, the sulfate 987-cm^{-1} band being taken as an external reference. The carbonyl stretching region (1620–1720 cm^{-1}) of the difference spectrum of Figure 4(1) can be interpreted simply by considering that illumination of untreated RCs induces a ca. 14-cm^{-1} downshift of one component at 1689 cm^{-1} of the 1685-cm^{-1} band and a slight

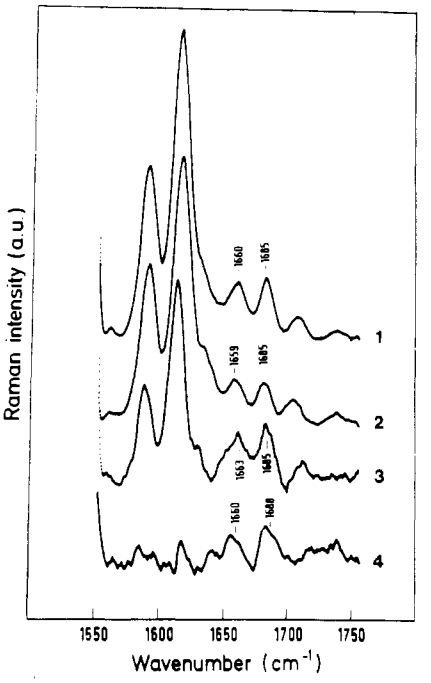

FIGURE 5: (1) Resonance Raman spectra of reaction centers from *Rhb. sphaeroides* R 26 (low-irradiance conditions, 1550–1750-cm^{-1} region). (2) Resonance Raman spectra of borohydride-treated R 26 reaction centers (same irradiance conditions, same spectral region). (3) Difference resonance Raman spectrum (1550–1750-cm^{-1} region): native reaction centers minus borohydride-treated reaction centers, both low-irradiance conditions. (4) Difference resonance Raman spectrum (1550–1750-cm^{-1} region): borohydride-treated reaction centers, low-irradiance minus high-irradiance conditions. T = 30 K; excitation wavelength = 363.8 nm. Normalization procedures, see text.

weakening of a band located at 1660 cm^{-1}. The two apparently S-shaped signals present near 1595 and near 1605 cm^{-1} most probably arise from a slight, asymmetric broadening of the very strong 1615-cm^{-1} band. All these light-induced changes appear to be reversible and are observed at temperatures as low as 18 K.

Figure 4(3) is a difference spectrum between RR spectra of untreated and of chemically oxidized R 26 RCs (both recorded at low irradiance). Figure 4(2) is a difference spectrum obtained by subtracting a RR spectrum obtained at low irradiance from chemically reduced R 26 RCs from a RR spectrum obtained at high irradiance from the same sample. It clearly appears that both of these difference spectra exhibit the same features as the difference spectrum of Figure 4(1) in the 1620–1700-cm^{-1} region.

Figure 5 compares RR spectra of borohydride-treated R 26 reaction centers (curve 2) and of nontreated reaction centers (curve 1). It has been shown (Marotti et al., 1985) that borohydride treatment results in the bleaching of one 800 nm absorbing BChl and in no major change in the mechanisms and kinetics of the first steps of the electron transfer. The change in the relative stoichiometry between BChl and Bpheo induces an 8% weakening of the intensity of the 1615-cm^{-1} band relative to the 1592-cm^{-1} one. Taking into account that there is a weak contribution from BChl in the 1592-cm^{-1} band (Robert & Lutz, 1985), this change in intensity may account for the bleaching of one BChl.

Figure 5(3) presents a difference between RR spectra of untreated and of borohydride-treated reaction centers. In order to obtain this difference, spectra have been appropriately weighted, taking into account for the normalization not only the 1592-cm^{-1} band, which arises largely but not exclusively from Bpheo, but also the 1708-cm^{-1} carbonyl band which arises

from the 535 nm absorbing Bpheo alone (Lutz, 1980; Lutz & Robert, 1985). Subtractions obtained after normalization on the 987-cm^{-1} band of $(NH_4)_2SO_4$ as an external standard are not qualitatively different from the one presented here. On this difference spectrum two positive bands may be observed at 1663 and 1685 cm^{-1}. In addition, two S-shaped components are present, with positive components near to 1625 and 1709 cm^{-1}. Comparison of the frequencies of these bands in curves 1 and 2 of Figure 5 allows measurement of the amplitude of these shifts: upon borohydride treatment, the 1708-cm^{-1} band should shift down to 1703 cm^{-1} while the 1625-cm^{-1} band should shift up by ca. 2 wavenumbers.

Figure 5(4) presents a difference calculated by subtracting a RR spectrum of borohydride-treated reaction centers obtained at high irradiance from a RR spectrum of borohydride-treated centers obtained at low irradiance. Normalization conditions were the same as those for obtaining difference spectra of Figure 4(1–3). Again, this difference spectrum is very similar to those presented in Figure 4(1–3).

Discussion

Differences between RR Spectra of RCs from 2.4.1 and from R 26 Strains of Rhb. sphaeroides. Through comparisons of electronic spectra of RCs from the R 26 strain of *Rhb. sphaeroides* with those either from the wild type (Lutz et al., 1978) or from the R 26 strain reconstituted with a carotenoid (Agalidis et al., 1980), it has been shown that the presence of a carotenoid in the center of *Rhb. sphaeroides* induces a slight bathochromism (1.5 nm) and a hyperchromism (10%) of the main component of the Soret band of the bacteriochlorin pigments. These studies however left unclear whether this change in the Soret region arose from the whole set of pigments contained in the RCs or only certain of them. Differences in the relative intensities of the RR bands of the RCs of the two *sphaeroides* strains as observed in Figure 3 are likely due to changes in resonance conditions induced by these electronic absorption changes. More precisely the main differences occurring between RR spectra of 2.4.1 and R 26 RCs concern carbonyl bands at 1637, 1660, and 1683 cm^{-1}, which arise, at least in part, from the primary donor. Hence, it seems further likely that the differences observed between the Soret bands of 2.4.1 and of R 26 strains mainly concern the Soret components of the primary donor BChls. These RR differences however do not concern the primary donor only: carotenoid-induced differences are also observed amid RR bands of Bpheo and of accessory BChl, when spectra of the 2.4.1 and R 26 strains are compared. For example, the 1592-cm^{-1} band, mostly arising from Bpheo, is weaker in R 26 spectra than in 2.4.1 spectra, relative to the 1615-cm^{-1} band which arises from both Bpheo and BChl. As the primary donor contributes only very weakly in R 26 spectra, one would have merely expected a uniform enhancement of all of the RR bands specifically arising from Bpheo relative to bands arising from the four BChl molecules. The relative weakening of the 1592-cm^{-1} band thus must arise from a distinct phenomenon, involving molecules other than those constituting P. However, such differences are on the whole smaller than the weakening of the primary donor contribution in RR spectra of R26 RCs excited at 363.8 nm, as compared to RR spectra of wild-type RCs.

Strong illumination of 2.4.1 RCs during recording of RR spectra excited at 363.8 nm induces a drastic change in the relative contribution of the neutral, ground-state primary donor. This fact is accounted for by the buildup of high concentrations of radical cation P^+, which does not significantly contributes in RR spectra at this wavelength (Robert & Lutz, 1986). This results in a change of the intensity ratio between the 1615- and the 1592-cm^{-1} bands. However, no change of this intensity ratio can be observed in RR spectra from R 26 RCs in the same conditions. This strongly confirms that the primary donor contributes only weakly in RR spectra of R 26 RCs excited at 363.8 nm. The photoinduced changes actually observed elsewhere in the spectrum of R 26 RCs, in particular in the carbonyl stretching frequency region, thus must essentially arise from the other four bacteriochlorin pigments. These changes may originate either directly from changes in pigment–pigment electronic couplings due to the formation of P^+ or more indirectly from changes in the proteic environment induced, e.g., by the formation of P^+.

RR spectra indicate that any electronic couplings involving the Soret transitions of the four non-P pigments are most probably not deeply perturbed by the presence of a positive charge on the primary donor: indeed, the 1615-cm^{-1} band arises from the four non-P pigments, and its intensity, relative, e.g., to that of the 1592-cm^{-1} band, hence should be affected by any change in the resonance conditions on any of these four molecules. Conversely, any change in resonance conditions affecting the Bpheo but not the accessory BChl would necessarily modify the integrated intensity of the 1592-cm^{-1} band relative to that of the 1615-cm^{-1} band. As the 1615: 1592-cm^{-1} intensity ratio does not depend on the irradiance values on the sample, it may thus be concluded that the photoinduced differences observed in the carbonyl region of RR spectra of R 26 RCs most probably arise from modifications in the local environments of one or more pigments rather than from changes in their electronic absorption spectra.

Spectral Changes following Borohydride Treatment of the RCs. The difference spectrum of Figure 5(3) is very similar to RR spectra of isolated BChl *a* excited at 363.8 nm. Moreover, the presence of only two positive bands in the 1620–1720-cm^{-1} region is consistent with the fact that a single population of BChl is significantly affected by borohydride treatment. The 1685-cm^{-1} frequency can only arise from a weakly interacting keto carbonyl group; the 1663-cm^{-1} band thus necessarily arises from a free-from-interaction acetyl carbonyl. In addition to the bleachings of the 1663- and 1685-cm^{-1} bands, which we assign to the acetyl and keto C=O stretching modes of the missing accessory BChl, borohydride treatment induces two bandshifts [Figure 5(3)]. The positive parts of these S-shaped signals are located at 1625 and 1709 cm^{-1}. These frequencies agree well with the values observed for the carbonyl stretching modes of the 535 nm absorbing Bpheo molecule (Lutz, 1980; Lutz & Robert, 1985), i.e., the Bpheo located along the M branch of the RCs. This fact constitutes additional evidence that the missing BChl in borohydride-treated RC is the one which is bound to the M branch (Marotti et al., 1985). The liganding on both carbonyl stretching bands of the 535 nm absorbing Bpheo is probably too small to correspond to any qualitative change in the liganding of the carbonyl groups: in particular, the keto group, which is free from bonding in untreated centers (1708 cm^{-1}), remains free upon borohydride treatment (1703 cm^{-1}). These changes in the local environment of Bpheo probably can be best described in terms of changes in local permittivity (or in van der Waals interactions as far as the keto group is concerned). These changes may be induced directly by the removal of the B_M molecule and its possible replacement by extraneous molecules. Alternatively, they may be induced indirectly by this removal and result from local rearrangment of the protein around the H_M molecule, involving, e.g., displacement of charged or aromatic residues.

Table I: Assignment of All the Carbonyl Vibrators Active in RR Spectra of *Rhb. sphaeroides*, 2.4.1 and R 26 Strains

frequency (cm^{-1})	assignment[a]	frequency (cm^{-1})	assignment[a]
1625	H_M (Ac)	1678	H_L (K)
1635	H_L (Ac)	1683	P (K)
1637	P (Ac)	1685	B_M (K)
1659	B_L (Ac)	1689	B_L (K)
1660	P (Ac and K)	1708	H_M (K)
1663	B_M (Ac)		

[a] Ac is acetyl. K is keto.

Assignment of All of the Carbonyl Vibrators Active in RR Spectra of R 26 Reaction Centers. As we have seen above, the set of carbonyl vibrators contributing in the 1620–1720-cm^{-1} region of R 26 RCs arise from four pigments only, i.e., from eight conjugated carbonyl groups. From in vitro studies, it is known that the stretching frequencies of the acetyl C=O groups of bacteriochlorophylls may vibrate between 1620 and 1665 cm^{-1} and that those of their keto groups may occur betwen 1640 and 1710 cm^{-1} (Lutz, 1984).

Excitation in each of the two Q_X electronic transition bands of the Bpheo molecules of R 26 RCs at low temperature allowed their carbonyl stretching modes to be observed independently (Lutz, 1980; Lutz & Robert, 1985): the acetyl and keto carbonyl groups of the 535 nm absorbing Bpheo vibrate at 1627 and 1708 cm^{-1} and those of the 545 nm absorbing Bpheo at 1635 and 1678 cm^{-1}, respectively. On the other hand, results obtained on borohydride-treated reaction centers allow the two bands present at 1663 and 1685 cm^{-1} in Figure 5(3) to be assigned to the two carbonyl stretching modes of the missing 800 nm absorbing BChl in borohydride RCs. The remaining band at 1659 cm^{-1} in RR spectra of borohydride-treated R 26 reaction centers arise neither from the Bpheo molecules nor from the primary donor and hence must be attributed to one of the carbonyl groups of the other 800 nm absorbing BChl, i.e., the accessory BChl located on the functional side of the RC, B_L.

Molecular Assignment of the Photoinduced Changes Observed in RR Spectra of R 26 Reaction Centers. A simple comparison of wavenumbers listed in Table I with the 1660- and 1675/1689-cm^{-1} values observed for photoinduced changes in RR spectra of R 26 RCs suggests that the B_M accessory molecule, the primary donor, or, at least in part, the B_L accessory molecule (1659-cm^{-1} band) might be involved in these changes. The accessory B_M molecule can be readily excluded as a candidate for this phenomenon inasmuch as the latter is essentially conserved in RR spectra of borohydride-treated RCs [Figure 5(4)]. Any involvement of the primary donor appears very unlikely as well. Indeed, as mentioned above, the I_{1615}/I_{1592} intensity ratio remains essentially constant in RR spectra of R 26 RCs (but not in those of 2.4.1 RCs) when P^+ or P^R is formed. If P^+ or P^R states contributed in RR spectra of R 26 RCs, they would most probably not both exactly compensate the bleaching of the 1615-cm^{-1} component of the neutral, ground species. Indeed, the wavenumber of the methine stretching mode is expected to shift down quite significantly upon the formation of a radical cation or triplet state (Lutz & Klêo, 1979; Kim et al., 1986). We are thus left with the conclusion that the accesssory BChl B_L alone is responsible for the light-induced changes observed in RR spectra of R 26 RCs. The variation observed at 1660 cm^{-1} is quite consistent with the known location of its $\nu_{2C=O}$ frequency at 1659 cm^{-1}. The present discussion shows that its $\nu_{9C=O}$ frequency must occur at 1689 cm^{-1}, in order to account for the light-induced 1689-cm^{-1} → 1675-cm^{-1} shift.

Table I summarizes the wavenumber assignments that we propose for the conjugated carbonyl stretching bands of the individual bacteriochlorin pigments present in *Rhb. sphaeroides* RCs.

Environmental Origin of the Light-Induced Changes in the RR Contributions of B_L. From the preceding discussion, the light-induced changes observed in the carbonyl stretching region of RR spectra of R 26 RCs most likely arise from the accessory B_L molecule alone. Another persistent light-induced change observed in these spectra concerns the 1615-cm^{-1} band. This change, which may be interpreted as a broadening, is particularly difficult to interpret. It is indeed not necessarily originating from molecule B_L alone and may reflect events concerning either the ground or excited electronic states of the pigments. Hence, we will not discuss it further and only consider, in the following, the carbonyl-related changes, which are clearly ascribed to molecule B_L.

(1) A 1689-cm^{-1} Component Shifts to 1675 cm^{-1}. As concluded above, this 14-cm^{-1} downshift specifically concerns the stretching mode of the keto carbonyl of the accessory BChl bound to the L branch (B_L), on the preferential pathway of electrons after the primary charge separation. Kirmaier et al. (1985) recently proposed that sizable electronic coupling should exist between the S^1 excited states of P and of accessory BChl molecules. When P is oxidized to P^+ or is excited to P^R, these couplings should be altered. If such a mixing also was sizable between the excited states involved in the resonance process yielding the present Raman spectra (i.e., essentially Soret excited states), it conceivably might be the origin for the light-induced changes observed in the resonance Raman contribution of B_L. However, P^+- or P^R-induced changes in the manifold of excited states of B_L should primarily affect the intensity of its RR scattering, rather than the frequencies of its Raman-active modes, which essentially are those of its ground electronic state. Also, such a phenomenon should also probably affect the Soret levels and hence the RR scattering of B_M as well as that of B_L, in view of the symmetrical structure of the B_MPB_L molecular system. For these two reasons, changes in electronic couplings between excited states of P and B_L do not appear as a very likely origin for the observed changes.

Hanson et al. recently performed INDO calculations which indicated that quite significant perturbations might be induced on the Q_x and Q_y electronic transitions of B_L by the presence of a net positive charge, either localized or delocalized over the two molecules of P (Hanson et al., 1987). Such perturbations, even if also affecting Soret transitions, most probably cannot explain the light-induced changes in the RR contributions of B_L, for the same two reasons indicated above. In addition, it appears unlikely that direct charge influence from either P^+ or P^R states might result in identical effects on the RR spectrum and hence on the electronic structure of B_L. We thus assign the shift of the keto carbonyl stretching mode to a local modification around the B_L molecule.

A change in the conformation of the keto carbonyl group of B_L relative, e.g., to the dihydrophorbin plane should primarily result in a change in the intensity of its stretching mode in the RR spectra. Such a 14-cm^{-1} downshift may more likely result from a significant increase in strength of a preexisting H-bond, or from formation of a H-bond, on this carbonyl. Zadorozhnyi et al. proposed that the downshift assumed by the stretching frequency $\nu_{C=O}$ of an aromatic carbonyl, upon formation of a H-bond, is related to the enthalpy H_{hb} of this latter bond, by the following Badger-type, empirical equation:

$$\frac{\Delta\nu_{C=O}}{\nu_{C=O}} = -K_{C=O}H_{hb}$$

According to this relationship, the 14-cm^{-1} light-induced downshift observed for the 9-keto stretching mode of B_L should correspond to a H_{hb} value of 2.1 kcal/mol. Such a value may account for the actual formation of an H-bond between the keto group of B_L and a suitable donor. The absolute 1689-cm^{-1} wavenumber measured for the stretching mode of this group when the RC is in the neutral, ground-state may correspond either to a H-bonded state or to a free state for this group. In the first case, the H-bond enthalpy would be also close to 2.3 kcal/mol according to eq 1 (the highest absolute wavenumber observed for a free keto group in BChl *a* is around 1710 cm^{-1}). The energy of this bonding would then be doubled upon formation of P^+. This might result either from strengthening of the existing bond or from formation of a second bond in addition to the preexisting one (Lutz et al., 1982). In the second case, the low 1689-cm^{-1} value for the $\nu_{9C=O}$ mode can be explained by a high local permittivity around the otherwise free group, as well as, possibly, by more complex effects, e.g., the influence of the presence of an imidazole ring as an axial ligand for the central Mg of the molecule.

(*2*) *The 1660-cm^{-1} Band Weakens.* The light-induced weakening of the acetyl carbonyl band of the accessory BChl bound to the L branch in RR spectra of R 26 RCs is not accompanied by any apparent decrease of the overall contribution of this pigment with respect to those of the other three bacteriochlorin pigments. Such a decrease in overall contribution might, e.g., be due to a variation of the electronic spectrum in the Soret region of B_L. For the same reasons as above, we ascribe this modification to a local conformational change around the B_L molecule and not to a direct, or protein-mediated, charge effect. The 1660-cm^{-1} value corresponds to the stretching of an interaction-free acetyl carbonyl. Changes in the intermolecular bonding of this group, or in the dielectric permittivity of its local environment, are expected to affect its frequency more than its Raman intensity. Such a hypochromism concerning a conjugated chemical group may result from a slight variation of its angle with the dihydrophorbin ring (Lutz et al., 1982). The fact that its frequency remains unchanged indicates that this motion is not induced by intermolecular bonding but rather by a steric repulsion from a nearby molecular site. Such a motion resulting from steric hindrance has indeed been observed for the acetyl group of a BChl molecule (Tronrud et al., 1986).

It may be worth noting at this point that Willems and Bocian (1984) have shown that the presence of an additional C=O group in deuteroporphyrin dimethyl ester could influence the frequency or intensity of the stretching mode of the other C=O of the molecule. It is thus conceivable that a change in the bonding strength of the 9-keto group of the B_L molecule might influence the RR intensity of the 2-acetyl one; in this case, changes affecting the two C=O vibrators would arise from a single environmental effect on the 9-keto vibrator only. However, comparisons between RR spectra of chlorophylls involving conjugated carbonyls at various positions and those of chlorophyll *a* showed that the frequency and relative intensity of the $\nu_{C=O}$ band, common to all of them, were not significantly different in all their spectra (Lutz, 1983; Lutz & Robert, 1988). Hence, the spectral changes observed on mode $\nu_{2C=O}$ are likely to correspond to an environmental change of this carbonyl.

Possible Origins of the Proteic Events following Charge Separation in R 26 RCs. From the previous discussion, it emerges that illumination of the reaction centers induces a modification in the local proteic environment of the accessory BChl bound to the L branch, i.e., the BChl which likely plays a role in the electron transfer, even if it does not constitute the primary electron acceptor (Woodbury et al., 1985). This protein conformational change occurs whether the primary donor is chemically or photochemically oxidized (compare curves 1 and 3 of Figure 4). This clearly shows that it is not the transfer of the electron per se that induces this protein change but, rather, the presence of a charge on the primary donor. It is worth noting that both chemical and photochemical oxidations of the primary donor induce an asymmetrical protein motion, concerning only the accessory BChl bound to the L branch.

Different mechanisms can be proposed for interpreting the fact that the variations observed in RR spectra of R 26 RCs when the P^+ state is accumulated also occur when P^R state is accumulated. First, the variation observed in the local environment of B_L may be induced by the 10-ns lifetime P^+I^- state with a similar time constant, and it may relax with a time constant longer than that of the P^R state (100 μs at 77 K): this hypothesis implies that the net charge of the primary donor induces a real protein conformational change that should have its own potential barrier.

Relaxation of the protein conformational changes around B_L may also occur only when the primary donor is back in its neutral ground state. Indeed, the triggering of the protein conformational change when P^+ is formed might conceivably originate from a slight motion of the two Bchls constituting P, e.g., relative to each other and/or from a motion of their Mg-bound histidines, rather than directly from the positive charge. Such triggering mechanisms have already been postulated in hemoproteins [see, e.g., Friedmann et al. (1982)]. It may be further speculated that in state P^R this deformation of P does not relax, hence delaying protein relaxation until P^R returns to the ground state.

Possible Natures of the Proteic Events following Charge Separation in R 26 RCs. Hypothetical molecular origins can be proposed from the present RR results about the local events occurring around B_L after charge separation. Recently refined crystallographic data from *Rps. viridis* (Michel et al., 1986) have allowed precise identification of the different amino acids present in the local environments of the accessory BChls. Comparisons of the sequences of RC polypeptides of *Rps. viridis* with those of *Rhb. sphaeroides* or *Rhb. capsulatus* show that many of these amino acids are conserved for the three species (Michel et al., 1986). It appears from these results that the amino acids proximal to the keto carbonyl of the accessory BChl bound to the L branch (via His L-153) are a glycine (residue M-201) and a tyrosine (residue M-208). The latter residue appears not to be able to provide a H-bond to the keto carbonyl of the B_L molecule in resting reaction centers (J. Deisenhofer, personal communication). It is nevertheless worth noting that this tyrosine residue is replaced on the M branch of the RCs by a phenylalanine (residue L-181) and moreover that its side chain is located between the special pair and accessory $BChl_L$. It is thus quite conceivable that it might play a role after ionization of P. A motion of the peptide and/or of the B_L molecule might bring the tyrosine side chain into a geometry allowing its liganding to the keto carbonyl. An alternative hypothesis is that a water molecule could be bound to an amino acid side chain located near the B_L molecule (such as M-208 Tyr itself). This water molecule could then be brought into H-bonding position with the 9C=O group of B_L by a mechanism similar to that just described for

Tyr M-208. In possible relevance with these two hypotheses, Vermeglio and Paillotin (1982) concluded that the LD changes accompanying charge separation in *Rps. viridis* RCs could not be interpreted without a change in the orientation of the Q_Y electronic transitions of at least one accessory BChl.

The local effects that we have described here for *Rhb. sphaeroides* R 26 have also been observed in RCs from *Rhb. capsulatus* (B. Robert, Q. Zhou, and M. Lutz, unpublished results). In these reaction centers, tyrosine M-208 is conserved. It is worth noting that, for both strains studied, no other change in the local environment of any other pigment may be evidenced by RR spectroscopy after charge separation. In particular, electron transfer from the 545 nm absorbing Bpheo to Q_A does not induce any permanent or long-lived change in the local environment of any pigment detectable by RR spectroscopy. Indeed, variations observed in RR spectra are similar whether the primary donor is chemically or photochemically oxidized: when chemically oxidized by $Fe(CN)_6^{3-}$, the reaction centers are in a P^+IQ state, and when photochemically oxidized, they are in a P^+IQ^- state. The fact that RR differences between states PIQ and P^+IQ on one hand and between state PIQ and P^+IQ^- on the other are the same (compare curves 1 and 3 of Figure 4) demonstrates the above proposal.

Current time-resolved resonance Raman experiments on the nanosecond scale should permit determination of whether the presence of a negative charge on $Bpheo_L$ is also accompanied by detectable environmental changes around other pigments. This should permit one to draw a more complete picture of the proteic events accompanying the primary steps of bacterial photosynthesis.

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