Fourier Transform Infrared Spectroscopy and Electrochemistry of the Primary Electron Donor in *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* Reaction Centers: Vibrational Modes of the Pigments in Situ and Evidence for Protein and Water Modes Affected by P^+ Formation†

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Received November 13, 1992; Revised Manuscript Received January 21, 1993

ABSTRACT: Protein electrochemistry in an ultra-thin-layer electrochemical cell suitable for UV/vis and IR spectroscopy has been used to characterize the vibrational modes of the primary electron donors of *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* reaction centers in their neutral and cation radical states (P and P^+, respectively). The $P \rightarrow P^+$ redox transitions could be well separated from redox reactions of other cofactors according to their redox midpoint potential. The IR difference bands of the primary electron donor bacteriochlorophylls all titrate in unison and exhibit the correct midpoint potential. Comparison of the difference spectra with those of isolated bacteriochlorophylls a and b in organic solvents of different polarity and proton activity [Mäntele, W., Wollenweber, A. M., Nabedryk, E., & Breton, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8468–8472] leads to similar conclusions on the binding and interaction of the pigments within the protein matrix as previously obtained from light-induced P^+Q^-/PQ difference spectra. Equilibration of the reaction centers in D_2O leads to few but distinct shifts of bands and changes of band intensities at 1662, 1634, and 1526 cm^{-1} (*Rhodobacter sphaeroides*) and 1694, 1664, 1648, 1630, and 1532 cm^{-1} (*Rhodopseudomonas viridis*) as well as to smaller deviations at other wavenumbers. The H→D-sensitive band at 1662 cm^{-1} is interpreted in terms of a histidine NH_2^+ bending mode. A second H/D-sensitive difference band around 1648 cm^{-1} in the *Rhodopseudomonas viridis* reaction center may be associated with the peptide C=O of one of the amino acids surrounding P [eventually of the histidine(s) ligating the Mg] which is affected by P^+ formation. Small negative signals at 1526 cm^{-1} (*Rhodobacter sphaeroides*) and 1532 cm^{-1} (*Rhodopseudomonas viridis*) sensitive to H→D exchange are tentatively assigned to a tyrosine residue, presumably Tyr_{M210} (*Rhodobacter sphaeroides*) and Tyr_{M208} (*Rhodopseudomonas viridis*), and could correspond to a change of hydrogen bonding of the phenyl hydroxy group or to a change of its conformation upon primary charge separation. A broad background signal in the 1620–1670-cm^{-1} range for *Rhodobacter sphaeroides* reaction centers disappearing in D_2O is assigned to a H–O–H bending mode. Together with two broad band features at 1480–1400 and 1250–1150 cm^{-1}, which can be assigned to the H–O–D and D–O–D bending modes, respectively, it is interpreted in terms of water molecule(s) in the primary donor vicinity affected by primary electron donor oxidation.

In bacterial photosynthetic reaction centers (RC),[1] an electron is rapidly transferred from a primary electron donor, a bacteriochlorophyll (BChl) a or b dimer, to a quinone electron acceptor upon light absorption. The structures of this dimer and its protein binding site have been characterized by X-ray crystallographic studies of the *Rhodopseudomonas* (Rps.) *viridis* and the *Rhodobacter* (*Rb.*) *sphaeroides* RC (Michel et al., 1986; Allen et al., 1988; El-Kabbani et al., 1991), and several interactions between the cofactors and their host sites in the quiescent state of the RC have been inferred from these studies. However, X-ray crystallography has not yet been able to pick up the structural changes in the active RC, where a cation radical ($BChl_2^{\bullet+}$) and a semiquinone anion ($Q_A^{\bullet-}$ or $Q_B^{\bullet-}$) are formed. In contrast, infrared (IR) difference spectroscopy, which nonselectively probes vibrational modes from all RC constituents, can be efficiently used to probe the interaction of the primary donor BChls in the quiescent *and* the active state of the RC. The current state of IR spectroscopic investigations on the photosynthetic RC is summarized in a recent review (Mäntele, 1993).

In previous investigations, light-induced FTIR difference spectroscopy under steady-state illumination (Mäntele et al., 1985, 1988; Hayashi et al., 1986; Nabedryk et al., 1990; Buchanan et al., 1992) or time-resolved IR spectroscopy using flash illumination (Hienerwadel et al., 1992a) have been used to characterize the changes of vibrational modes in the RC upon charge separation. The difference spectra thus obtained are composed of contributions from pigment and quinone bonds, from their respective binding sites, and from effects of radical formation and charge separation on polypeptide backbond and amino acid side chain modes. The dominant contributions in the 1750–1620-cm^{-1} range of these spectra are those of the carbonyl C=O groups of the primary electron donor BChls. These modes can be closely approximated as group vibrations and have been used as probes for the solvation state of the BChls in the neutral and radical state (Mäntele et al., 1988). A considerable overlap of modes from the pigments, the quinone(s), and the protein especially in the

† W.M. was funded by the Deutsche Forschungsgemeinschaft through several grants and by a Heisenberg fellowship.

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[1] Abbreviations: P, primary electron donor; P_L and P_M, primary electron donor bacteriochlorophylls associated with the L and the M subunit of the reaction center, respectively; BChl, bacteriochlorophyll; RC, reaction center(s); Q_A and Q_B, primary and secondary quinone electron acceptor, respectively; SHE, standard hydrogen electrode.

0006-2960/93/0432-4532$04.00/0

conformationally sensitive 1600–1700-cm^{-1} range has prevented an assignment of modes specifically to the protein environment of the primary electron donor. However, from comparison of light-induced FTIR difference spectra (P^+Q^-/PQ) with appropriate ($BChl^+/BChl$) model compound spectra (Mäntele et al., 1988), it appeared that only few protein modes are associated with P^+ formation and that conformational changes, if present at all, are small and probably on the level of individual peptide bonds and side chain groups instead of affecting larger protein domains.

Using redox reactions in a spectroelectrochemical cell suitable for IR spectroscopy as demonstrated for cytochrome *c* (Moss et al., 1990), this overlap of contributions from different cofactors can be circumvented. Unlike light-induced FTIR difference spectroscopy, redox-induced FTIR difference spectroscopy can be used to study transitions of *single* redox cofactors in proteins. The difference spectra thus obtained are as sensitive as are light-induced FTIR difference spectra, but provide the additional possibility of potential titration of the bands, thus ensuring that a band is related to a specific redox transition. In previous work on the *Rb. sphaeroides* RC, we have reported electrochemical titrations of the near-infrared absorbance bands (Moss et al., 1991), and have used redox reactions to analyze the vibrational modes of the Q_A electron acceptor (Mäntele et al., 1990). Here, we report redox-induced FTIR difference spectra of the *Rb. sphaeroides* and the *Rps. viridis* RC, which offer the unique possibility to separate the P^+/P from the Q^-/Q difference spectra, and provide vibrational IR spectra of the pigments in situ and their protein host site.

MATERIALS AND METHODS

Sample Preparation. *Rb. sphaeroides* R26 RC were prepared essentially as described by Feher (1971) and *Rps. viridis* RC as described by Welte et al. (1983). For electrochemical measurements, RC were transferred to a 12.5 mM phosphate buffer, pH 7.4, containing 0.1% octyl glucoside, 62.5 mM KCl, and 0.25 mM ferrocyanide for *Rb. sphaeroides* or to a 50 mM phosphate buffer, pH 7.4, containing 0.3% octyl glucoside, 250 mM KCl, and 1 mM ferrocyanide for *Rps. viridis*. RC were concentrated to approximately 0.3–1 mM using ultrafiltration cells (Centricon 30 from Amicon, Witten).

Electrochemistry. The ultra-thin-layer electrochemical cell for UV/vis/IR spectroscopy has been described in detail (Moss et al., 1990; Baymann et al., 1991). Briefly, it consists of a transparent gold minigrid working electrode (obtained from Buckbee-Mears Co., St. Paul, MN) between two CaF_2 windows, a Pt counter electrode, and an Ag/AgCl/3 M KCl reference electrode. All potentials are quoted vs this reference; for values vs SHE, add +208 mV. The RC protein sample (ca. 5 μL of a 0.3–1 mM solution) forms a layer on the gold minigrid at a path length of ca. 10 μm, thus giving access to the full spectral range from 190 nm to 10 μm even in spectral regions of strong water absorbance. Constant potential electrolysis, chronoamperometry, coulometry, and cyclic voltammetry were performed with a potentiostat of local design (Sevenich-Gimbel Model II) interfaced to a computer. Quoted potentials are accurate within ca. 5 mV.

Spectroscopy. FTIR spectra were recorded on a modified Bruker FTIR spectrophotometer equipped with an MCT detector of selected sensitivity. Absorbance spectra of the RC sample in the vis/NIR spectral region were recorded simultaneously with a beam set up coaxially with the IR beam. FTIR spectra, vis/NIR spectra, and the potentiostat were controlled through interfaces from a data acquisition and treatment program (MSPEK) developed by D. A. Moss and S. Grzybek in our laboratory. All measurements were performed with the electrochemical cell thermostated at 7 °C.

RESULTS

Rhodobacter sphaeroides Reaction Centers. A *Rb. sphaeroides* RC sample equilibrated at 0 V (all potentials are quoted vs Ag/AgCl, add +208 mV for potentials vs SHE) exhibits the well-known vis/NIR absorbance spectrum (Figure 1 inset, solid line). When the potential is switched to +0.4 V at $t = 0$, an oxidative current is observed which decays to zero within ca. 20 s to 1 min (data not shown). The new equilibrium is that of a RC with P fully oxidized (Figure 1 inset, dashed line). Intermediate potentials lead to titration of the primary donor band [cf. Moss et al. (1991)]; these titrations can be repeated in many cycles. The difference spectrum (+0.4 minus 0 V) of a *Rb. sphaeroides* RC in the 700–1000-nm region [data not shown, cf. Mäntele et al. (1990)] is identical to that obtained by chemical titration (Reed & Ke, 1973).

The FTIR difference spectrum (+0.4 minus 0 V) in the 1800–1000-cm^{-1} region recorded under the same conditions is shown in Figure 1. Highly detailed band structures are obtained, with amplitudes of at most 5×10^{-3} of the sample amide I absorbance. Prominent bands are observed throughout the spectrum, with half-widths much smaller than those of the main protein absorbance bands (data not shown). The sensitivity achieved in these difference spectra corresponds to those of light-induced FTIR difference spectra. We note a strong similarity with previously published light-induced FTIR difference spectra of charge separation (P^+Q^-/PQ) (Mäntele et al., 1985, 1988; Hayashi et al., 1986; Nabedryk et al., 1990). However, we would like to emphasize that the electrochemically-generated FTIR difference spectra exclusively represent the changes of vibrational modes upon the $P \rightarrow P^+$ transition, without any contributions from redox transitions of other cofactors. In fact, application of intermediate potentials yields a titration of the IR difference bands (data not shown). All IR bands were found to titrate in unison and to exhibit the correct midpoint potential.

The spectral region between 1600 and 1750 cm^{-1} is dominated by a strong differential band at 1682 cm^{-1}(–)/1702 cm^{-1}(+) with shoulder structures on the high-wavenumber side. At higher frequencies, a positive band at 1748 cm^{-1} without a strong negative counterpart (which would be characteristic for a differential band caused by a band shift) is observed. Three further negative bands in the region of the protein amide I absorption at 1662, 1648, and 1634 cm^{-1} (with a shoulder at 1622 cm^{-1}) are observed. In the 1600–1300-cm^{-1} region, two strong positive features with peaks at 1566, 1546, 1474, and 1456 cm^{-1} are detected, with three smaller negative peaks (the highest frequency one at 1526 cm^{-1}) in between.

Equilibration of the RC in D_2O induces a few but pronounced changes of band intensities and small shifts of band positions. In order to correct for the different sample concentrations of the H_2O and D_2O samples, both spectra were normalized to the same concentration. We would like to emphasize that a *single* normalization factor was sufficient to match both samples in the whole IR range and in the NIR. At frequencies above ca. 1680 cm^{-1}, the D_2O difference spectrum nearly perfectly matches that in H_2O. Below 1680 cm^{-1}, the difference spectrum in D_2O shows the negative band

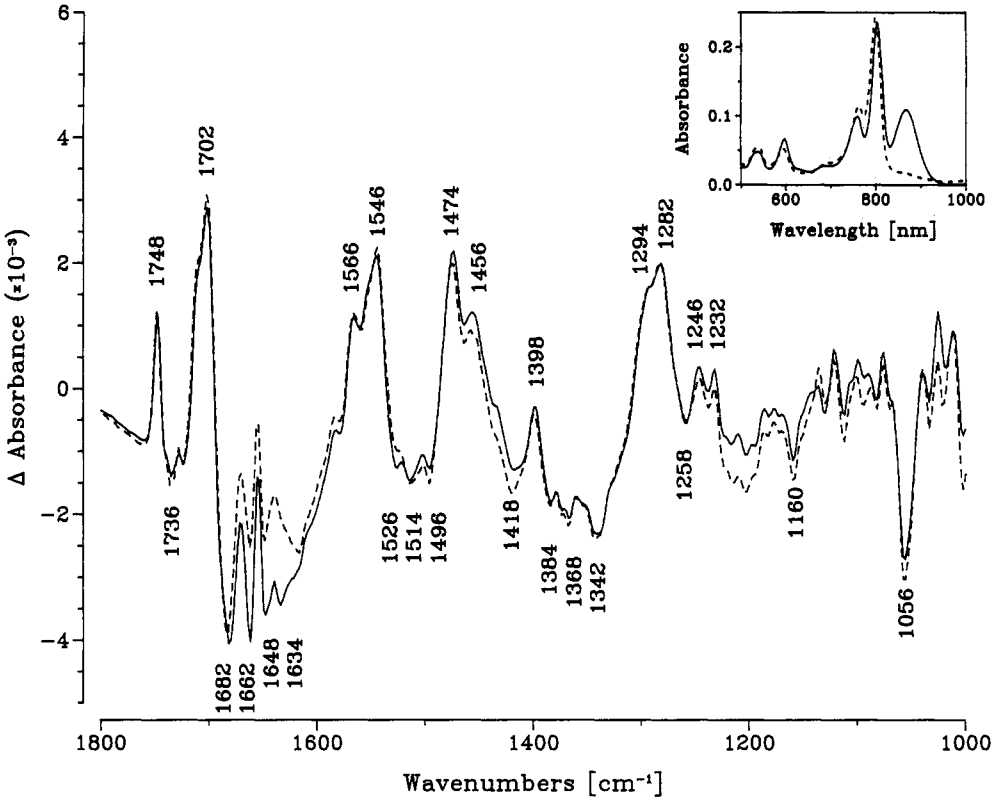

FIGURE 1: Electrochemically-induced FTIR difference spectra (+0.4 minus 0 V) of *Rb. sphaeroides* R26 reaction centers. Solid line: RC sample in H_2O. Dashed line: RC equilibrated in D_2O. Inset: vis/NIR absorbance spectra of the same sample recorded at 0 V (solid line) and at +0.4 V (dashed line). For the composition of the sample and the conditions of the measurement, see the text.

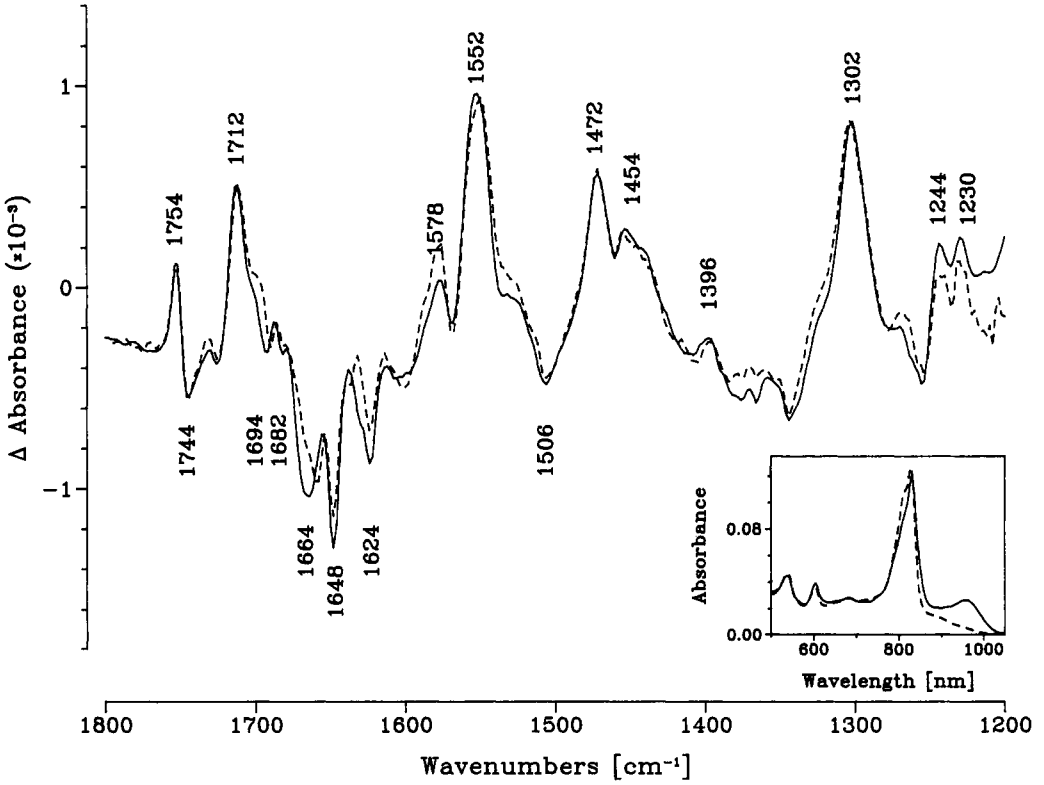

FIGURE 2: Electrochemically-induced FTIR difference spectra (+0.5 minus +0.24 V) of *Rps. viridis* reaction centers. Solid line: RC sample in H_2O. Dashed line: RC equilibrated in D_2O. Inset: vis/NIR absorbance spectra of the same sample recorded at +0.24 V (solid line) and at +0.5 V (dashed line). For the composition of the sample and the conditions of the measurement, see the text.

at 1662 at slightly reduced intensity and the 1648-cm^{-1} band almost unchanged as compared to the H_2O spectrum. The broad absorbance decrease between ca. 1670 and ca. 1610 cm^{-1} seen in the H_2O spectrum, however, is lacking in D_2O. Furthermore, the band structures between ca. 1640 and 1620 cm^{-1} are changed, indicating a new negative band in D_2O at around 1616 cm^{-1}. Another effect of D_2O is seen at 1526 cm^{-1}, where a pronounced band in H_2O becomes a shoulder

in D_2O. Further effects of deuteration are observed between 1460 and 1420 cm^{-1} and between 1220 and 1130 cm^{-1}.

Rhodopseudomonas viridis Reaction Centers. *Rps. viridis* RC were equilibrated at +0.5 V in the electrochemical cell to record P^+ spectra. In order to generate the neutral-state P without any interference from cytochrome reduction, the potential was set to +0.24 V [cf. Fritz et al. (1992)]. Figure 2 shows the P^+/P FTIR difference spectra in H_2O (solid line) and D_2O (dashed line). The inset shows the vis/NIR absorbance spectra obtained under the same conditions. We note that no redox change from the cytochrome subunit is evident from these spectra.

As in the case of the *Rb. sphaeroides* RC, the spectra of the $P \rightarrow P^+$ transition show a strong similarity with the light-induced P^+Q^-/PQ difference spectra previously obtained (Mäntele et al., 1985) and tabulated (Mäntele et al., 1988). The highest frequency band is a differential feature at 1744 $cm^{-1}(-)/1754\ cm^{-1}(+)$. At 1712 cm^{-1}, a strong positive band appears, followed by negative signals at 1694, 1664, 1648, 1624, and 1602 cm^{-1}. In the region from 1600 to 1200 cm^{-1}, mostly positive signals, notably at 1552, 1472, and 1302 cm^{-1}, are detected. In the region around 1480–1540 cm^{-1}, where three smaller negative signals had been detected in the *Rb. sphaeroides* RC, only one negative signal is evident at 1506 cm^{-1}.

As in the case of *Rb. sphaeroides* RC, deuteration induces only a few but pronounced shifts and changes of band intensities. The negative 1694- and 1664-cm^{-1} bands are shifted to lower wavenumbers by 4 cm^{-1}. In addition, the intensity of the 1648-cm^{-1} band is reduced. We note, however, that, unlike in the *Rb. sphaeroides* RC, deuteration leaves the background signal in the region of water H–O–H absorbance almost unchanged. Further changes of the band pattern are observed around 1260 cm^{-1}.

DISCUSSION

The formation of the π cation radical of BChl a or b results in significant changes of its vibrational spectrum (Mäntele et al., 1988), a consequence of removal of the electron from the conjugated system, redistribution of the charges, and the resulting changes of bond order. In addition, bonds not involved in conjugation as well as hydrogen bonds to the pigment carbonyls are affected (Mäntele et al., 1988). In previously obtained light-induced FTIR difference spectra of *Rb. sphaeroides* and *Rps. viridis* RC, we have attributed the difference features in the 1750-1620-cm^{-1} region to the changes in the vibrational spectrum of the primary electron donor and its environment upon charge separation (Mäntele et al., 1988). Although this attribution neglected possible contributions from the quione(s) in this range, the data presented here confirm that it was a reasonable approximation. Furthermore, "pure" Q_A^-/Q_A or Q_B^-/Q_B difference spectra obtained recently by electrochemical techniques (Mäntele et al., 1990; Bauscher 1991; Bauscher et al., 1992) or under illumination in the presence of reductants (Breton et al., 1991a,b) indicated that the contributions of the quinones to the light-induced FTIR difference spectra are considerably weaker than those of the primary donor BChls. Nevertheless, Q_A^-/Q_A or Q_B^-/Q_B difference spectra show difference bands at above 1700 cm^{-1}, arising from Asp and Glu side chain residues (Hienerwadel et al., 1992b,c), which complicate an assignment of pigment modes in light-induced FTIR difference spectra. We emphasize again that the difference spectra presented *here* represent exclusively the modes of the primary electron donor and those of its environment affected by P^+ formation.

In line with the vibrational spectra of isolated BChl a and its π cation (Mäntele et al., 1988; Leonhard et al., 1989; Leonhard, 1992), we identify the positive bands at 1748 cm^{-1} (*Rb. sphaeroides*) and 1754 cm^{-1} (*Rps. viridis*) with the 10a-ester C=O of the BChl a (BChl b) molecule(s) in the cation radical state of the primary electron donor. In the neutral state of P (negative bands in the difference spectrum), only the *Rps. viridis* RC spectra show a counterpart at 1744 cm^{-1}, whereas the P^+/P spectra of the *Rb. sphaeroides* RC only exhibit a local minimum. In agreement with the assignment from light-induced difference spectra, we thus conclude that for *Rb. sphaeroides* RC this 10a-ester C=O mode arises from a H-bonded C=O group in the P state, and from a nonsolvated 10a-ester C=O group in P^+. For *Rps. viridis* RC, we conclude that the 10a-ester C=O group is nonsolvated in both P^+ and P.

The 7c-ester C=O group of BChl a or b absorbs at ca. 1735 cm^{-1} in tetrahydrofuran (Leonhard et al., 1989) and could principally contribute with a difference signal in this spectral region. Leonhard et al. (1989) have compared electrochemically-generated cation-minus-neutral IR difference spectra of BChl a and its derivative pyro-BChl a. Pyro-BChl a lacks the 10a-ester C=O group and exhibits no differential signal in the 1730–1760-cm^{-1} region. We thus conclude that the signals observed in the 1730–1760-cm^{-1} range in Figures 1 and 2 exclusively arise from the 10a-ester C=O group.

The second band in the P^+/P difference spectra at 1702 cm^{-1} (*Rb. sphaeroides*) and 1712 cm^{-1} (*Rps. viridis*) can be identified with the 9-keto C=O group(s) of the primary electron donor in the P^+ state, shifted from 1682 cm^{-1} (*Rb. sphaeroides*) in the P state. This assignment was also proposed on the basis of polarized-light measurements on oriented films of *Rb. sphaeroides* RC (Thibodeau et al., 1991). In the case of the *Rps. viridis* RC, the assignment of the 9-keto C=O in the P state is less clear, since the negative 1694-cm^{-1} band and the negative 1664-cm^{-1} band both are in the appropriate frequency range. Only the 1664-cm^{-1} band, however, shows an amplitude comparable to the positive 1712-cm^{-1} band. The X-ray data of the *Rps. viridis* RC suggest a H bond from Thr_{L248} to the 9-keto C=O group of P_L; the 9-keto C=O of P_M should not be H-bonded (Michel & Deisenhofer, 1988). According to EPR and ENDOR data and to molecular orbital calculations, the positive charge of the oxidized electron donor appears to be localized predominantly on the P_L BChl (Davis et al., 1979; Lendzian et al., 1988; Plato et al., 1986). We would thus expect this P_L BChl to identify itself in the IR difference spectrum. This leads us to assign the negative 1664-cm^{-1} band and the positive 1712-cm^{-1} band to the bond order changes of the solvated 9-keto C=O of P_L in the *Rps. viridis* RC.

In the *Rps. viridis* RC, the obvious downshift of the negative 1664-cm^{-1} band (as well as of the 1694-cm^{-1} band, which was a less likely candidate for the 9-keto C=O group as judged from its smaller oscillator strength) by 4 cm^{-1} upon deuteration might question this assignment: since no exchangeable protons are present, the 9-keto C=O group should experience no H–D shift. One might argue, however, that the 9-keto C=O bond, which is involved in strong H-bonding in *Rps. viridis* RC, is downshifted as a consequence of the H–D exchange. Another possibility would be that the negative 1664-cm^{-1} band is composed of a 9-keto C=O mode and a protein signal, the latter being shifted upon deuteration.

The 2a-acetyl C=O group should give rise to a difference band between ca. 1660 and 1620 cm^{-1}. The bands at 1648 and 1634 cm^{-1} as well as a shoulder at 1622 cm^{-1} are

appropriate candidates in the P^+/P difference spectrum of *Rb. sphaeroides* RC, and those at 1648 and 1624 cm^{-1}, including a shoulder at 1630 cm^{-1}, for the *Rps. viridis* RC. In the case of *Rb. sphaeroides* RC, X-ray data suggest H-bonding of the 2a-acetyl C=O group of P_L to His_{L168}; that of P_M does not appear to be solvated (El-Kabbani et al., 1991). We thus tentatively assign the negative band at 1634 cm^{-1} to the solvated 2a-acetyl C=O group of P_L, and assume that part of the 1648-cm^{-1} band arises from the free 2a-acetyl C=O group of P_M. For the 2a-acetyl C=O groups of *Rps. viridis*, the X-ray data suggesting H-bonding to His_{L168} and Tyr_{M195} lead us to the attribution of the negative band at 1624 cm^{-1} and its shoulder at 1630 cm^{-1} to the 2a-acetyl C=O groups.

The H-bonding of the pigment carbonyls in the quiescent state of the RC as deduced from the position of the IR signals should be taken as a measure of pigment solvation independent of the X-ray data. At present, there is no conflict with the different sets of X-ray data available. Different bonding properties, however, have been proposed for the 7c-ester C=O of the P_L BChl in *Rb. sphaeroides* RC. A H bond to Tyr_{M208} has been proposed by Yeates et al. (1988), which could not be observed by El-Kabbani et al. (1991). Furthermore, no H bonds were observed for the 2a-acetyl C=O groups of P by Yeates et al. (1988), while a H bond from His_{L168} to the 2a-acetyl C=O group of P_L was proposed by El-Kabbani et al. (1991). Further high-resolution structures of the *Rb. sphaeroides* Y strain RC (Reiss-Husson, personal communication) and *Rb. sphaeroides* R26 RC (Michel, personal communication) are about to be completed and will probably help to clarify the questions on pigment carbonyl–protein interactions.

In the spectral region below 1600 cm^{-1}, monomeric BChl a or b in organic solvents investigated thus far represents insufficient models, since the modes in this range (mostly modes of the macrocycle and coordination-state markers) should be strongly affected by dimer formation. Although it appears possible to use these modes to monitor the properties of P or P^+, we refrain from this attempt until further model compound spectra (for example from covalently-bound dimers) have been investigated. We shall refer to this spectral range only inasfar as deuteration effects are concerned.

Effects of H→D Substitution and Nonpigment Vibrational Modes. Genuine P or P^+ modes should show the same band features both in H_2O and in D_2O, since no exchangeable protons are present. However, P^+/P difference spectra in D_2O show significant deviations. For the difference bands assigned to the 10a-ester C=O, and even down to 1700 cm^{-1}, the difference spectra in H_2O and in D_2O both for *Rb. sphaeroides* and for *Rps. viridis* show remarkable agreement. We take this as further confirmation for the above assignment.

In the *Rps. viridis* difference spectrum, a clear shift of the negative bands from 1694 to 1690 cm^{-1} and from 1664 to 1660 cm^{-1} has been discussed above in connection with the assignment to the 9-keto C=O in P. For the 1664-cm^{-1} band, the possibility of an isotope shift might be explained by strong H-bonding. In the case of *Rb. sphaeroides* RC, the negative 1682-cm^{-1} difference band assigned to the free 9-keto C=O group(s) of P is unshifted and at almost identical intensity in H_2O and in D_2O. This absence of a shift might be associated with the absence of H-bonding.

A broad negative signal in the range from ca. 1680 to almost 1600 cm^{-1} in the *Rb. sphaeroides* spectrum (Figure 1) appears removed in D_2O, affecting the intensities of all signals in this spectral region. We have found this broad signal (and its disappearance in D_2O) reproducibly, but only for *Rb. sphaeroides* RC, hand in hand with appearing broad negative signals at 1250–1150 and 1480–1400 cm^{-1} in D_2O. These frequencies are well within the range of the H–O–H bending (around 1650 cm^{-1}), D–O–D bending (around 1220 cm^{-1}), and H–O–D bending (around 1455 cm^{-1}) vibrations, respectively, of water molecules (Zundel, 1969). We are thus tempted to attribute the broad signal between 1680 and 1600 cm^{-1} to the bending mode of one (or more) water molecule(s) in the environment of P. The X-ray coordinates (Michel & Deisenhofer, 1988; Yeates et al., 1988) suggest the presence of water molecules in the RC, a large number of them at fixed positions, and possibly even between the primary donor BChls. At present, however, these locations seem to be less well-defined for the *Rb. sphaeroides* RC, so that we cannot rely on a sufficiently accurate map to attempt a more previse assignment.

The lack of the broad negative feature in the *Rps. viridis* RC (or at least the fact that a broad feature does not disappear upon deuteration as in *Rb. sphaeroides* RC) is puzzling, since water molecules were thought to be present near or between the primary donor BChl b molecules. It could be possible, however, that the $H_2O \rightarrow D_2O$ exchange is considerably slower or even impossible for this "hard core" of the *Rps. viridis* RC, thus leading to unchanged spectral features in this range, even when extended exchange was performed over the period of days. El-Kabbani et. al. (1991) have suggested a more dense packing of the pigments and the protein matrix in the case of the *Rps. viridis* RC, which might explain the differences reported here.

In *Rb. sphaeroides* RC, deuteration strongly reduces the intensity of the 1662-cm^{-1} band. The negative 1634-cm^{-1} band disappears, and the intensity of the negative 1526-cm^{-1} band is reduced. In *Rps. viridis* RC, deuteration shifts the position of the negative 1694-cm^{-1} band and of the 1664-cm^{-1} band by 4 cm^{-1} to lower wavenumbers and reduces the intensity of the negative 1648-cm^{-1} band and of the 1630-cm^{-1} shoulder.

At 1662 cm^{-1} (*Rb. sphaeroides*) and 1664 cm^{-1} (*Rps. viridis*), contributions from the NH_2^+ deformation mode of histidine(s)—presumably the ligand(s) of the central Mg—appear probable. This mode should shift to approximately 1620 cm^{-1} upon deuteration, and could account for the changes in the difference spectra in that wavenumber range upon deuteration. In fact, double difference spectra [$P^+/P(H_2O)$ minus $P^+/P(D_2O)$] (data not shown) suggest a band at 1616 cm^{-1} in D_2O, which is not present in H_2O. As for the negative 1648-cm^{-1} bands for both *Rb. sphaeroides* and *Rps. viridis* RC, attribution to the peptide C=O stretching mode of the histidine appears possible. In this case, we only would expect shifts of 1–6 cm^{-1} to lower frequencies, and effects on the oscillator strength upon H–D substitution.

The question arises whether changes of protonation of carboxylic groups from Asp or Glu side chains in the RC are associated with the $P \rightarrow P^+$ transition. Q_A^-/Q_A and Q_B^-/Q_B difference spectra (Mäntele et al., 1990; Breton et al., 1991a,b, Bauscher 1991, 1992) clearly exhibit such signals in the spectral range from 1700 to 1750 cm^{-1}. A preliminary assignment of one band at 1728 cm^{-1} to Glu_{L212} was given by Hienerwadel et al. (1992c) on the basis of a Glu→Gln mutation in *Rb. spaeroides* RC. Since the P^+/P spectra exhibit strong signals from the ester and keto C=O groups in the 1750–1700-cm^{-1} range, an answer to the question of Asp or Glu protonation cannot be given simply by the presence of signals in that range, but has to rely on isotope shifts upon H→D exchange. In the spectral range considered, deviations

of the P^+/P difference spectra in D_2O are extremely small, and only discernible (if at all) around 1736 cm^{-1}, both in *Rb. sphaeroides* and in *Rps. viridis* RC. With the given signal-to-noise ratio, however, we cannot confidently attribute them to protolytic reactions. At frequencies somewhat lower than those expected for Asp or Glu side chain C=O modes, isotope shifts upon H→D exchange become stronger. For example, the 1694-cm^{-1} band which shifts to 1690 cm^{-1} in D_2O in the *Rps. viridis* P^+/P difference spectra might be a candidate for a protolytic reaction.

The side chain of Tyr_{M208} of *Rb. sphaeroides* seems to be within van der Waals distance of the macrocycles of P_L, the monomeric BChl, and the bacteriopheophytin on the L side (El-Kabbani et al., 1991). However, El-Kabbani et al. (1991) could not confirm a H bond from this tyrosine to the 7c-ester C=O group of P_L as suggested by Yeates et al. (1988), nor to the monomeric BChl on the L side. Robert and Lutz (1988) have discussed the possibility that Tyr_{M208} is affected upon the oxidation of P and might then form a H bond to the 9-keto C=O group of the monomeric BChl on the L side. No data from X-ray crystallography are available for such a "switching". However, a Tyr signal in the NMR spectra of RC enriched with [^{13}C]Tyr was found to change upon light-induced P^+Q^- formation, thus suggesting that charge separation may have an impact on a tyrosine (J. Lugtenburg, personal communication).

The C=C ring vibration of tyrosine absorbs in the 1510–1520-cm^{-1} range (Venyaminov & Kalnin, 1990). In redox-induced FTIR difference spectra of cytochrome *c*, a differential signal centered at ca. 1515 cm^{-1} was attributed to a tyrosine changing its H-bonding upon the redox transition (Moss et al., 1990; Schlereth & Mäntele, 1992). Two signals observed in P^+/P difference specra at 1526 cm^{-1} (*Rb. sphaeroides* RC) and at 1532 cm^{-1} (*Rps. viridis* RC) are affected by H→D exchange, as would be expected for a tyrosine changing its H-bonding character as a consequence of P^+ formation. We thus tentatively attribute these signals to Tyr_{M210} or Tyr_{M208}, respectively. However, a final assignment will have to await P^+/P difference spectra of reaction centers with isotopically labeled tyrosines.

CONCLUSIONS

The electrochemically induced P^+/P FTIR difference spectra of the *Rb. sphaeroides* and the *Rps. viridis RC* represent the first vibrational IR spectra of the primary electron donor in situ and its protein environment. The majority of the bands in the seemingly complex spectra can be explained by the change of charge density and of solvation for the primary donor pigment C=O groups upon cation radical formation, thus implying a very small involvement of the protein. The presence of only a few and distinct H→D isotope shifts supports this view. At present, we only can provide evidence for changes of the vibrational intensity of a peptide C=O and a NH_2^+ signal from a histidine, and of a tyrosine ring C=C mode. A final assignment of these signals will probably await data from RC with isotopically labeled amino acids. As for the involvement of H_2O molecule(s) inferred from the broad background signal, a more accurate map will be needed to locate it precisely within the primary donor site.

The FTIR spectra presented here suggest a rather rigid protein environment for the primary electron donor, allowing only small rearrangements and adjustments for the radical ion state. In contrast to this, the impact of Q_A and Q_B reduction on the respective protein environment appears to be considerably stronger; in this case, the FTIR difference spectra (Mäntele et al., 1990; Bauscher, 1991; Breton et al., 1991a,b; Bauscher & Mäntele, 1992) reveal several strong signals in the peptide C=O region and in the region of aromatic amino acid side chains. On the other hand, the nearly perfect matching of the P^+/P difference spectra in H_2O and D_2O in the 1700–1750-cm^{-1} region makes significant changes of protonation of Asp and Glu side chain groups, as observed with Q_A and Q_B reduction, rather unlikely.

ACKNOWLEDGMENT

We thank Drs. D. A. Moss, J. Breton, and E. Nabedryk for stimulating discussions and Dr. M. Bauscher, F. Fritz, and S. Grzybek for their intense collaboration in this project.

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