Electrostatic Influence of Q_A Reduction on the IR Vibrational Mode of the 10a-Ester C═O of H_A Demonstrated by Mutations at Residues Glu L104 and Trp L100 in Reaction Centers from *Rhodobacter sphaeroides*†

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ABSTRACT: The light-induced Q_A^-/Q_A FTIR difference spectrum of the photoreduction of the primary quinone (Q_A) in reaction centers (RCs) from *Rhodobacter sphaeroides* exhibits a set of complex differential bands between 1750 and 1715 cm^{-1}. Several of these features correspond in frequency to bands that bleach in the H_A^-/H_A FTIR difference spectra of the photoreduction of the bacteriopheophytin electron acceptor (H_A). Since the 10a-ester C═O from H_A and the side chains of protonated carboxylic acids would be expected to contribute in this spectral region, mutations were designed at Trp L100 and Glu L104, which have been proposed to form hydrogen bonds to the 10a-ester and the 9-keto carbonyls on ring V of H_A, respectively. The Q_A^-/Q_A spectra measured in ^1H_2O and ^2H_2O of RCs from wild type (WT) were compared to those of RCs with the mutation Trp to Phe at L100 [WF(L100)], Glu to Leu at L104 [EL(L104)], or both mutations [EL(L104)/WF(L100)]. The spectra of the mutants in the 1800−1400 cm^{-1} frequency range exhibit only limited perturbations compared to those of WT, indicating the absence of significant structural changes due to the mutations. Part of a differential signal centered around 1732 cm^{-1} in the spectrum of WT RCs is downshifted by ≈7 cm^{-1} in EL(L104), while it is upshifted by ≈11 cm^{-1} in WF(L100). This upshift of the differential signal is assigned to the frequency change of the 10a-ester C═O of H_A induced by the rupture of the hydrogen bond with Trp L100. The ^1H_2O-minus-^2H_2O double-difference spectrum of WT RCs exhibits a characteristic differential signal positive at 1730 cm^{-1} and negative at 1724 cm^{-1} that is absent in the corresponding spectra of EL(L104) and of the double mutant, implicating Glu L104 in the Q_A^-/Q_A spectral changes. This differential signal is strongly modified in frequency and amplitude in the ^1H_2O-minus-^2H_2O spectrum of WF(L100), indicating that it does not correspond to a direct response of the C═O mode of the Glu L104 side chain upon Q_A reduction. Instead, perturbation of the hydrogen bond of the 9-keto C═O with Glu L104 is proposed to induce a change of electron density on ring V of H_A, thereby altering the frequency of the 10a-ester C═O that is in partial conjugation with ring V. The loss of the hydrogen bond to the 9-keto C═O of H_A due to the Glu L104 to Leu mutation or the alteration of the strength of the hydrogen bond by $^1H/^2H$ exchange on Glu L104 appears to produce such effects. Thus, the Q_A^-/Q_A spectra above 1700 cm^{-1} are dominated by contributions from the 10a-ester C═O of H_A, with most of the differential signals assigned to a small frequency downshift of the 10a-ester C═O of H_A in response to Q_A reduction. The complexity of the signals implies a structural heterogeneity of the conformation and hydrogen bonding of the 10a-ester C═O of H_A, which may be related to the functional heterogeneity observed in electron transfer kinetics. The present FTIR results show that the reduction of Q_A can induce a pronounced electrostatic effect on molecular vibrations of chemical groups located about 10 Å away from Q_A. They also demonstrate that, within experimental limits, the proton uptake observed at pH 7 upon Q_A photoreduction [McPherson, P. H., Okamura, M. Y., & Feher, G. (1988) *Biochim. Biophys. Acta 934*, 348−368] involves none of the exchangeable carboxylic groups of the RC.

In the photosynthetic bacterial reaction center (RC),[1] light induces the rapid transfer of an electron from a bacteriochlorophyll dimer to a molecule of bacteriopheophytin (H_A) and then to the primary quinone acceptor (Q_A). The precise role of the protein in assisting the electron transport and in preventing wasteful back reactions is still poorly understood, although the interactions evident in the crystal structure of the RC (Michel et al., 1986; Deisenhofer & Michel, 1989; Lancaster et al., 1995; Allen et al., 1988; Yeates et al., 1988; Feher et al., 1989; El-Kabbani et al., 1991; Ermler et al., 1994; Arnoux & Reiss-Husson, 1996) are probably important both for the geometrical organization of the cofactors participating in the electron transfer pathway and for fine-tuning the energy levels of the cofactors. Nuclear rearrangements of the RC protein and cofactors are expected to provide

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[1] Abbreviations: RC, reaction center; H_A, bacteriopheophytin electron acceptor; Q_A and Q_B, primary and secondary quinone acceptor, respectively; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*; FTIR, Fourier transform infrared; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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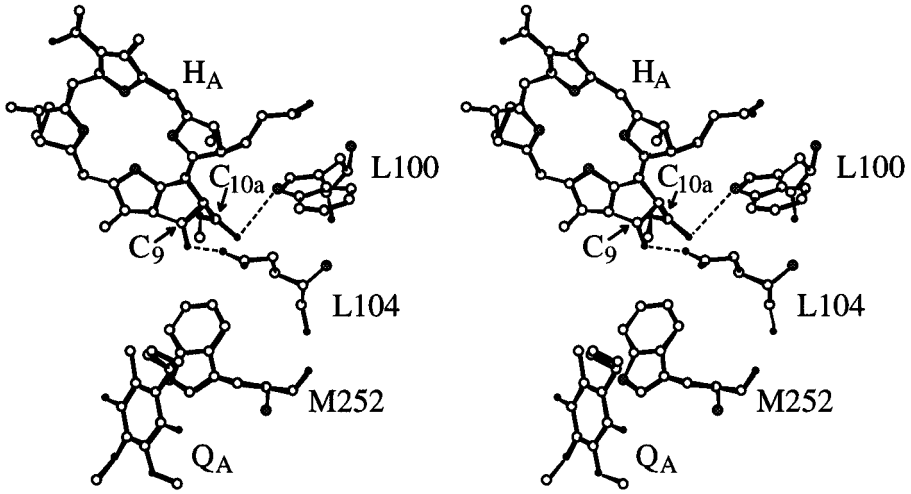

FIGURE 1: Stereoviews of the environment of ring V of the bacteriopheophytin electron acceptor H_A in the photosynthetic reaction center of *Rb. sphaeroides*, depicting hydrogen bonds between the 9-keto and 10a-ester C═O of H_A and the residues Glu L104 and Trp L100, respectively. Also shown are the primary quinone acceptor Q_A and the residue Trp M252. The structure is oriented with the C_2 symmetry axis approximately vertical. The phytyl and isoprenoid chains of H_A and Q_A have been truncated for clarity. Coordinates shown are from Brookhaven National Laboratory file 4RCR.

specific contributions to the reorganization energy, which is associated with long-range electron transfer. These structural changes have yet to be identified and could be either localized in the close vicinity of the cofactors involved in the electron transfer reaction or distributed at more distant sites, depending upon the strength of the electrostatic interactions.

Light-induced FTIR difference spectroscopy affords the sensitivity for investigating changes of the individual atomic bonds that are altered by the charge separation process. While this technique in itself is rather unspecific, since it provides information concerning all bonds of both the cofactors and protein that are affected by the photoreaction, selectivity can be achieved in a few favorable situations such as the photoreduction of the primary and secondary quinones in *Rhodobacter sphaeroides*. Notably, the Q^-/Q FTIR difference spectra of quinone-depleted RCs reconstituted with isotopically labeled ubiquinones have allowed the quinone modes perturbed by the photoreaction to be discriminated from those of the RC protein for both Q_A and the secondary quinone acceptor Q_B (Breton et al., 1994a−c, 1995, 1996a; Breton & Nabedryk, 1996; Brudler et al., 1994, 1995, 1996). The challenge then remains to assign the vibrations observed in these spectra that are not due to quinones. One way to probe possible contributions from specific amino acid residues or other cofactors is to perturb particular protein−cofactor interactions that may influence the vibrational spectra by site-directed mutagenesis. The IR spectral region extending between 1770 and 1700 cm^{-1} is very amenable to such a study as the C═O modes of the protonated carboxylic amino acids and of the ester groups from the pigments and the lipids are the only potential contributors in this region. Furthermore, $^1H/^2H$ exchange induces a frequency downshift of the C═O mode of an accessible protonated carboxylic group while leaving the ester groups unaffected. For example, the combination of IR difference spectroscopy, site-directed mutagenesis, and $^1H/^2H$ exchange has allowed the protonation state of several carboxylic residues involved in the Q_B to Q_B^- transition in RCs of *Rb. sphaeroides* to be determined (Hienerwadel et al., 1992, 1995; Nabedryk et al., 1993, 1995a,b).

Well-defined signals are observed in the region between 1750 and 1715 cm^{-1} of the Q_A^-/Q_A FTIR difference spectra of *Rb. sphaeroides* (Mäntele et al., 1990; Breton et al., 1991a) and *Rhodopseudomonas viridis* (Breton et al., 1991b), and parts of these signals are affected by $^1H/^2H$ exchange (Breton & Nabedryk, 1995). Such signals are also present in the Q_A^-/Q_A spectra of several other species of photosynthetic bacteria such as *Rhodospirillum rubrum*, *Chromatium vinosum*, and *Chloroflexus aurantiacus* (Breton et al., 1991a,b) as well as of the photosystem II of plants (Berthomieu et al., 1990, 1992). The possibility of correlating the observed Q_A^-/Q_A IR signals in this region with changes of the protonation state of carboxylic residues is suggested by reports of proton uptake upon Q_A^- formation in isolated RCs of *Rb. sphaeroides* and *Rb. capsulatus* (McPherson et al., 1988; Maroti & Wraight, 1988; Maroti et al., 1995; Sebban et al., 1995). One of the closest carboxylic residues to Q_A is Glu L104 (Figure 1). This residue is also within hydrogen-bonding distance of the 9-keto group on ring V of H_A. The existence of a hydrogen bond between Glu L104 and the 9-keto C═O of H_A was first proposed on the basis of X-ray crystallography of RCs (Michel et al., 1986; Deisenhofer & Michel, 1989; Lancaster et al., 1995; Yeates et al., 1988; Feher et al., 1989; El-Kabbani et al., 1991; Ermler et al., 1994; Arnoux & Reiss-Husson, 1996) and is also fully consistent with a variety of spectroscopic data such as low-temperature absorption (Bylina et al., 1988), linear dichroism (Breton et al., 1989), resonance Raman (Lutz & Robert, 1985; Robert & Lutz, 1988; Palaniappan et al., 1993; Palaniappan & Bocian, 1995), and H_A^-/H_A FTIR difference spectra (Mäntele et al., 1988; Nabedryk et al., 1986, 1988, 1995c) and ENDOR of H_A^- (Feher et al., 1988; Lubitz, 1991; Lubitz et al., 1995). Furthermore, ENDOR experiments have shown that the proton of the COOH group of Glu L104 exchanges in ^2H_2O (Feher et al., 1988; Lubitz et al., 1995).

Several observations open the possibility of an electrostatic contribution in the Q_A^-/Q_A FTIR spectra from the 10a-ester C═O of the electron acceptor H_A (Mäntele et al., 1990; Breton et al., 1992). This group is located about 10 Å away from Q_A and is within hydrogen-bonding distance of Trp L100 (Figure 1). The side chain of Glu L104 is also located

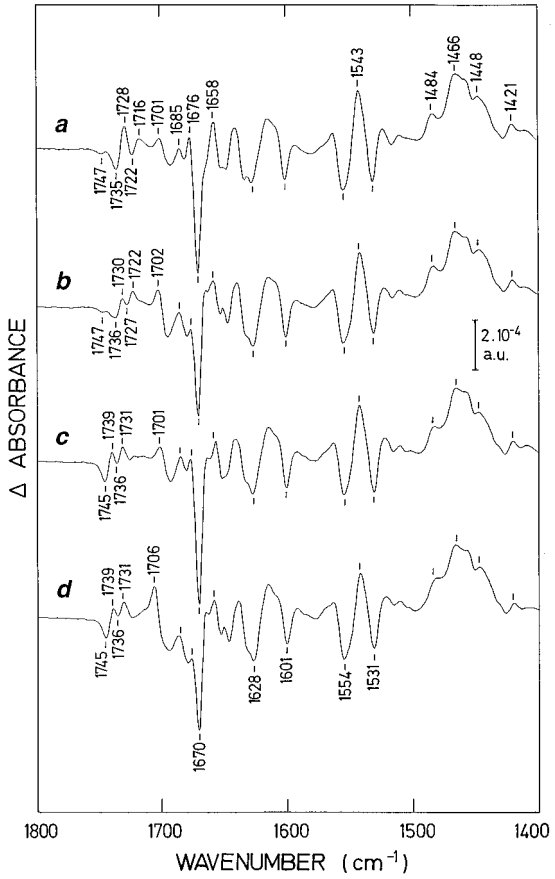

FIGURE 2: Light-induced Q_A^-/Q_A FTIR difference spectra in ^1H_2O at 5 °C of *Rb. sphaeroides* RCs: (a) WT, (b) the EL(L104) mutant, (c) the WF(L100) mutant, and (d) the EL(L104)/WF(L100) double mutant. The spectral resolution was 4 cm^{-1}. About 100 000 interferograms were added. The frequency of the IR bands is given at ± 1 cm^{-1}. a.u. is absorbance units.

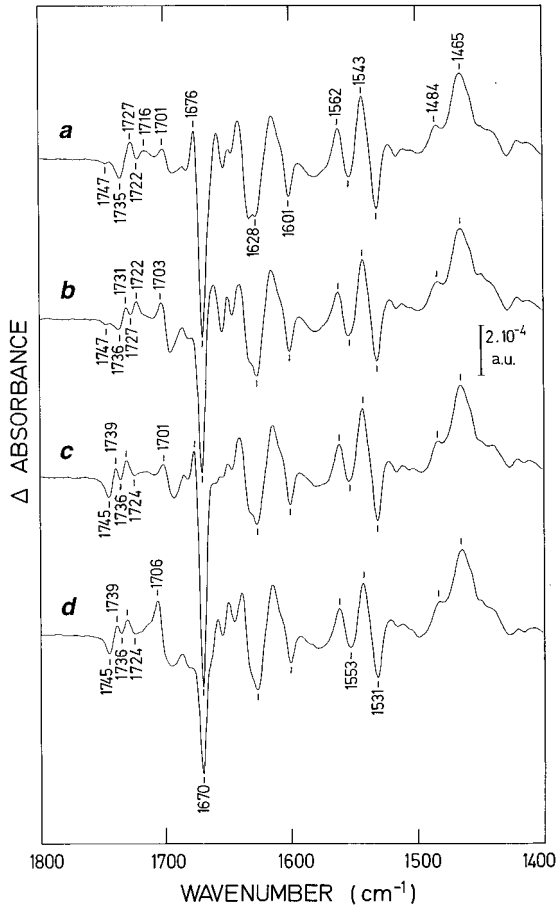

FIGURE 3: Light-induced Q_A^-/Q_A FTIR difference spectra in ^2H_2O at 5 °C of *Rb. sphaeroides* RCs: (a) WT, (b) the EL(L104) mutant, (c) the WF(L100) mutant, and (d) the EL(L104)/WF(L100) double mutant. Same conditions as for Figure 2.

close to the 10a-ester but clearly is not hydrogen bonded to this group on the basis of both the distance and geometry observed in the structure. It has long been established that the electric field generated by Q_A^- affects the electronic absorption properties of H_A (Verméglio & Clayton, 1977; Shopes & Wraight, 1985; Tiede & Hanson, 1992; Tiede et al., 1996). The electrochemically induced FTIR difference spectra for the reduction of isolated bacteriopheophytin, pheophytin, and pyropheophytin show that the 10a-ester C═O downshifts by ≈10 to 15 cm^{-1} upon one-electron reduction while the 7c-ester C═O is unaffected (Mäntele et al., 1988; Leonhard et al., 1990; Nabedryk et al., 1990), demonstrating that changes of electron density on ring V induce pronounced effects on the frequency of the 10a-ester C═O mode. Although the 10a-ester C═O is formally not conjugated to ring V, the vibrational properties of isolated phorbins show that this ester group influences the 9-keto C═O mode (Andersson et al., 1989; Heald & Cotton, 1990). Several mechanisms such as strain-induced interaction of the 9-keto and 10a-ester C═O or orbital overlap between these groups and bonds from the adjacent macrocycle have been proposed to explain the partial conjugation of the 10a-ester C═O (Andersson et al., 1989). This partial conjugation leads to weak contributions of the 10a-ester C═O vibration in the resonance Raman spectra of chlorophyll pigments in solution (Lutz & Mäntele, 1991). In addition, interactions with the protein can affect this conjugation. Notably, the enhancement of a resonance Raman band at 1726 cm^{-1}, tentatively assigned to the 10a-ester C═O mode of H_A in *Rb. sphaeroides* RCs, has been explained by a protein-induced redistribution of charges in ring V without enolization (Bocian et al., 1987). Furthermore, molecular orbital calculations on bacteriopheophytin anions have predicted that hydrogen bond formation to the two carbonyls of ring V induces significant changes of spin density on these C═O groups (Hanson et al., 1988).

Replacement of Glu L104 and Trp L100 with non-hydrogen bonding analogues will result in removal of the C═O of the Glu L104 side chain and the hydrogen bonds to the 9-keto and 10a-ester C═O groups of H_A. In this paper, site-directed mutagenesis at the Glu L104 and Trp L100 sites is combined with light-induced FTIR difference spectroscopy and $^1H/^2H$ exchange to assess possible contributions from the C═O of the Glu L104 side chain and of the 10a-ester of H_A in the Q_A^-/Q_A spectra and to investigate the long-range electrostatic response of H_A to Q_A reduction. A preliminary account of part of this work has been presented (Breton et al., 1996b).

MATERIALS AND METHODS

The mutant strains EL(L104) and WF(L100) contain the changes Glu to Leu at residue L104 and Trp to Phe at residue L100, respectively, and the double mutant EL(L104)/WF-(L100) contains both mutations. Oligonucleotide-directed mutagenesis was performed either using polymerase III holoenzyme (Stratagene) or by the method of Barik (1993)

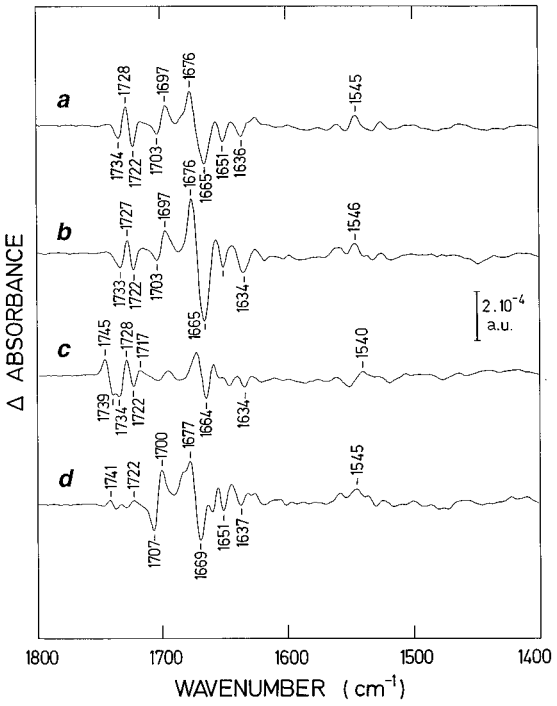

FIGURE 4: Double-difference spectra calculated from Q_A^-/Q_A spectra shown in Figures 2 and 3: (a) WT-minus-EL(L104) in ^1H_2O, (b) WT-minus-EL(L104) in ^2H_2O, (c) WT-minus-WF(L100) in ^1H_2O, and (d) WF(L100)-minus-EL(L104)/WF(L100) in ^1H_2O.

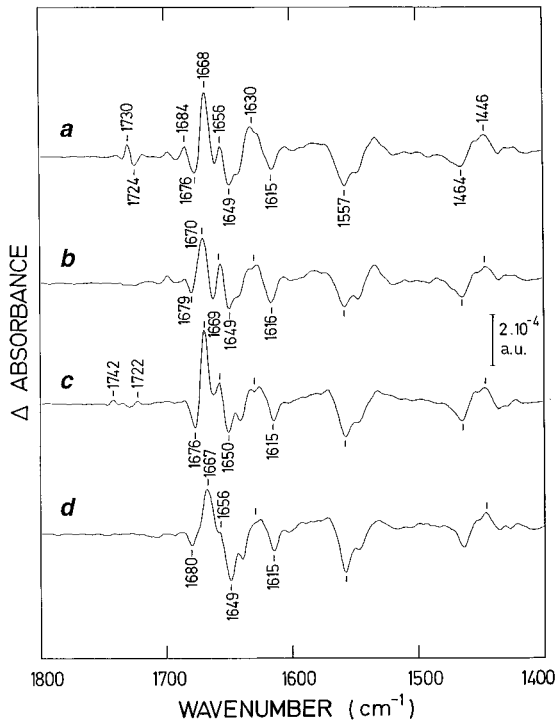

FIGURE 5: ^1H_2O-minus-^2H_2O double-difference spectra calculated from Q_A^-/Q_A spectra shown in Figures 2 and 3: (a) WT, (b) the EL(L104) mutant, (c) the WF(L100) mutant, and (d) the EL(L104)/WF(L100) double mutant.

using Vent DNA polymerase (New England Biolabs). The vectors contained a *Cla*I site immediately before the start of the L subunit gene (Lin et al., 1994), enabling the use of the *Cla*I-*Asp*718 fragment for mutagenesis. After DNA sequencing, this fragment was cloned into the shuttle vector pRKSCHΔK, replacing a fragment that contains a deletion of the ≈180 bp *Kpn*I-*Kpn*I segment. The mutated genes were expressed in the *Rb. sphaeroides puf*LM deletion strain ΔLM1.1, which is derived from the wild-type 2.4.1 strain (Paddock et al., 1989). The WT RCs were those isolated from the ΔLM1.1 deletion strain complemented with wild-type genes.

The RC samples were isolated in 15 mM Tris-HCl (pH 8), 0.025% lauryldimethylamine *N*-oxide, and 1 mM EDTA as previously described (Lin et al., 1994). For $^1H/^2H$ exchange, the RCs were resuspended three times in ^2H_2O at 20 °C and the solvent was evaporated under argon. This procedure leads to about 70% ^2H labeling of the NH peptide groups. Control experiments were performed under the same conditions with the RCs suspended in ^1H_2O. Light-induced FTIR measurements (Nicolet 60SX instrument) were performed under steady state illumination at 5 °C in the presence of TMPD and ascorbate at pH 7 as reported previously (Breton et al., 1994a) except that stigmatellin (2 mM) was used instead of *o*-phenanthroline to inhibit electron transfer to Q_B. For the ^2H_2O samples, the TMPD/ascorbate/stigmatellin mixture was prepared in ^2H_2O. The Q_A^-/Q_A spectra, which represent an average of measurements on at least three separate samples, have been corrected for the small contribution from $TMPD^+$/TMPD (Breton et al., 1992).

RESULTS

The Q_A^-/Q_A light-minus-dark FTIR difference spectra in ^1H_2O of RCs isolated from *Rb. sphaeroides* WT and from the mutants are shown in Figure 2. The corresponding spectra after the RCs have been incubated in ^2H_2O are depicted in Figure 3. In these spectra, the negative bands pertain to the vibrations from the Q_A state and the positive bands belong to those of the Q_A^- state. The spectra of WT RCs are almost indistinguishable from those previously reported for RCs isolated from the carotenoid-less strain R26 of *Rb. sphaeroides* (Breton et al., 1991a, 1992, 1994a−c; Breton & Nabedryk, 1995). The spectra of the mutants differ from those of WT, notably between 1750 and 1650 cm^{-1}. These differences are best visualized by calculating a double-difference spectrum (WT-minus-mutant) between the two individual Q_A^-/Q_A spectra upon minimization of the residual signals in the whole spectral range of the measurement (1800−1000 cm^{-1}). Such spectra are depicted in Figure 4 in the 1800−1400 cm^{-1} frequency range, and the ^1H_2O-minus-^2H_2O double-difference spectra are shown in Figure 5. In these double-difference spectra, interactive subtraction of the two parent spectra is used to obtain the smallest possible peak-to-peak amplitude for all the bands assigned to quinone vibrations (Breton et al., 1994a,c). It is observed that this normalization of the quinone bands also corresponds to an overall minimum for most of the non-quinone bands. The shape of the double-difference spectra was consistently reproduced when different sets of Q_A^-/Q_A spectra obtained for RC samples corresponding to identical experimental conditions were used. Finally, variations by ±10% of the optimum coefficient selected from the interactive subtraction do not lead to a significant perturbation of the double-difference spectra (data not shown), in particular for those bands that bear a frequency label (Figures 4 and 5) and which are discussed below.

The mutant EL(L104), in which Glu L104 has been replaced by a leucine residue, should eliminate the possible contribution to the IR spectra of the carboxylic side chain and any indirect effect brought about by the hydrogen bond

to the 9-keto C═O of H_A. The most noticeable differences between the Q_A^-/Q_A spectra of WT and the EL(L104) mutant (Figure 4a) are the small amplitude changes at ≈1545, 1636, and 1651 cm^{-1}, an apparent band shift responsible for the large 1665/1676 cm^{-1} differential signal, the amplitude increase of a differential signal at 1697/1703 cm^{-1}, and a signal with a second-derivative shape centered at 1728 cm^{-1}. While the parent spectra (Figure 2a,b) are quite complicated in the frequency region corresponding to the 1728 cm^{-1} signal, the second-derivative shape of this signal (Figure 4a) suggests that it could originate from the sharpening upon Q_A reduction of a band centered close to 1728 cm^{-1} in WT which would be absent in the mutant. Alternatively, this feature could be interpreted as resulting from the overlap of two derivative signals of opposite signs and centered at ≈1732 and 1725 cm^{-1}, reflecting the shift upon mutation of a differential signal present at 1735(−)/1728(+) cm^{-1} in WT (Figure 2a) and at 1727(−)/1722(+) cm^{-1} in the mutant (Figure 2b).

The Q_A^-/Q_A spectra for RCs that have been incubated in ^2H_2O (Figure 3a,b) are significantly different from those of RCs that have been incubated in ^1H_2O (Figure 2a,b) for both WT and the EL(L104) mutant. Further information on the effect of the $^1H/^2H$ exchange from these four spectra can be obtained by comparison of the WT-minus-mutant double-difference spectra and the ^1H_2O-minus-^2H_2O double-difference spectra. The WT-minus-mutant double-difference spectrum calculated for the RCs incubated in ^2H_2O (Figure 4b) is close in shape to that measured in ^1H_2O (Figure 4a). The main difference is the amplitude increase in ^2H_2O of the differential signal at 1665/1676 cm^{-1}. In the region around 1730 cm^{-1}, $^1H/^2H$ exchange induces only a 1 cm^{-1} downshift of the bands at 1734 and 1728 cm^{-1} and a slight decrease of the amplitude of the 1728/1722 cm^{-1} differential signal. The ^1H_2O-minus-^2H_2O double-difference spectra for WT and for the EL(L104) mutant (Figure 5a,b) are very close in the 1700−1400 cm^{-1} frequency region but differ strongly in the region above 1700 cm^{-1}. They display a differential signal positive at 1446 cm^{-1} and negative at 1464 cm^{-1} underlying the semiquinone modes of the Q_A^-/Q_A spectra, a negative band at 1557 cm^{-1} in the region of the amide II absorption, as well as bands positive at ≈1630, 1656, and 1668 cm^{-1} and negative at ≈1615 and 1649 cm^{-1}. Notably, a mostly differential signal, positive at 1730 cm^{-1} and negative at 1724 cm^{-1}, is observed in the ^1H_2O-minus-^2H_2O double-difference spectrum for WT (Figure 5a) but is essentially absent in the corresponding spectrum for the mutant (Figure 5b). The disappearance upon EL(L104) mutation of the 1730/1724 cm^{-1} differential signal induced by $^1H/^2H$ exchange in WT (Figure 5a,b) inescapably points to an involvement of Glu L104 in these IR spectral changes. However, these data do not clearly indicate whether these changes should be related simply to an electrostatic perturbation of the C═O mode of the Glu L104 side chain in WT RCs or if they are rather due to some indirect effect of $^1H/^2H$ exchange or of the mutation at Glu L104 on a nearby vibration.

The possibility that the 10a-ester C═O mode of H_A contributes to the IR changes observed above 1700 cm^{-1} has been tested by investigating the WF(L100) mutant in which the Trp residue that has been proposed to engage in hydrogen bonding interaction with the 10a-ester C═O of H_A is replaced by the non-hydrogen bonding analogue Phe. Compared to the Q_A^-/Q_A spectra of WT (Figures 2a and 3a), pronounced perturbations brought about by the WF-(L100) mutation are observed above 1700 cm^{-1} while only a very weak effect of $^1H/^2H$ exchange can be noticed in this region (Figures 2c and 3c). A new differential signal appears at 1745(−)/1739(+) cm^{-1} in the spectra of the mutant with a smaller one remaining at 1736(−)/1731(+) cm^{-1}. The WT-minus-WF(L100) double-difference spectrum in ^1H_2O (Figure 4c) reveals the appearance of the former differential signal in the mutant mainly at the expense of the 1735(−)/1728(+)/1722(−) cm^{-1} feature in the spectrum of WT. The observation that the WF(L100) mutation induces large changes in the region above 1700 cm^{-1} of the Q_A^-/Q_A spectrum provides strong evidence for an electrostatic response of the 10a-ester C═O of H_A upon Q_A reduction.

The effects of changes at both L100 and L104 positions can be observed in the Q_A^-/Q_A spectra of RCs from the double mutant EL(L104)/WF(L100). In the region from 1750 to 1715 cm^{-1}, the spectra of the double mutant measured in ^1H_2O (Figure 2d) and in ^2H_2O (Figure 3d) are very similar to those of the WF(L100) mutant (Figures 2c and 3c). The double-difference spectrum calculated between the Q_A^-/Q_A spectra of the WF(L100) single mutant and of the EL(L104)/WF(L100) double mutant in ^1H_2O reveals only small signals centered around 1741 and 1722 cm^{-1} (Figure 4d). These signals are very different in amplitude and in frequency from those corresponding to the effect of the single EL(L104) mutation (Figure 4a). Thus, the effect of the EL-(L104) mutation on the Q_A^-/Q_A spectra above 1700 cm^{-1} differs when the residue at the position L100 is Trp or Phe.

The ^1H_2O-minus-^2H_2O double-difference spectra of both the WF(L100) (Figure 5c) and the double mutant (Figure 5d) display all the bands that are characteristic of $^1H/^2H$ exchange in WT RCs (Figure 5a) in the region below 1700 cm^{-1}. In contrast, the 1730/1724 cm^{-1} differential signal in the ^1H_2O-minus-^2H_2O spectrum of WT RCs is replaced by small signals centered around 1742 and 1722 cm^{-1} in the ^1H_2O-minus-^2H_2O spectrum of the WF(L100) mutant that disappear in the ^1H_2O-minus-^2H_2O spectrum of the EL-(L104)/WF(L100) double mutant. Thus, the effect of $^1H/^2H$ exchange on the IR signature of the Glu L104 residue in the Q_A^-/Q_A spectra above 1700 cm^{-1} is distinctly different in WT and in the WF(L100) mutant.

DISCUSSION

The Q_A^-/Q_A spectra of RCs of WT *Rb. sphaeroides* and of the EL(L104), WF(L100), and EL(L104)/WF(L100) mutants are similar with regard to the overall shape but have distinct differences in the 1700−1750 cm^{-1} region (Figure 2). The bands previously assigned to the vibrations of the neutral quinone (e.g., at 1628 and 1601 cm^{-1}) and of the semiquinone (at 1484, 1466, and 1448 cm^{-1}) (Breton et al., 1994a,c) are present in all the mutant RCs, demonstrating that the mutation does not affect the quinone−protein interactions. Furthermore, a number of the non-quinone bands in these spectra are also essentially unaffected by the mutations which demonstrates that the structural changes accompanying the reduction of Q_A are not significantly perturbed by the mutations. Although secondary effects can occur upon mutation, these observations are taken to warrant an analysis of the observed FTIR spectral differences primarily in terms of the direct effect of the substitution of

chemical groups at the site of the mutation. This notion is further strengthened by a comparison of the main features of the double-difference spectra. The observation of rather large differences between the spectra obtained for the two single mutants (Figure 4a,c) is consistent with the view that each WT-minus-mutant spectrum primarily reveals the vibrations of the chemical groups that both differ between the two types of RCs and are affected by Q_A reduction. On the other hand, the overall similarity of all the ^1H_2O-minus-^2H_2O spectra (Figure 5a-d), which select the subset of vibrations of the chemical groups that are both sensitive to $^1H/^2H$ exchange and affected by Q_A reduction, shows that the spectral features are dominated by the effect of $^1H/^2H$ exchange rather than that of the mutations. These observations provide further evidence that the investigated mutations lead to only limited perturbations of the overall response of the RC vibrations to Q_A photoreduction.

Effect of $^1H/^2H$ Exchange on the Quinone Vibrations. The effect of $^1H/^2H$ exchange on the Q_A^-/Q_A spectra of WT *Rb. sphaeroides* (Figure 5a) is highly comparable to that reported recently for RCs isolated from the R26 strain (Breton & Nabedryk, 1995). Notably, bands previously assigned to C═O and C═C vibrations of the neutral quinones, i.e., the strongly downshifted C_4═O mode at 1601 cm^{-1} and the 1628 cm^{-1} mode of mixed C═O and C═C character (Breton et al., 1994a,c, 1996a; Breton & Nabedryk, 1996), are insensitive to the $^1H/^2H$ isotope effect. In contrast, the semiquinone band at 1448 cm^{-1} in *Rb. sphaeroides* is significantly shifted in ^2H_2O, giving rise to the 1464/1446 cm^{-1} differential signal in Figure 5a. This is in good agreement with the results from a detailed study of the Q_A^- ENDOR spectra indicating hydrogen bonding interactions of exchangeable protons with the carbonyls of Q_A^- (Feher et al., 1985). Comparison with the effect of $^1H/^2H$ exchange on the Q_A^- FTIR bands of *Rp. viridis* and of Q_A-depleted *Rb. sphaeroides* RCs reconstituted with vitamin K_1 led to the conclusion that it is a semiquinone C•••O mode in strong interaction with the protein that is predominantly affected by $^1H/^2H$ exchange (Breton & Nabedryk, 1995, 1996; Breton et al., 1996a). This C•••O mode is responsible for the positive band at 1446 cm^{-1} in the ^1H_2O-minus-^2H_2O double-difference spectra in *Rb. sphaeroides* (Figure 5). These observations do not agree with the assignment of the 1448 cm^{-1} semiquinone band to a C•••C mode proposed by Brudler et al. (1994) but concur with a preliminary assignment of this band to the C_4•••O mode in strong interaction with the protein (Breton et al., 1994c). One interesting observation is the different behavior of the C_4═O and C_4•••O modes upon $^1H/^2H$ exchange. As discussed previously (Breton et al., 1994c), the large frequency downshift by ≈50 cm^{-1} of the 1601 cm^{-1} C_4═O mode of the neutral Q_A compared to the frequency of ubiquinone in solution is best interpreted in terms of a strong hydrogen bond to the imidazole $N_{\delta 1}$ atom of His M219. The absence of a shift of the C_4═O vibration upon $^1H/^2H$ exchange for the neutral state while the corresponding mode of the semiquinone is significantly affected may indicate at least a partial shift of the hydrogen bonding partner from His M219 in Q_A to another group in Q_A^-. Such a group could be the OH side chain of Thr M222 that has been proposed to interact with the C_4═O carbonyl of Q_A in a number of the X-ray structures (Allen et al., 1988; Feher et al., 1989; El-Kabbani et al., 1991; Arnoux & Reiss-Husson, 1996). This change in the hydrogen bond direction between Q_A and Q_A^- would have to correspond to an electrostatic effect and not to a quinone displacement as it has been shown that Q_A does not move significantly upon reduction (Breton et al., 1994c).

Indirect Contribution of Glu L104 in Q_A^-/Q_A Spectra. The loss of the 1730/1724 cm^{-1} signal in the ^1H_2O-minus-^2H_2O double-difference spectra of the EL(L104) mutant (Figure 5b) compared to that of WT (Figure 5a) provides compelling evidence that the residue Glu L104 is protonated and sensitive to $^1H/^2H$ exchange. One way to interpret the 1730/1724 cm^{-1} differential signal induced by $^1H/^2H$ exchange in WT RCs would be in terms of an electrostatic response upon Q_A reduction of the side chain C═O mode of Glu L104 that downshifts in ^2H_2O (Breton & Nabedryk, 1995; Breton et al., 1996b). In this scheme, the spectral feature with a second-derivative shape that is centered at 1728 cm^{-1} in the WT-minus-mutant spectra (Figure 4a) would be assigned to the sharpening upon Q_A reduction of the band from the C═O mode of Glu L104. However, several observations are not consistent with this interpretation. Part of this feature downshifts by ≈1 cm^{-1} in ^2H_2O (Figure 4b).[2] The magnitude of this shift appears small for an exchangeable carboxylic residue for which values of 4 to 12 cm^{-1} are more typical [Rothschild, 1992; Siebert, 1993, and references therein; see also Nabedryk et al. (1995a) and Hellwig et al. (1996)]. In addition, a drastic change of frequency and amplitude of the 1730/1724 cm^{-1} differential signal in the ^1H_2O-minus-^2H_2O double-difference spectrum of WT RCs (Figure 5a) is observed in the corresponding spectrum for the WF(L100) mutant (Figure 5c). Furthermore, the changes in the spectra due to the L104 mutation observed by the comparison between WT and EL(L104) (Figure 4a) are different than those observed for the comparison between WF(L100) and EL(L104)/WF(L100) (Figure 4d). To reconcile the changes due to substitution at L100 with a direct electrostatic response of the carboxylic C═O mode of Glu L104 upon Q_A reduction, one would have to invoke the possibility that the presence of Trp or Phe at the L100 position can have a large influence both on the C═O frequency of Glu L104 and on its spectral response to Q_A reduction. This appears quite improbable considering that the C═O mode of the side chain of Glu L104 is **not** conjugated to ring V of H_A. The partial conjugation of the 10a-ester C═O to ring V of H_A provides a simpler rationale for a frequency shift of this ester group upon perturbation of the 9-keto C═O that is also conjugated to ring V.

Comparison between Q_A^-/Q_A and H_A^-/H_A Spectra of WT RCs. The possibility that vibrations of H_A are involved in the Q_A^-/Q_A FTIR difference spectra has been suggested (Mäntele et al., 1990; Breton et al., 1992) on the basis of the known electrochromic effect of Q_A^- on the electronic transitions of H_A (Verméglio & Clayton, 1977; Shopes & Wraight, 1985; Tiede & Hanson, 1992; Tiede et al., 1996). The H_A^-/H_A FTIR difference spectrum of the photoreduction of H_A in *Rb. sphaeroides* exhibits two negative bands at 1732 and 1745 cm^{-1} (Nabedryk et al., 1995c). It is interesting to note that for each of these features of the H_A^-/H_A spectrum

[2] The 1730/1724 cm^{-1} differential signal in Figure 5a must originate from the downshift by ≈1 cm^{-1} upon $^1H/^2H$ exchange of part of the feature centered at 1728 cm^{-1} in Figure 4a since (i) the difference between spectra a and b in Figure 4 is strictly identical to the difference between spectra a and b in Figure 5 and (ii) spectrum b in Figure 5 is essentially flat in this spectral range.

one can associate a signal at about the same frequency in the Q_A^-/Q_A FTIR spectrum. The 1732 cm^{-1} band in the H_A^-/H_A spectrum corresponds to the large differential signal centered at the same frequency in the Q_A^-/Q_A spectrum, while the 1745 cm^{-1} band corresponds to the signal of very small amplitude at 1747 cm^{-1} in the Q_A^-/Q_A spectrum (Figure 2a). The same observation applies to the H_A^-/H_A and Q_A^-/Q_A spectra of *Rp. viridis* RCs, where an additional shoulder at ≈1725 cm^{-1} in the H_A^-/H_A spectrum (Nabedryk et al., 1988; 1995c) corresponds to a small differential signal centered at the same frequency in the Q_A^-/Q_A spectrum (Breton et al., 1994a). Similarly, a correlation has been made (Breton & Nabedryk, 1995) between the presence of a large differential signal at 1724/1719 cm^{-1} in the Q_A^-/Q_A spectrum (Berthomieu et al., 1990, 1992) and the presence of a negative band at 1721 cm^{-1} in the H_A^-/H_A spectrum (Nabedryk et al., 1990) of photosystem II. Thus, there is a close correlation between the H_A^-/H_A signals above 1700 cm^{-1} and signals centered at the same frequencies in the Q_A^-/Q_A spectra.

Comparison of the H_A^-/H_A spectra of bacterial RCs with the anion-minus-neutral FTIR difference spectra of isolated bacteriopheophytin has led to the conclusion that at least part of the signals above 1700 cm^{-1} in the H_A^-/H_A spectra originates from a downshift upon reduction of the 10a-ester C═O of H_A (Nabedryk et al., 1986, 1988, 1995c; Mäntele et al., 1988). Therefore, at least part of the Q_A^-/Q_A signals above 1700 cm^{-1} could also correspond to an electrostatic perturbation of the 10a-ester C═O of H_A upon Q_A reduction. Although it would be useful to correlate the effect of the investigated mutations on the Q_A^-/Q_A and H_A^-/H_A spectra, all our attempts at generating H_A^- in RCs of *Rb. sphaeroides* mutants under the reversible conditions required for FTIR studies have been unsuccessful so far.

It should be noted that the 9-keto C═O mode of H_A has been identified at ≈1680 cm^{-1} on the basis of resonance Raman and FTIR studies (Lutz & Robert, 1985; Robert & Lutz, 1988; Nabedryk et al., 1986, 1988, 1995c; Palaniappan et al., 1993; Palaniappan & Bocian, 1995). Thus, the Q_A^-/Q_A signals observed in the 1750−1715 cm^{-1} frequency range cannot originate from the 9-keto C═O of H_A. No assignment is proposed in the present FTIR experiments that would correspond to vibrations of the 9-keto C═O of H_A, since the Q_A^-/Q_A FTIR difference spectra appear congested in this frequency range, where peptide C═O and side chain modes are also expected to absorb. The contribution of the 9-keto C═O of H_A in the Q_A^-/Q_A spectra is being investigated in *Rp. viridis* by comparing the Q_A^-/Q_A and H_A^-/H_A spectra of WT and EQ(L104) RCs (J. Breton, M. Bibikova, E. Nabedryk, and D. Oesterhelt, manuscript in preparation).

Q_A Reduction Affects the 10a-Ester C═O of H_A. The observation that the Trp to Phe mutation at L100 induces large changes in the region above 1700 cm^{-1} of the Q_A^-/Q_A spectrum (Figure 4c) provides strong evidence that the 10a-ester C═O of H_A is directly involved in the Q_A^-/Q_A spectra of RCs from both WT and the mutants. This result is fully consistent with the existence of a hydrogen bond between this C═O group and Trp L100 in WT RCs (Figure 1) as proposed in all the available X-ray structures (Michel et al., 1986; Deisenhofer & Michel, 1989; Lancaster et al., 1995; Yeates et al., 1988; Feher et al., 1989; El-Kabbani et al., 1991; Ermler et al., 1994; Arnoux & Reiss-Husson, 1996). The prominent 1745/1739 cm^{-1} differential signal in the Q_A^-/Q_A spectra of RCs from the WF(L100) mutant is thus assigned to a frequency downshift of the free 10a-ester C═O of H_A upon Q_A reduction.

For RCs with the Trp to Phe mutation at L100, a small signal around 1742 cm^{-1} is generated both by the additional Glu to Leu mutation at L104 in the double mutant (Figure 4d) and by $^1H/^2H$ exchange (Figure 5c). These observations indicate a shift of the vibrational mode of the 10a-ester C═O upon perturbation of the 9-keto C═O of H_A. Therefore, $^1H/^2H$ exchange of the COOH side chain of Glu L104 in WT or replacement of the COOH of Glu L104 by the two methyl groups of the Leu side chain in the EL(L104) mutant appears to influence the frequency of the 10a-ester C═O, most probably by altering the electron density on ring V of H_A.

The signals induced by the EL(L104) substitution or by $^1H/^2H$ exchange are very different in WT RCs and in WF-(L100), and the amplitude of these induced signals is larger when the 10a-ester C═O of H_A is bound to Trp L100 than when it is free. The partial conjugation of the 10a-ester C═O to ring V of H_A provides a rationale for this difference since one can assume that the response of the 10a-ester C═O of H_A to a perturbation of the electron density on ring V depends on the extent of conjugation, and thus on the conformation of this ester group. The absence of any effect of $^1H/^2H$ exchange on the Q_A^-/Q_A spectrum of the EL(L104) mutant above 1700 cm^{-1} shows either that the side chain NH of Trp L100 does not exchange or that, if it exchanges, the $^1H/^2H$ substitution does not alter the frequency of the 10a-ester C═O of H_A.

The signals observed in the 1750−1715 cm^{-1} range of the Q_A^-/Q_A spectra of RCs from the WT and the mutants (Figure 2) exhibit mainly a derivative shape. According to the analysis presented above, these derivative signals are taken to represent the frequency downshift of the 10a-ester C═O mode of specific populations of H_A upon Q_A^- formation. The frequency difference between a given negative transition and the closest positive one ranges between 5 and 7 cm^{-1}. A quantitative analysis of these derivative signals that accounts for the relative amplitude of the Q_A^-/Q_A and H_A^-/H_A signals is made difficult by the strong congestion of the spectra and by the lack of the H_A^-/H_A spectra for the mutants. Nevertheless, a crude graphic estimate based on a band width (FWHM) of 8 cm^{-1} suggests that a downshift of 2−4 cm^{-1} would be sufficient to explain the spectra. This value can be compared to the ≈10−15 cm^{-1} downshift of the 10a-ester C═O mode of isolated (bacterio)pheophytins upon one-electron reduction (Mäntele et al., 1988; Leonhard et al., 1990; Nabedryk et al., 1990). Although comparison of the extent of these downshifts could in principle be used to relate the perturbation of the 10a-ester C═O mode of H_A to the strength of the local electric field generated by Q_A^- (Chattopadhyay & Boxer, 1995; Hush & Reimers, 1995), one should also consider that geometrical changes of the carbomethoxy group or even of the whole ring V, leading to a change of conjugation of the 10a-ester C═O, could occur upon Q_A reduction.

While an indirect contribution of Glu L104 to the Q_A^-/Q_A spectra via a perturbation of ring V of H_A is supported by the present data, one cannot rule out a small additional response of the side chain C═O mode of this residue upon Q_A reduction. As discussed above, the signals observed above 1700 cm^{-1} in the ^1H_2O-minus-^2H_2O double-difference spectra of WT RCs (Figure 5a) and WF (L100) mutant

(Figure 5c) are best explained as resulting from a small shift (by ≈1 cm^{-1}) of the frequency of the 10a-ester C═O of H_A upon $^1H/^2H$ exchange of Glu L104. A direct contribution from Glu L104 to the two spectra can only have a very small amplitude compared to that of the 10a-ester C═O of H_A. This question will be further addressed by using *Rb. sphaeroides* (R26) RCs in which H_A has been exchanged for ^{12}C- or ^{13}C-labeled plant pheophytin (Meyer & Scheer, 1995; Meyer et al., 1996).

Multiple Conformations of the 10a-Ester C═O of H_A. The main differential signal at 1735/1728 cm^{-1} in the Q_A^-/Q_A spectrum of WT RCs (Figure 2a) decreases in amplitude upon the different mutations investigated here. However, a residual differential signal remains at ≈1736/1730 cm^{-1} in all the mutant spectra (Figure 2b-d). The close correspondence in frequency and amplitude of this differential signal in the Q_A^-/Q_A spectra of EL(L104) and of WF(L100) suggests that the 1735/1728 cm^{-1} signal of WT RCs is made of two overlapping signals, with one at ≈1736/1730 cm^{-1} being insensitive to the investigated mutations and to $^1H/^2H$ exchange and the other at ≈1734/1728 cm^{-1} (Figure 4a,c) being sensitive to $^1H/^2H$ exchange as well as to the mutations. The latter population would give rise to the 1727/1722 and 1745/1739 cm^{-1} differential signals upon the EL(L104) and WF(L100) mutations, respectively.

The large 1745/1739 cm^{-1} differential signal in the Q_A^-/Q_A spectrum of WF(L100) is assigned to the downshift upon Q_A reduction of the 10a-ester C═O of H_A. The negative peak at 1745 cm^{-1} in the Q_A state is consistent with a 10a-ester C═O free from interaction with the protein (Lutz & Mäntele, 1991). The observation that the 1745/1739 cm^{-1} signal of WF(L100) is affected both by the additional EL(L104) mutation (Figure 4d) and by $^1H/^2H$ exchange (Figure 5c) provides evidence that the interaction between the 9-keto group of H_A and the COOH of Glu L104 is retained for this dominant population of H_A in the WF(L100) mutant.

In principle, the Q_A^-/Q_A spectrum of WF(L100) between 1750 and 1715 cm^{-1} (Figure 2c) should contain only the contribution from a free 10a-ester C═O of H_A. It is thus somewhat surprising to observe in the spectra of this mutant a residual ≈1736/1730 cm^{-1} differential signal as also found in the spectra of the other RCs. The 1736/1730 cm^{-1} signal could correspond to a 10a-ester C═O population belonging to a fraction of H_A with both the 10a-ester and the 9-keto C═O groups free from interaction with the protein. Alternatively, it could represent a different group such as the 10a-ester C═O of the inactive bacteriopheophytin (H_B) close to Q_B, a 7c-ester C═O (of H_A or H_B) that would respond to a change of local electrostatic brought about by Q_A reduction, or even the contribution of an unexchangeable carboxylic group.

The Q_A^-/Q_A spectra of WT and EL(L104) *Rb. sphaeroides* RCs (Figure 2a,b) show a weak signal at 1747 cm^{-1}, while a band of larger amplitude is observed at the same frequency in the Q_A^-/Q_A spectrum of *Rp. viridis* RCs (Breton et al., 1992, 1994a). The H_A^-/H_A spectra of WT RCs of both species exhibit a negative band at 1745 cm^{-1} (Nabedryk et al., 1995c). In view of the large amplitude increase of a signal at 1745 cm^{-1} in the Q_A^-/Q_A spectrum when the hydrogen bond to Trp L100 is released (Figure 4c), the 1747 cm^{-1} band in the Q_A^-/Q_A spectrum of WT RCs is assigned to a minor population of H_A with its 10a-ester C═O free from interaction with Trp L100.

The small differential signal at 1722/1716 cm^{-1} in the Q_A^-/Q_A spectrum of WT RCs (Figure 2a) is affected by the two mutations (Figure 4a,c) and appears sensitive to $^1H/^2H$ exchange (Figure 5a,c). This signal is thus tentatively assigned to a 10a-ester C═O belonging to a population of H_A having hydrogen bonds to both the 9-keto and the 10a-ester groups. This proposal concurs with a previous one based on resonance Raman data on *Rb. sphaeroides* RCs (Bocian et al., 1987), although it has also been proposed that the 1722 cm^{-1} band could correspond to a nonfundamental mode (Palaniappan et al., 1993). It should also be noted that resonance Raman bands at 1750 and 1742 cm^{-1} have been assigned to 10a-ester C═O contributions from the two bacteriopheophytins (Palaniappan et al., 1993).

The origin of the heterogeneity of the 10a-ester C═O mode of H_A in WT RCs indicated by the present data is unknown. Also unknown is whether this heterogeneity is static or dynamic. Such heterogeneity can be rationalized in terms of multiple conformations of the 10a-carbomethoxy group or even of the whole ring V of H_A. Small changes in the strength or the orientation of the hydrogen bonds engaged with the 9-keto and 10a-ester C═O groups could have a large impact on the extent of conjugation and therefore the frequency of the 10a-ester C═O. Due to steric constraints, only a few conformations of the 10a-carbomethoxy group will be favored. In this respect, direct evidence that Trp M252, an important residue located between Q_A and H_A (Figure 1), affects such conformations has been recently obtained by investigating the WF(M252) and WY(M252) mutants of *Rb. sphaeroides* and the analogous mutants of *Rp. viridis* (J. Breton, M. Bibikova, E. Nabedryk, and D. Oesterhelt, unpublished).

Implications for Electron and Proton Transfer. The presence of several discrete conformations of the 10a-ester C═O of H_A in WT RCs indicated by the present FTIR study would cause a heterogeneity of the electronic structure of H_A and hence in the electron transfer properties of this cofactor. A heterogeneity of conformation has been frequently invoked to explain various distributions of functional properties of electron transfer of native RCs. For example, a distribution of rates has been observed for the forward electron transfer step from H_A^- to Q_A (Kirmaier & Holten, 1990) as well as for the charge recombination between the oxidized primary donor and H_A- or Q_A^- (Woodbury & Parson, 1984; Ogrodnik et al., 1994; Schoepp et al., 1992; Baciou & Sebban, 1995). It has been proposed that H_A was involved in this heterogeneity as both electron transfer reactions to and from H_A were affected (Kirmaier & Holten, 1990). Such heterogeneity of electron transfer kinetics and the structural heterogeneity of the conformation of the 10a-ester C═O of H_A deduced from the present FTIR experiments might bear some common origin. Notably, both the H_A^- to Q_A electron transfer rate (Bylina et al., 1988) and the conformation of the 10a-ester C═O of H_A are affected by the EL(L104) mutation. Furthermore, the different conformations of the 10a-ester C═O, by affecting the conformation of ring V, may play a role in tuning the redox potential of H_A (Hanson et al., 1988).

The nonexponential kinetics observed for absorption changes involving H_A^- may also partially arise from heterogeneity in the reorganization energy for electron transfer. Specific contributions of the protein and cofactors to the reorganization energy have yet to be identified. The

data presented show that electrostatic interactions introduced due to electron transfer to Q_A are sufficiently strong to alter the vibrational properties of H_A. The alteration of the vibrational properties of the bonds would contribute to the reorganization energy associated with the electron transfer steps. The possible involvement of heterogeneity of the energetics in the electron transfer process of the RC has been proposed by several groups (Small et al., 1992; Jia et al., 1993; Peloquin et al., 1994; Beekman et al., 1995; Kirmaier et al., 1995).

Coupled to electron transfer to the quinones is the transfer of protons [reviewed in Okamura and Feher (1995), Maroti et al. (1995), and Sebban et al. (1995)]. Experimental evidence for proton uptake upon Q_A^- formation has been obtained in the pH range of 4−10 for *Rb. sphaeroides* and *Rb. capsulatus* RCs (McPherson et al., 1988; Maroti & Wraight, 1988; Maroti et al., 1995; Sebban et al., 1995). Furthermore, electrostatic calculations have suggested proton uptake by carboxylic groups upon Q_A reduction for both *Rb. sphaeroides* (Gunner & Honig, 1992; Beroza et al., 1995) and *Rp. viridis* (Lancaster et al., 1996). The absence of an effect of $^1H/^2H$ exchange in the 1770−1700 cm^{-1} range of the Q_A^-/Q_A spectra of the EL(L104) and EL(L104)/WF-(L100) mutants (Figure 5b,d) demonstrates that **no** accessible protonated carboxylic residue other than Glu L104 contributes directly or indirectly to the Q_A^-/Q_A FTIR spectra of WT RCs at pH 7. The present study shows that the protonation state of Glu L104 does not change upon Q_A reduction, in agreement with electrostatic calculations in *Rb. sphaeroides* (Beroza et al., 1995) and *Rp. viridis* (Lancaster et al., 1996). Under the reasonable assumption that carboxylic groups that participate in proton uptake should be sensitive to $^1H/^2H$ exchange of the solvent, it can thus be proposed that the ≈0.4 H^+ uptake per Q_A^- measured in *Rb. sphaeroides* WT RCs at pH 7 (McPherson et al., 1988) originates from chemical groups other than those involving carboxylic side chains.[3] This conclusion contrasts with the result of electrostatic calculations in *Rb. sphaeroides* indicating proton uptake by Glu L212 (≈0.2 H^+/Q_A^- at pH 7) upon Q_A reduction (Beroza et al., 1995). A similar discrepancy between the result of calculations on the protonation of carboxylic groups and the FTIR results is also found for Q_A^- and Q_B^- formation in *Rp. viridis*. More specifically, electrostatic calculations have predicted that three Glu residues (L212, H177, and M234) from the Q_B cluster were responsible for the bulk of the proton uptake (≈0.35 H^+/Q_A^- and ≈0.5H^+/Q_B^- at pH 7) upon quinone reduction (Lancaster et al., 1996). However, the FTIR spectra of quinone photoreduction in *Rp. viridis* RCs show **no** evidence for proton uptake by an accessible carboxylic residue in the 1770−1700 cm^{-1} range for both Q_A^- (J. Breton, M. Bibikova, E. Nabedryk, and D. Oesterhelt, manuscript in preparation) and Q_B^- formation (Breton et al., 1996a). On the other hand, a qualitative agreement has been found for Q_B reduction in *Rb. sphaeroides* WT RCs between the stoichiometry of proton uptake by Glu L212 (0.3−0.4 H^+/Q_B^- at pH 7) determined by FTIR (Nabedryk et al., 1995a) and the calculated uptake of ≈0.7−1 H^+/Q_B^- (Gunner & Honig, 1992; Beroza et al., 1995). Thus, with the notable exception of the protonation of Glu L212 upon Q_B reduction in *Rb. sphaeroides* RCs, the FTIR observations cannot be reconciled with the idea that carboxylic residues are the main contributors to the proton uptake experimentally observed upon quinone reduction at pH 7 in *Rb. sphaeroides* and *Rp. viridis* RCs.

A broad positive band at 2900−2500 cm^{-1} has been consistently observed in the Q_A^-/Q_A and Q_B^-/Q_B spectra of both *Rb. sphaeroides* and *Rp. viridis* RCs (J. Breton and E. Nabedryk, unpublished). Importantly, this band shifts to 2200−2000 cm^{-1} upon $^1H/^2H$ exchange. It is tentatively proposed that the band at 2900−2500 cm^{-1} could correspond to the electrostatic response upon quinone reduction of the so-called "Zundel polarizability" of the protons shifting within a network of polarizable hydrogen bonds (Zundel, 1992). Interaction of the protons with their environment as they fluctuate in the double potential well of the hydrogen bonds leads to a characteristic IR continuum, notably in the 3000−2000 cm^{-1} frequency range (Olejnik et al., 1992; Borgis et al., 1992). The existence of a network of interacting chemical groups of the cofactors H_A, Q_A, and Q_B, the amino acid side chains, and the array of organized water molecules that have been resolved in some recent crystallographic structures of the RCs (Ermler et al., 1994; Lancaster et al., 1995) is consistent with a number of our FTIR results. This network appears to include at least the ring V of H_A, Trp L100, Glu L104, Trp M252, Thr M222, the C_4═O mode of Q_A and Q_A^-, His M219, the non-heme iron, His L190, the C_4═O mode of Q_B and Q_B^-, the side chains of the many polar residues in the Q_B pocket, as well as the connections of these groups to the water chains. Such a network could play an important role in the coupled electron/proton transfer reactions involving the quinone acceptors. Further FTIR experiments are currently under way to test this hypothesis.

In conclusion, the electrostatic response of a vibrational mode of the bacteriopheophytin electron acceptor H_A upon photoreduction of the primary quinone Q_A has been identified by FTIR difference spectroscopy in *Rb. sphaeroides* RCs. The WF(L100) mutation, designed to remove the proposed hydrogen bond to the 10a-ester C═O on ring V of H_A, has a pronounced effect on a differential signal centered at 1732 cm^{-1} in the Q_A^-/Q_A spectrum of WT RCs. This strengthens the existence of the hydrogen bond and shows that the 10a-ester C═O of H_A experiences a small frequency downshift upon Q_A reduction. The EL(L104) mutation, which breaks the hydrogen bond to the 9-keto C═O on ring V of H_A, also perturbs the 10a-ester C═O vibrations. This is most probably the result of the partial conjugation of the 10a-ester C═O to ring V and of the change of electron density on ring V induced by the mutation. In contrast, no direct contribution of the C═O mode of the protonated side chain of Glu L104 or of any other carboxylic residue is detected in the Q_A^-/Q_A spectra. Several conformations of the 10a-ester C═O of H_A are observed in WT RCs. If these different conformations are related to the well-established heterogeneity of electron transfer kinetics, this would provide a link between structural and functional heterogeneity in bacterial RCs.

[3] An upper limit of 0.05 H^+/Q_A^- can be estimated for the protonation change of carboxylic residues that would be compatible with the noise level in the 1770−1700 cm^{-1} spectral range. This is obtained by comparing spectrum b in Figure 5 to the spectrum of the effect of $^1H/^2H$ exchange on Q_B reduction in WT *Rb. sphaeroides* for which proton uptake by Glu L212 of 0.3−0.4 H^+/Q_B^- has been reported (Nabedryk et al., 1995a). For this comparison, the Q_A^-/Q_A and Q_B^-/Q_B spectra have been normalized on the integrated intensity of the main semiquinone band.

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