Proton Uptake by Carboxylic Acid Groups upon Photoreduction of the Secondary Quinone (Q_B) in Bacterial Reaction Centers from *Rhodobacter sphaeroides*: FTIR Studies on the Effects of Replacing Glu H173[†]

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ABSTRACT: In the photosynthetic reaction center (RC) from *Rhodobacter sphaeroides*, Glu H173, located ∼7 Å from the center of the secondary quinone acceptor Q_B, is expected to contribute to proton uptake upon Q_B^- formation in response to the movement of an electron in its vicinity. Steady-state FTIR difference spectroscopy provides a method to monitor proton uptake by carboxylic acids upon photochemical changes. The FTIR spectra corresponding to the photoreduction of Q_B were obtained at pH 7 for RCs containing Glu (native), Gln (EQ H173), or Asp (ED H173) at the H173 site. No new bands were observed in the carboxylic acid region (1770−1700 cm^{-1}) in any of the mutant RCs compared to native RCs. In addition, the positive band at 1728 cm^{-1}, previously assigned to Glu L212 [Nabedryk, E., Breton, J., Hienerwadel, R., Fogel, C., Mäntele, W., Paddock, M. L., and Okamura, M. Y. (1995) *Biochemistry 34*, 14722−14732], remained present in all of the mutant RCs. This result shows that Glu H173 is not a major contributor to proton uptake upon Q_B^- formation and further strengthens the assignment of the 1728 cm^{-1} band to Glu L212. An increase in the 1728 cm^{-1} band was observed in the EQ H173 RCs compared to that of either the ED H173 or native RCs. These changes are consistent with Glu and Asp at H173 remaining ionized in the Q_B and Q_B^- states. Changes in the absorption regions of the semiquinone and amide or side chain groups in the spectra of the mutant RCs suggest slight changes in the protein structure compared to those of native RCs, which could contribute to the altered kinetics observed in the mutant RCs.

In the photosynthetic bacterial reaction center (RC[1]), light induces a sequence of electron- and proton-transfer reactions that results in the double reduction and protonation of the secondary quinone Q_B to Q_BH_2, which then dissociates from the RC (*1*). Formation of the quinol is a key step in the bioenergetics of photosynthetic purple bacteria, and many details of the mechanism and energetics of proton-coupled electron-transfer events are still unknown. Upon the first electron transfer to Q_B leading to the formation of the semiquinone, it has been established that substoichiometric proton uptake by the protein occurs in response to the electrostatic influence of the semiquinone charge (*2*−*4*). At least three possible proton-transfer pathways connecting the Q_B site to the surface of the protein can be identified in the most recent high-resolution crystal structures of the RC from *Rhodobacter (Rb.) sphaeroides* (*5*−*7*). These paths include ionizable amino acid side chains of the L, M, and H subunits and bound internal water molecules that form a network of hydrogen bonds. In particular, the three residues Ser L223, Asp L213, and Glu L212 that have been earlier shown by site-directed mutagenesis work to be crucial for proton-transfer events (*8*, *9*) are connected to the cytoplasmic surface via these paths (see Figure 1). Moreover, six carboxylic acid residues form an acidic cluster in the vicinity of Q_B, each within 4.5 Å of a neighboring carboxylic acid or of a bridging water molecule (*6*), which may be important for the energetics and kinetics of protonation of reduced Q_B. This acidic cluster includes Asp L213, Asp L210, Asp M17, and three amino acid residues of the H subunit (Glu H173, Asp H170, and Asp H124). Transient optical spectroscopy (*8*−*11*) as well as steady-state FTIR (*12*−*14*) and kinetic IR (*15*) studies of RCs with mutations of L subunit amino acids have been reported. More recently, electron-transfer kinetic studies on site-directed mutants of the H subunit showed that acid residues at H173 and H170 are important for rapid proton-coupled electron transfer to the reduced quinone (*16*−*19*).

Knowledge of the protonation state of carboxylic acid groups near Q_B is crucial for a clear understanding of the details of the mechanism and energetics of proton-coupled electron transfer to Q_B^-. This can be obtained from knowledge of the position and occupancy of the protein hydrogen atoms, as has been recently structurally determined for lysosyme using neutron diffraction (*20*). However, the resolution of the X-ray crystal structure of the bacterial RC is insufficient to yield such details at present. Thus, several

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[1] Abbreviations: FTIR, Fourier transform infrared; RC, reaction center; Q_B/Q_A, secondary/primary quinone acceptor; D, primary electron donor; WT, wild type; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*.

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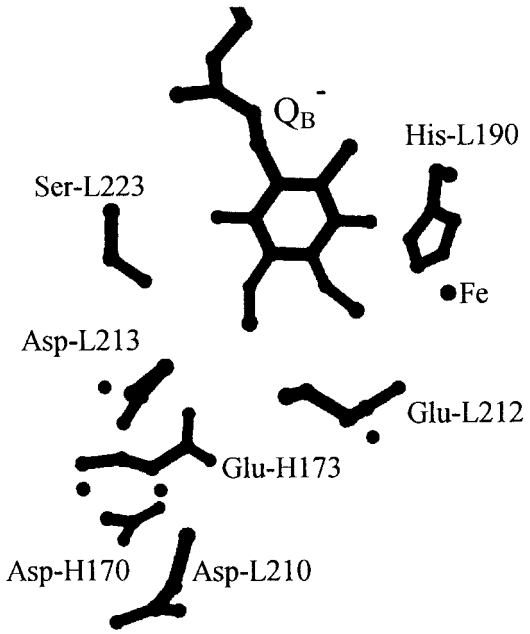

FIGURE 1: Structure of the Q_B binding pocket in the bacterial photosynthetic RC from *Rb. sphaeroides* (*5*). Shown are the positions of the semiquinone Q_B^- and the non-haem Fe^{2+}, the potential hydrogen bonding residues His L190 and Ser L223, the nearby acid groups Glu L212, Asp L213, Glu H173, Asp L210, and Asp H170, and nearby bound water molecules (solid circles).

other experimental approaches have been used to estimate the protonation state of acid residues near Q_B. One method is to measure the electron-transfer rates in native and mutant RCs as function of pH (see, e.g., refs *8* and *9*). The kinetic results were interpreted in terms of changes in the electrostatic potential near Q_B due to amino acid titrations and site-specific mutations. From these studies, estimates were obtained for the protonation state of amino acid residues near Q_B and their pK_as assuming a classic Henderson−Hasselbach acid titration. Detailed electrostatic calculations have predicted the titration curves of amino acid residues near Q_B for both *Rb. sphaeroides* and *Rhodospeudomonas* (*Rp.*) *viridis* RCs and have suggested nonclassic titration of many internal acid groups located near Q_B (*21*−*24*). Experimental support for nonclassic titration behavior comes from IR data (*12*, *15*). These results suggest that previous analysis of the kinetic data may be oversimplified.

A more direct experimental method for probing changes of the protonation states of Asp and Glu residues upon photo- or redox-induced reactions is provided by IR difference spectroscopy. For energy-transducing systems, such as retinal proteins (*25*, *26*), cytochrome oxidase (*27*, *28*), and bacterial RCs (*12*−*15*), absorption changes of Asp and Glu side chains in the 1770−1700 cm^{-1} IR spectral range have been revealed upon changes in the isomerization, redox, or ionization state of cofactors. For *Rb. sphaeroides* RCs, the Q_B^- minus Q_B FTIR difference spectrum (Q_B^-/Q_B) of native RCs at pH 7 shows a positive band at 1728 cm^{-1} that is sensitive to $^1H/^2H$ isotopic exchange, as expected for a carboxylic acid group. This 1728 cm^{-1} signal in native RCs has been attributed to substoichiometric proton uptake by Glu L212 upon Q_B^- formation based on its absence when Glu L212 was replaced with Gln (*12*). Thus, Glu L212 is partially ionized in the Q_B neutral state and becomes more protonated upon Q_B photoreduction, consistent with electrostatic calculations (*21*, *22*). However, in view of the strong electrostatic interactions expected among many nearby carboxylic acids forming a cluster near Q_B (*6*), it cannot be excluded that some other acid, in addition to Glu L212, contributes to the 1728 cm^{-1} absorption peak in native RCs as has been recently suggested based on electrostatic calculations performed in *Rp. viridis* RCs (*24*). The three structurally closest residues to Glu L212 are Asp L213, Glu H173, and Asp L210 (Figure 1). Previous steady-state FTIR results have shown that there is no significant contribution of either Asp L213 or Asp L210 to the 1728 cm^{-1} peak (*12*, *13*). To examine the involvement of Glu H173 in proton uptake and to estimate its state of ionization, we have investigated the effects of the mutations Glu H173 → Gln and Glu H173 → Asp on the light-induced steady-state Q_B^-/Q_B FTIR difference spectra in *Rb. sphaeroides* RCs. In addition to monitoring changes in the protonation state of amino acid residues, FTIR provides a method to assess structural changes that may result from site-specific amino acid replacements. A preliminary account of part of this work has been presented (*29*).

MATERIALS AND METHODS

The *Rb. sphaeroides* H173 mutations were constructed and incorporated into *puhA*, the gene coding for the H subunit polypeptide, essentially as previously described in ref *30*. The modified *puhA* was transferred into pRK404 with flanking DNA to reconstruct the *puhA* operon on a 1.3 kb BamHI fragment (*31*). This plasmid was used to complement the *Rb. sphaeroides* deletion strain PUHA1 as described in ref *31*. RC isolation was performed as described in ref *10*. To 10 μl of an RC sample (∼0.2 mM) containing a 10-fold excess of ubiquinone-10, 10 μl of ascorbate 10 mM and diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine) 20 mM in Tris-HCl, pH 7, 90mM, was added. The solution was dried under argon to a thin paste on a CaF_2 window. The RC sample was then covered with 2 μl of ^1H_2O and sealed with another CaF_2 window. For $^1H/^2H$ exchange, the RC sample containing the mediators was resuspended at least three times in ^2H_2O at 20 °C (total incubation time of ∼2 h). This procedure leads to the deuteration of about 60−70% peptide NH groups. The corresponding buffer with ascorbate and mediator was made in ^2H_2O. The long-lived Q_B^- state ($t_{1/2} \approx 30$ s) was generated under single saturating flash excitation (Nd:YAG laser, 7 ns, 530 nm). Light-induced FTIR difference spectra were acquired at 15 °C with a Nicolet 60 SX spectrometer. A detailed description of the measurements is found in ref *12*.

RESULTS

The Q_B^-/Q_B FTIR difference spectra of EQ H173 [Glu H173 → Gln] (Figure 2a) and ED H173 [Glu H173 → Asp] (Figure 2b) display several typical absorption changes associated with Q_B reduction in native RCs (Figure 2c) (*12*, *32*−*34*). In particular, the three main positive bands at ∼1728 cm^{-1} (carboxylic acid region), 1651 cm^{-1} (protein backbone region), and 1479−1480 cm^{-1} (semiquinone region), as well as the negative bands at 1640 cm^{-1} (protein and quinone carbonyls) and at 1265 cm^{-1} and ∼1290 cm^{-1} (Q_B methoxy groups) appear in all of the spectra.

In the spectra of the EQ H173 and ED H173 mutants, several differences are however observed for the position,

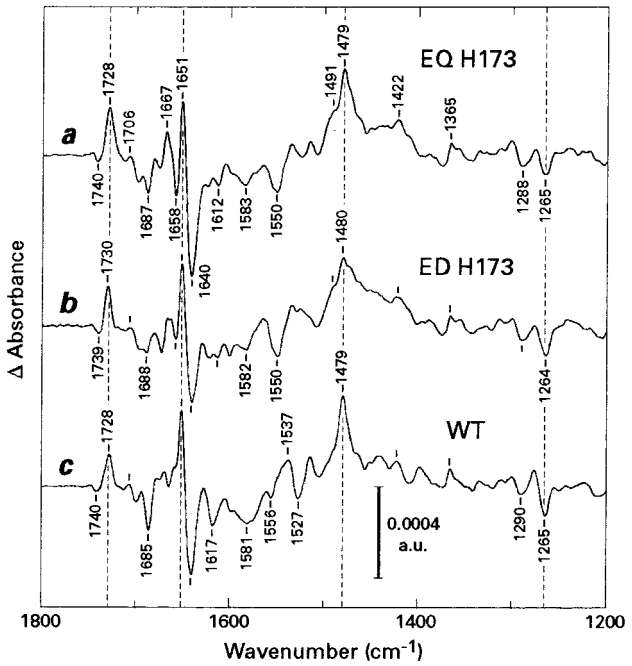

FIGURE 2: Light-induced Q_B^-/Q_B FTIR spectra of *Rb. sphaeroides* RCs at pH 7 in ^1H_2O: (a) Glu H173 → Gln mutant (EQ H173), (b) Glu H173 → Asp mutant (ED H173), (c) wild type (WT); 15 °C, 4 cm^{-1} resolution, a.u. is absorbance units. About 80 000 and 50 000 scans were averaged for mutant and wild-type RCs, respectively.

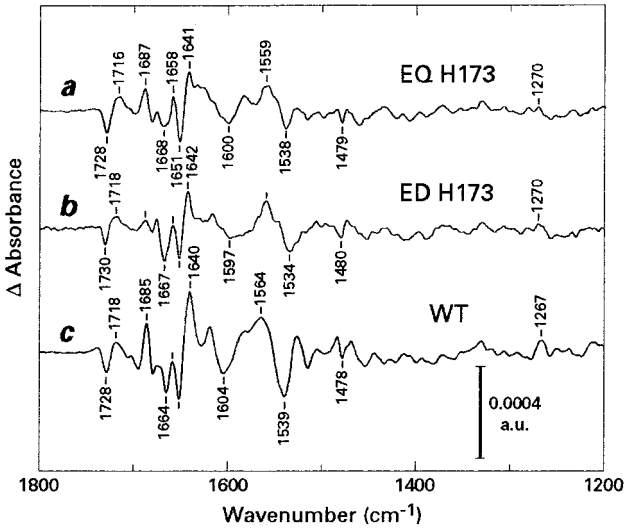

FIGURE 3: Calculated double-difference spectra between Q_B^-/Q_B difference spectra of *Rb. sphaeroides* RCs obtained in ^2H_2O and ^1H_2O (i.e., ^2H_2O minus ^1H_2O): (a) Glu H173 → Gln mutant (EQ H173); (b) Glu H173 → Asp mutant (ED H173); (c) wild type (WT).

half-width, and amplitude of some of the bands described above, compared to those for native RCs. The most obvious differences are in the absorption region of the semiquinone between ~1500 and 1430 cm^{-1}. In particular, both Q_B^-/Q_B mutant difference spectra display an additional shoulder at 1491 cm^{-1} on the main quinone anion band, which peaks at 1479 cm^{-1} in EQ H173 (Figure 2a) and at 1480 cm^{-1} in ED H173 (Figure 2b).[2] In addition, the 1480 cm^{-1} band in ED H173 is significantly perturbed on the low-wavenumber side. When Q_B^-/Q_B difference spectra from mutant and native RCs are compared, the spectra are normalized based on the semiquinone and methoxy bands by minimizing the net difference between the mutant and native RC spectra in these regions; the normalization factor can be varied by ±10% without affecting significantly the shape of the bands in the double-difference spectra (data not shown).

Changes in the Q_B^-/Q_B difference spectra of mutant RCs compared to native RCs are also apparent between 1700 and 1500 cm^{-1} (Figure 2). For example, a differential signal at 1667/1658 cm^{-1}, which is in the absorption range of peptide/side chain groups, is observed in the spectrum of EQ H173. In the amide II region, a negative band is present at 1550 cm^{-1} in both EQ H173 and ED H173 spectra in contrast to a positive band at 1537 cm^{-1}, a negative band at 1527 cm^{-1}, and a small negative band at 1556 cm^{-1} in native RCs.

In the carboxylic acid absorption region between 1770 and 1700 cm^{-1} (Figure 2), native and mutant RCs show common signals with a main positive band at 1728−1730 cm^{-1} and small signals at ~1740 (−) and 1706 (+) cm^{-1}. In EQ H173, the amplitude of the 1728 cm^{-1} band is larger than that in native RCs with a comparable fwhm (~13 cm^{-1}). In ED H173, the main carboxylic band peaks at 1730 cm^{-1} and is narrower (~10 cm^{-1} fwhm) than the 1728 cm^{-1} band in native RCs. The integrated absorption over the width of the 1728−1730 cm^{-1} band determined for all RCs yields a larger value (45 ± 10%) in the EQ H173 spectrum compared to that for wild-type RCs. Given the broadness of the semiquinone band in the ED H173 spectrum and hence the possible uncertainty in the normalization factor of the Q_B^-/Q_B spectra, the intensity of the 1730 cm^{-1} band in the Q_B^-/Q_B spectrum of ED H173 is considered to be comparable to that of the 1728 cm^{-1} band for the native RCs.

The Q_B^-/Q_B difference spectra were also obtained in ^2H_2O to identify bands that are sensitive to isotopic exchange. Figure 3 shows the Q_B^-/Q_B double-difference spectra (Q_B^-/Q_B difference spectra obtained in ^2H_2O minus Q_B^-/Q_B difference spectra obtained in ^1H_2O). In these double-difference spectra, only the IR modes that both are affected by the reduction of Q_B *and* are sensitive to $^1H/^2H$ isotopic exchange will give rise to shifted bands and/or differential signals (*12*). Note that the overall shapes of the three double-difference spectra for EQ H173, ED H173, and native RCs are comparable with a differential signal at ~1651/1641 cm^{-1} and a negative band at 1534−1539 cm^{-1}, most probably due to shifts of protein modes upon $^1H/^2H$ exchange. Importantly, a similar frequency downshift of the carboxylic acid band at 1728−1730 cm^{-1} in ^1H_2O to ~1718−1716 cm^{-1} in ^2H_2O is observed for mutant and native RCs; the amplitude of the band is larger in EQ H173 and somewhat smaller in ED H173 compared to that in native RCs. In native RCs, the differential signal at 1728/1718 cm^{-1} in the double-difference spectra ^2H_2O minus ^1H_2O has been previously attributed to the $^1H/^2H$ isotope shift of the C═O mode of the carboxylic group of Glu L212 that becomes protonated upon Q_B^- formation (*12*).

[2] Reconstitution of Q_B-depleted RCs with uniformly ^{13}C-labeled ubiquinones has previously allowed the whole 1479 cm^{-1} band to be assigned to semiquinone modes in native RCs from *Rb. sphaeroides* (*33*).

DISCUSSION

In this work, we report the steady-state Q_B^-/Q_B FTIR difference spectra at pH 7 from mutant RCs in which Glu H173 was replaced with Gln and Asp (EQ H173 and ED H173, respectively). From a comparison of mutant spectra to the native spectrum, information about changes in protonation and/or the local environment of carboxylic acid residues that occur upon Q_B^- formation was obtained. In addition, some information about changes in the interaction between the semiquinone and the protein and in the backbone structure/flexibility in the mutant RCs could be revealed. We start with a discussion of the carboxylic acid region of the steady-state FTIR difference spectra and the implications on proton uptake by carboxylic acid groups in response to the formation of Q_B^-. We end with a comparison of electron-transfer kinetics and FTIR data to probe electrostatic changes, structural changes, and/or alterations of semiquinone−protein interactions following photoreduction of Q_B.

Protonation of Carboxylic Acid Groups in Response to the Formation of Q_B^-. The FTIR data show that Glu H173 does not significantly change its protonation state upon reduction of Q_B to Q_B^-. The positive band at 1728 cm^{-1}, previously assigned to protonation of Glu L212 (*12*, *13*), is observed in the Q_B^-/Q_B FTIR difference spectrum of the EQ H173 mutant (Figure 2a) and is similarly shifted to ∼1716 cm^{-1} upon $^1H/^2H$ exchange (Figure 3a) as is observed in native RCs (Figure 3c). In this mutant, any signal due to protonation of Glu H173 should be absent. The similarity of the 1728 cm^{-1} band in the EQ H173 spectrum to that of the native suggests that Glu H173 does not contribute directly to the 1728 cm^{-1} band. Thus, the assignment of the 1728 cm^{-1} band to proton uptake by Glu L212 is further strengthened by these observations. In the ED H173 mutant, the comparable amplitude of the peak at 1730 cm^{-1} to that observed at 1728 cm^{-1} in native RCs suggests a comparable protonation of Glu L212 to that observed in native RCs. The 2 cm^{-1} frequency upshift and the small narrowing of the carboxylic band at 1730 cm^{-1} can be attributed to slight differences in the environment of the COOH side chain group of Glu L212 in the ED H173 mutant compared to that in the native RCs. The only other signals at ∼1740 (−) and 1706 (+) cm^{-1} are not significantly influenced by the mutation of Glu H173 to Gln or Asp. Thus, no band can be assigned to a significant change ($\geq$0.05 H^+/Q_B^-) in the protonation of Glu H173 in the Q_B^-/Q_B spectrum of native RCs at pH 7. Consequently, we conclude that Glu H173 does not change ionization state upon Q_B^- formation.

To obtain information about the ionization state of Glu H173 in the ground state of native RCs, we examine the effect of mutation on the amplitude of the 1728 cm^{-1} band, which should respond to changes in the local electrostatic environment. The larger amplitude of the 1728 cm^{-1} band in the EQ H173 compared to that in native RCs (45 ± 10% larger) is reminiscent of results from mutant RCs in which Asp L213 was replaced with Asn, Leu, His, or Ser (*12*, *13*), which was attributed to the replacement of a negatively charged Asp with a neutral residue. The larger amplitude has been attributed to increased proton uptake by Glu L212 in these mutant RCs upon Q_B reduction. (*12*, *13*). The greater protonation of Glu L212 in the EQ H173 mutant RCs can similarly be taken to indicate that a negatively charged acid residue at H173 in native RCs has been replaced by a neutral side chain in the mutant, leading to a greater protonation of Glu L212 upon Q_B reduction. In contrast to the EQ H173 data, the FTIR difference spectrum of ED H173 shows no significant change in the proton uptake by Glu L212 since the amplitude of the 1728−1730 cm^{-1} band is roughly equivalent in both ED H173 and native RCs. Using a simple electrostatic explanation, this observation leads to the conclusion that Asp H173 in ED H173 and Glu H173 in native RCs are negatively charged. Thus, from the FTIR data, we propose that in native *Rb. sphaeroides* RCs at pH 7, Glu H173 is mostly ionized in both the Q_B and Q_B^- states.

The experimental data are in qualitative agreement with the results of the two available electrostatic calculations of the proton uptake upon Q_B reduction in the RC from *Rb. sphaeroides* (*21*, *22*), which predict that the proton uptake is dominated by Glu L212 (>95% of the total calculated proton uptake). The titration curve calculated for Glu H173 by Beroza et al. (1995) indicates that Glu H173 is mostly ionized in the Q_B state at pH 7, titrating only in the low pH range (*22*). Since the calculated proton uptake from Glu H173 is estimated to change by less than 0.1 H^+/Q_B^- (*22*), it follows that Glu H173 remains mostly ionized in the Q_B^- state as deduced from the present FTIR data.

More recently, two sets of detailed electrostatic calculations of the protonation state of several residues near Q_B in the RC from *Rp. viridis* have been performed (*23*, *24*). These calculations predict proton uptake by both Glu L212 and Glu H177, the homologous residue to Glu H173 in *Rb. sphaeroides*. Lancaster et al. (*23*) calculated that most of the proton uptake associated with Q_B^- formation could be attributed to a carboxylic acid cluster consisting of Glu L212, Glu H177, and Glu M234. Almost equal proton uptake (0.12 and 0.17 H^+/Q_B^-) was estimated for Glu H177 and Glu L212, respectively. In contrast, Rabenstein et al. (*24*) calculate that Glu L212 does not contribute significantly to the proton uptake at pH 7.5, where the main contributor is Glu H177 (0.56 H^+/Q_B^-). These authors further suggest that in *Rb. sphaeroides* RCs, the kinetic IR transient at 1725 cm^{-1} assigned to protonation of Glu L212 (*15*) could alternatively be due to protonation of Glu H173. However, the present FTIR data on the EQ H173 and ED H173 mutant RCs support our previous attribution of the 1728 cm^{-1} signal in the Q_B^-/Q_B spectrum of native RCs to protonation of Glu L212 and rule out the possibility that significant proton uptake by Glu H173 occurs at pH 7 at 1728 cm^{-1} upon reduction of Q_B in *Rb. sphaeroides.* It should be noted that large differences are seen in the Q_B^-/Q_B FTIR difference spectra of *Rp. viridis* and *Rb. sphaeroides* (*32*, *33*, *35*, *36*). Thus, calculations performed on *Rp. viridis* may not be applicable to *Rb. sphaeroides*. In particular, the Q_B^-/Q_B spectra of *Rp. viridis* RCs in ^1H_2O and ^2H_2O show no evidence for proton uptake by carboxylic groups upon Q_B reduction (*35*, *36*) and thus do not provide support for the results of electrostatic calculations in *Rp. viridis* RCs (*23*, *24*).

The Q_B^-/Q_B FTIR spectra of native *Rb. sphaeroides* RCs only show a large positive band at 1728 cm^{-1} in the carboxylic acid region, which is clearly attributed to protonation of Glu L212. Part of the small negative signal at 1740 cm^{-1} has been tentatively assigned to a change of environment of protonated Asp L210 based on the effects

of the mutation Asp L210 → Asn and of $^1H/^2H$ exchange (*12*). More generally, the FTIR spectra of mutant and native RCs do not show evidence for protonation of another carboxylic acid group. Does this mean that other carboxylic acid groups do not change protonation state in response to the formation of Q_B^-? The FTIR results indeed suggest this possibility. It cannot be excluded, however, that some carboxylic acid groups have a small contribution to proton uptake (≤ 0.05 H^+/Q_B^-) or that their bands are significantly shifted out of the normal carboxylic acid region, possibly in response to strong interactions with other groups (e.g., another carboxylic acid). Such groups if shifted to lower wavenumbers would be difficult to identify, since their bands would overlap with bands from the protein. It is also possible that some carboxylic acid groups have a broader line, making their observation more difficult. Finally, still another possibility would be that transient protonation of carboxylic groups occurs, but it is not detected in the steady-state measurements reported here. However, kinetic IR measurements on native RCs and the EQ L212 (Glu L212 → Gln) mutant with microsecond resolution (*15*) do not favor this hypothesis.

Comparison of FTIR and Kinetic Electron-Transfer Data. A simple electrostatic model consisting of two interacting point charges, i.e., Glu L212 and Glu H173, can explain the FTIR results of the H173 mutant RCs (as discussed above). However, such a simple model is insufficient to explain all of the optical kinetic data. The rate of charge recombination k_{BD} between the photooxidized primary electron donor D^+ and Q_B^- ($D^+Q_AQ_B^- \rightarrow DQ_AQ_B$) has been taken as a measure of the electrostatic environment of Q_B, i.e., the more negative the electrostatic potential near Q_B the faster the observed rate (*11*). Identical and elevated values for k_{BD} were obtained for the two mutants in the pH range from 5 to 8 compared to those for native RCs (unpublished data) suggesting a more negative electrostatic potential around Q_B. This is seemingly inconsistent with the presence of a neutral Gln side chain in the EQ H173 RCs. Thus, the simple electrostatic model seems insufficient to explain the kinetic data. A more complicated model involving many coupled acids acting as a single group was presented to explain the larger value of k_{BD} observed in the EQ H173 RCs (*18*). This model, however, does not easily accommodate the larger value of k_{BD} observed in the ED H173 RCs (unpublished data). It is likely that factors other than the electrostatic environment can influence k_{BD}, such as structural changes around Q_B or altered bonding interactions for Q_B^- (*37*). It thus appears that FTIR difference spectroscopy, as reflected by variations in the proton uptake by Glu L212 monitored at 1728 cm^{-1}, and transient kinetic experiments, such as the measurement of k_{BD}, can probe different properties of the Q_B environment.

The Q_B^-/Q_B FTIR difference spectra provide, in addition to the electrostatic-sensitive 1728 cm^{-1} band, information on semiquinone−protein interactions and backbone/side chain rearrangements (*14, 33*). We have recently reported FTIR studies of double mutant RCs carrying a suppressor mutation of the Asp → Asn L213 lesion, for example a Asn M44 → Asp, Arg M233 → Cys, or Arg H177 → His compensatory mutation (*14*). FTIR difference spectra of Q_B reduction in these suppressor mutants have provided direct evidence of structural changes accompanying the restoration of efficient proton transfer in addition to the changes in the electrostatic environment of Q_B (*14, 37*). In particular, perturbations of several protein and semiquinone modes were observed in these suppressor mutants. In the EQ H173 and ED H173 mutant RCs presented in this work, the new shoulder seen at 1491 cm^{-1} on the main semiquinone band at 1479 cm^{-1} of the EQ H173 spectrum possibly reflects a slight perturbation of the interactions of the semiquinone with the protein. Larger perturbations of the semiquinone−protein interactions are expected in the ED H173 mutant as indicated by the large change observed in the shape of the semiquinone band. Studies using mutant RCs reconstituted with isotope-labeled quinones will be necessary to investigate such alterations in detail. It is also worth mentioning that slight changes in the amide/side chain modes can be observed in the Q_B^-/Q_B spectra of the EQ H173 and ED H173 mutant RCs, with respect to native RCs. Notably, absorption changes are different in the 1620−1535 cm^{-1} region of mutant and native RCs (Figure 2). In the EQ H173 spectrum, the differential signal at 1667/1658 cm^{-1} could involve a shift of a peptide carbonyl group. All these effects could be related to small structural changes occurring in the EQ H173 and ED H173 mutants upon Q_B reduction, which can contribute to changes in the electron-transfer rate constants measured in optical kinetic experiments. In the high-resolution crystal structures of the native RC from *Rb. sphaeroides* (*Rp. viridis*), Glu H173 (Glu H177) is hydrogen bonded to a structural water molecule (*5*−*7, 38, 39*). Possible rearrangements could be directly or indirectly caused by the loss of the hydrogen bond between the water molecule and H173 upon replacement with Gln or Asp.

From analysis of the present FTIR results from RCs with mutations at the H173 site and previous FTIR results from RCs with mutations at other sites in the L subunit (*12*−*14*), Glu H173 and Asp L213 appear to be mostly ionized in both the Q_B and Q_B^- states and do not significantly contribute to proton uptake in native RCs at pH 7. It thus appears that protonation of Glu L212, which gives rise to the 1728 cm^{-1} band in the steady-state Q_B^-/Q_B FTIR difference spectra, is the main contributor to proton uptake upon Q_B^- formation in *Rb. sphaeroides*.

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