Accelerated Publications

Identification of a Novel Protonation Pattern for Carboxylic Acids upon Q_B Photoreduction in *Rhodobacter sphaeroides* Reaction Center Mutants at Asp-L213 and Glu-L212 Sites†

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ABSTRACT: In the reaction center from the photosynthetic purple bacterium *Rhodobacter sphaeroides*, light energy is rapidly converted to chemical energy through coupled electron-proton transfer to a buried quinone molecule Q_B. Involved in the proton uptake steps are carboxylic acids, which have characteristic infrared vibrations that are observable using light-induced Fourier transform infrared (FTIR) difference spectroscopy. Upon formation, Q_B^- induces protonation of Glu-L212, located within 5 Å of Q_B, resulting in a IR signal at 1728 cm^{-1}. However, no other IR signal is observed within the classic absorption range of protonated carboxylic acids (1770−1700 cm^{-1}). In particular, no signal for Asp-L213 is found despite its juxtaposition to Q_B and importance for proton uptake on the second electron-transfer step. In an attempt to uncover the reason behind this lack of signal, the microscopic electrostatic environment in the vicinity of Q_B was modified by interchanging Asp and Glu at the L213 and L212 positions. The Q_B^-/Q_B FTIR spectrum of the Asp-L212/Glu-L213 swap mutant in the 1770−1700 cm^{-1} range shows several distinct new signals, which are sensitive to $^1H/^2H$ isotopic exchange, indicating that the reduction of Q_B results in the change of the protonation state of several carboxylic acids. The new bands at 1752 and 1747 cm^{-1} were assigned to an increase of protonation in response to Q_B reduction of Glu-L213 and Asp-L212, respectively, based on the effect of replacing them with their amine analogues. Since other carboxylic acid signals were observed, it is concluded that the swap mutations at L212 and L213 affect a cluster of carboxylic acids larger than the L212/L213 acid pair. Implications for the native reaction center are discussed.

In the reaction center (RC)[1] from the photosynthetic purple bacterium *Rhodobacter* (*Rb.*) *sphaeroides*, proton-coupled electron-transfer reactions that lead to the reduction of the secondary quinone molecule Q_B are an essential step for energy conversion (*1*−*3*). Light-induced electron transfer is initiated from the primary electron donor D (a dimer of bacteriochlorophyll) through a series of electron acceptors to the loosely bound quinone Q_B (ubiquinone-10). The double reduction of Q_B to quinol (Q_BH_2) takes place in a two-step process and requires the transfer of two electrons coupled to the uptake of two protons from the solution (*4*). The first electron transfer to Q_B is accompanied by proton uptake by the RC protein (*5*−*7*); however, no measurable protonation of the semiquinone occurs. The second electron transfer leads to the direct protonation of the quinone and the formation of Q_BH_2.

The details of the proton-transfer pathway(s) were investigated by structural (*4*, *8*−*12*), mutational (*1*−*3*), and metal binding (*13*−*16*) studies in isolated RCs. Three residues, Asp-L213 (*17*−*19*), Ser-L223 (*20*, *21*), and Glu-L212 (*18*, *22*, *23*), were shown to be crucial for rapid electron-proton transfer to reduced Q_B (Figure 1). Delivery of the first proton to reduced Q_B involves Asp-L213 and Ser-L223, whereas delivery of the second proton involves Asp-L213 and Glu-L212 (*22*−*24*). The pathways share the involvement of the surface residues His-H126 and His-H128 (*25*), which constitute the entry point as shown by the inhibition of proton uptake upon binding Zn^{2+} or Cd^{2+} (*13*, *14*, *16*). The proton pathways also share the involvement of the intermediate carboxylic acids Asp-L210 and Asp-M17 (*26*, *27*). Bound water molecules facilitate proton transfer between the acid groups (Figure 1).

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[1] Abbreviations: RC, reaction center; Q_B, secondary quinone acceptor; UQ_n, ubiquinone-*n*, 2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone; D, primary electron donor; FTIR, Fourier transform infrared; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodospeudomonas.*

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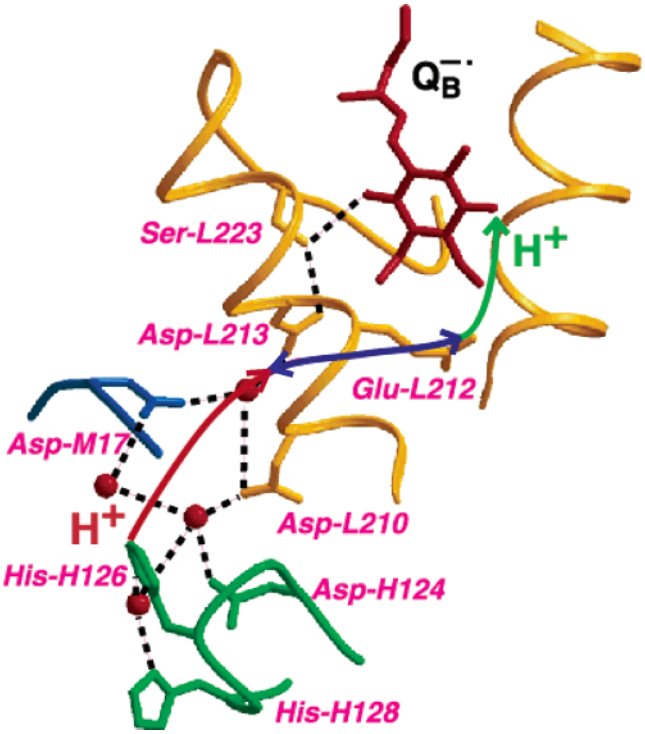

FIGURE 1: Structure of the reaction center from *Rb. sphaeroides* near the secondary quinone Q_B. The arrows represent the general proton flow to Asp-L213 and Glu-L212. As discussed in the text, the extent of proton sharing between the carboxylic acids depends on the details of the electrostatic interactions of these acids near Q_B. More specifically, the blue double-sided arrow concerns the Asp-L212/Glu-L213 swap mutant where the proton is clearly shared between several carboxylic acids, in particular L212 and L213. The red spheres represent bound water molecules.

In *Rb. sphaeroides*, although extensive structural, experimental, and theoretical work has been performed, there is no consensus on the protonation states of the two key residues Asp-L213 and Glu-L212. The earliest indications on the protonation state of Asp-L213 came from kinetic measurements of the charge recombination rate $D^+Q_B^- \rightarrow DQ_B$ (k_{BD}). Upon replacing Asp-L213 with Asn, k_{BD} was greatly decreased showing a more stable $D^+Q_B^-$ state and indicating that Asn stabilized Q_B^-. Thus, it was inferred that Asp-L213 must be ionized in the native RC and does not contribute to proton uptake (*17*−*19*). The simplest interpretation of early kinetic measurements made on native RCs and mutants with modifications at Glu-L212 indicated that Glu is protonated at neutral pH and contributes to proton uptake only at high pH (*18*, *22*, *28*−*30*). Subsequent electrostatic calculations have suggested that upon Q_B reduction both Glu-L212 and Asp-L213 should protonate (at least partially) because of the relatively large electrostatic interaction between them and Q_B^- (*31*−*39*); the exact amount of proton uptake is quite variable and depends on the details of the structure and theoretical treatment. However, in general, the calculations show that a cluster of acids composed of Asp-L210, Asp-L213, and Glu-L212, share one proton in the Q_B state and two protons in the Q_B^- state, although the exact location of the protons is not well-established. It becomes difficult to rationalize the calculated protonation changes with the early interpretation of the kinetic results.

In an attempt to directly measure the protonation changes of the carboxylic acids, we used Fourier transform infrared (FTIR) difference spectroscopy (*40*, *41*). Bands arising from the C═O stretching mode of protonated side chains of Asp and Glu are found in a clear region between 1770 and 1700 cm^{-1} and are sensitive to $^1H/^2H$ isotopic exchange (*42*). The earliest experiments showed that when Q_B^- is formed, substoichiometric protonation of Glu-L212 occurs at pH 7 (*43*, *44*). This was shown by the appearance of a positive band at 1728 cm^{-1} in the $Q_B^- - Q_B$ FTIR difference spectrum (henceforth referred to as the Q_B^-/Q_B spectrum) that was sensitive to $^1H/^2H$ isotopic exchange and absent when Glu-L212 was replaced with Gln or Ala (*43*−*47*). Therefore, it appears that Glu-L212 is at least partially ionized at neutral pH in the Q_B ground state and becomes fully protonated upon Q_B reduction. However, no other spectral changes were found in the FTIR spectrum between 1770 and 1700 cm^{-1}. Thus, no signal from any other carboxylic acid could be identified in the Q_B^-/Q_B spectra of the native or of a number of mutant RCs (*43*−*50*). Although these results are consistent with theoretical electrostatic calculations by showing evidence for nonclassic titration behavior, they do not provide evidence for the extent of proton uptake that the calculations indicate.

The possibility of changing the microscopic electrostatic environment near Q_B without perturbing the overall macroscopic environment offers a novel strategy to probe protonation patterns of internal carboxylic acids. This was accomplished by interchanging Asp and Glu at L212 and L213 (*51*). Kinetic measurements on the Asp-L212/Glu-L213 mutant RC suggested that the overall electrostatic environment near Q_B was similar to the native RC, but there was a difference in the microscopic electrostatic environment attributed to differences in the state of ionization of Asp and Glu at either L212 or L213 (*51*). This mutant became the basis of the present study and led to the identification of a novel protonation pattern of carboxylic acids near Q_B.

In this paper, the effect of replacing Glu-L212 with Asp and Asp-L213 with Glu in the *Rb. sphaeroides* RC was investigated by monitoring the light-induced FTIR absorption changes associated with the photoreduction of Q_B. The Asp-L212/Glu-L213 mutant RC (henceforth referred to as the swap mutant) provided several new previously unobserved features in the 1770−1700 cm^{-1} IR region of protonated carboxylic acids. To assign these new bands, two additional double mutant RCs were constructed, which retained only one of the mutant carboxylic acids with the other one replaced by its amine analogue. The Q_B^-/Q_B spectra of the double mutants **Asn**-L212/Glu-L213 and Asp-L212/**Gln**-L213 were thus compared to that of the Asp-L212/Glu-L213 RC. Information about the changes in ionization state of Asp-212 and Glu-L213 in the swap mutant is provided by examining the effects of both the mutations and $^1H/^2H$ isotopic exchange in the 1770−1700 cm^{-1} IR region. A brief account of part of this work has been presented (*52*).

EXPERIMENTAL PROCEDURES

The construction of the site-directed mutant Asp-L212/Glu-L213 was previously described in refs *19* and *51*. The construction of the Asp-L212/**Gln**-L213 and **Asn**-L212/Glu-L213 mutants was performed as described (*27*) using oligonucleotides carrying the desired nucleic acid changes. RCs were isolated in *N*,*N*′-dimethyldodecylamine *N*-oxide

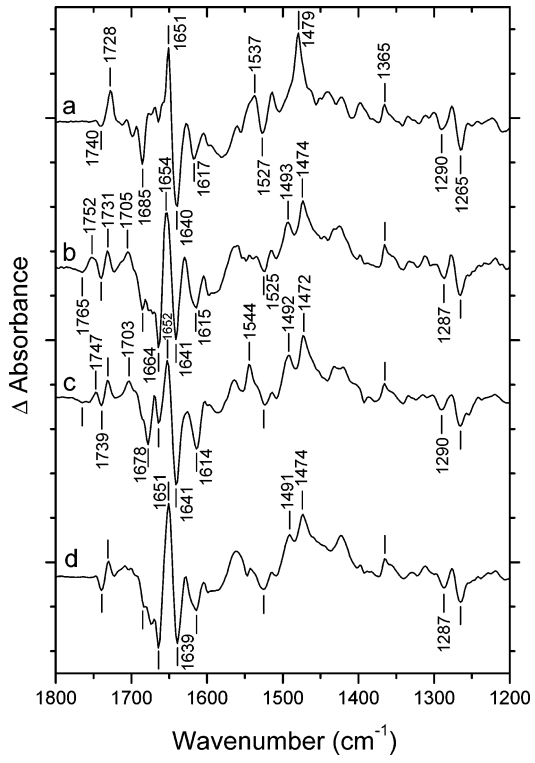

FIGURE 2: Light-induced Q_B^-/Q_B FTIR difference spectra at pH 7 and 15 °C of *Rb. sphaeroides* RCs in ^1H_2O: (a) native, (b) Asp-L212/Glu-L213, (c) Asp-L212/**Gln**-L213, (d) **Asn**-L212/Glu-L213. Spectral resolution was 4 cm^{-1}. Between 50 000 and 100 000 interferograms were averaged. The frequency of the peaks is given at ±1 cm^{-1}. The minor tick marks on the vertical axis are separated by 2×10^{-4} absorbance units. For the native RC, data are taken from Nabedryk et al. (*43*).

(LDAO) as previously described (*53*). A detailed description of the preparation of RC samples for FTIR experiments is given in refs *43* and *49*); 10 μL of an RC sample (~0.2 mM) containing a 10-fold excess of UQ_{10} for Q_B reconstitution was deposited on a CaF_2 disk, and then 10 μL of sodium ascorbate (10 mM) and 20 mM diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine) in 90 mM Tris-HCl at pH 7 was added. The RC sample was partially dried under argon to a thin paste and then covered with 2 μL of ^1H_2O and sealed with another CaF_2 disk. The preparation of RC samples in ^2H_2O was carried out as reported in ref *49*.

Steady-state light-induced FTIR difference spectra of the $Q_B \rightarrow Q_B^-$ transition in mutant RCs were recorded at 15 °C in ^1H_2O or ^2H_2O, with a Nicolet 60SX spectrometer, as described in refs *43* and *49*. The Q_B^- state was generated by excitation with a single-turnover saturating flash (Nd:YAG laser, 7 ns, 530 nm). Difference spectra were calculated from each 128 scans (acquisition time = 23 s) recorded before and after laser-flash excitation. For a given sample, these measurements were repeated over 15−20 h. Spectra are an average of 2−3 samples.

RESULTS

The Q_B^-/Q_B light-induced FTIR difference spectra for the mutant RCs were measured in ^1H_2O (Figures 2 and 3A) and ^2H_2O (Figure 3B). In these spectra, negative bands originate from the neutral Q_B state and positive bands arise from the Q_B^- state. Vibrational bands from the neutral quinone C=O and C=C modes are expected in the range 1660−1600 cm^{-1} (*54*−*57*), the semiquinone Q_B^- modes in the range 1500−1400 cm^{-1} (*54*−*57*), and the protein contribution (backbone and side chains) in the ranges ~1700−1600 cm^{-1} (amide I), 1520−1570 cm^{-1} (amide II) (*58*), and 1770−1700 cm^{-1} (protonated side chains of Asp and Glu carboxylic acids) (*42*, *59*, *60*). Because the protonation signals are of particular interest, we will examine them separately.

Q_B^-/Q_B FTIR Spectra of the Asp-L212/Glu-L213 Swap Mutant. The Q_B^-/Q_B spectrum of the Asp-L212/Glu-L213 RC (Figure 2b) displays several differences with respect to that of the native RC (Figure 2a). In the semiquinone range, it has two peaks at 1493 and 1474 cm^{-1}, instead of the main single anion band seen at 1479 cm^{-1} (Figure 2a). In the protein-absorption range, there are noticeable changes at 1664, 1654, and ~1637 cm^{-1}. The most remarkable differences appear in the protonated carboxylic acid region in which new signals are observed; three positive peaks are observed at 1752 (with a clear shoulder at ~1747 cm^{-1}), 1731, and 1705 cm^{-1} compared with the single prominent positive peak at 1728 cm^{-1} in the native RC (*43*). In addition, a new broad negative feature occurs at 1765 cm^{-1}.

To assess the protonation signals from other protein or cofactor contributions in the 1770−1700 cm^{-1} range, the sensitivity of the signal to ^2H_2O was tested. A detailed comparison of the spectra obtained in ^1H_2O and ^2H_2O is shown in parts A and B of Figure 3, respectively. In ^2H_2O, the Q_B^-/Q_B spectrum of the swap mutant (part b of Figure 3B) shows two positive signals at 1746 and 1731 cm^{-1}, with a trough between them at 1739 cm^{-1}. The calculated double-difference spectrum $^1H_2O - {}^2H_2O$ (part b of Figure 3C) reveals two differential signals at 1753(+)/1743(−) cm^{-1} and 1733(+)/1724(−) cm^{-1}; i.e., the bands are shifted by ~10 cm^{-1}. Since a shift of this magnitude is expected for protonated carboxylic acids accessible to the solvent (*42*), these bands are assigned to an increase in protonation of carboxylic acids in response to Q_B reduction. To determine if L212 or L213 contribute to the new signals, we constructed analogous mutants with either Gln-L213 (Asp-L212/**Gln**-L213) or Asn-L212 (**Asn**-L212/Glu-L213) and measured their Q_B^-/Q_B FTIR spectra. Initial attempts to assign the new protonation signals in the swap mutant involved FTIR studies of the corresponding single mutants, which retain only either Asp-L212 or Glu-L213. However, Q_B^- at pH 7 is unstable in the Asp-L212 mutant RC (*51*) making the measurement of steady-state FTIR Q_B^-/Q_B spectra unreliable. On the other hand, the carboxylic acid region from 1770 to 1700 cm^{-1} of the single Glu-L213 mutant RC is very similar to that of the native RC, and shows a single 1728 cm^{-1} band (*52*) that is downshifted by ~10 cm^{-1} in ^2H_2O (unpublished). This band is attributed to proton uptake by Glu-L212 upon Q_B^- formation. Thus, information essential for an FTIR assignment in the swap mutant could not be obtained from the studies of the single Asp-L212 and Glu-L213 mutant RCs.

*Q_B^-/Q_B FTIR Spectra of the Double Mutant Asp-L212/**Gln**-L213.* To overcome this problem, we created a double mutant RC, which retained Asp-L212, but has replaced Glu-L213 with Gln (Asp-L212/**Gln**-L213). The Q_B^-/Q_B spectrum of the Asp-L212/**Gln**-L213 mutant RC in ^1H_2O (Figure 2c) has many features in common with that of the swap mutant (Figure 2b). In particular, it shows a similar contribution of the Q_B^- anion modes at 1492 and 1472 cm^{-1}. Only small differences were observed in the protein-absorption regions

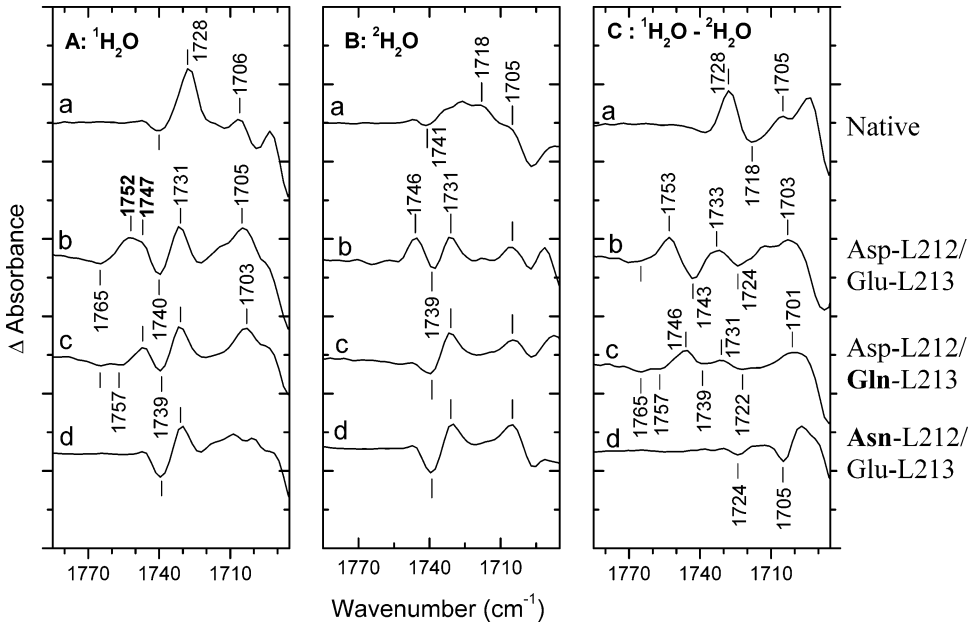

FIGURE 3: Comparison of the 1785−1685 cm^{-1} spectral region of the Q_B^-/Q_B spectra in (A) ^1H_2O and (B) ^2H_2O for (a) native, (b) Asp-L212/Glu-L213, (c) Asp-L212/**Gln**-L213, and (d) **Asn**-L212/Glu-L213 RCs. (C) Corresponding calculated double-difference spectra ^1H_2O − ^2H_2O. The minor marks on the vertical axis are separated by 10^{-4} absorbance units.

around 1678, 1664, and 1544 cm^{-1}. The main difference between these two mutants occurs in the region of protonated carboxylic groups with the loss of the peak at 1752 cm^{-1} and the appearance of an isolated positive signal at 1747 cm^{-1} (Figure 2c); this latter signal most probably corresponds to the shoulder seen at the same frequency on the low-energy side of the 1752 cm^{-1} peak in the Asp-L212/Glu-L213 RC (Figure 2b). In ^2H_2O, the 1747 cm^{-1} signal as well as the broad continuum at around 1770−1755 cm^{-1} disappear (part c of Figure 3B). Only a differential signal at 1739(−)/1731(+) cm^{-1} and a small positive signal at ∼1705 cm^{-1} are observed. The corresponding ^1H_2O − ^2H_2O double-difference spectrum (part c of Figure 3C) displays two small differential signals at 1746(+)/1739(−) and 1731(+)/1722(−) cm^{-1}, small negative features at 1765 and 1757 cm^{-1}, and a broad positive feature at ∼1701 cm^{-1}. Thus, compared to the previous swap mutant, which showed several new carboxylic acid signals, the replacement of Glu-L213 with Gln resulted in the loss of the peak at 1752 cm^{-1} and the appearance of a clearly isolated signal at 1747 cm^{-1}.

*Q_B^-/Q_B FTIR Spectra of the Double Mutant **Asn**-L212/Glu-L213.* We further studied a mutant that retains Glu-L213 but has replaced Asp-L212 with Asn (**Asn**-L212/Glu-L213). The Q_B^-/Q_B spectra of the **Asn**-L212/Glu-L213 mutant (Figure 2d) is very comparable to that of the swap mutant (Figure 2b), notably in the quinone, semiquinone, and protein amide regions. However, it is very different in the 1770−1700 cm^{-1} carboxylic acid range, which shows only a differential signal at 1739(−)/1731(+) cm^{-1} (Figure 2d) that is not sensitive to ^2H_2O (Figure 3d). Such a differential signal at 1739/1731 cm^{-1} has been previously observed in the Q_B^-/Q_B spectra of Q_B mutants, where Glu-L212 is substituted with Gln or Ala (*43*, *46*, *47*). Thus, the broad positive signal at 1752 cm^{-1}, its shoulder at 1747 cm^{-1}, and the small negative feature at 1765 cm^{-1}, which are present in the Asp-L212/Glu-L213 RC, are absent in the **Asn**-L212/Glu-L213 RC.

DISCUSSION

In this paper, we investigated protonation of internal carboxylic acids in response to Q_B reduction using FTIR difference spectroscopy in the isolated RC from *Rb. sphaeroides*. The native system was perturbed by replacing Glu-L212 with Asp and Asp-L213 with Glu.[2] The FTIR spectra are rich in information on the quinone and semiquinone interactions, protein changes, and protonation of internal carboxylic acids. Of particular interest is the carboxylic acid region of the modified system where several new bands appear in response to Q_B reduction. The sensitivity of these bands to $^1H/^2H$ exchange shows that they correspond to protonation of several internal acids. The strategy used to investigate these new carboxylic acid signals was to study two additional double mutant RCs, i.e., Asp-L212/**Gln**-L213 and **Asn**-L212/Glu-L213. The IR signals of the quinone, semiquinone, and general protein response are similar in the three double mutants. The most significant differences are in the carboxylic acid region. Below, we focus on possible assignments of the new carboxylic acid bands in the Asp-L212/Glu-L213 RC and the implications for the native system.

Protonation Changes of Carboxylic Acids at L212 and L213 Sites. In the carboxylic acid region from 1770 to 1700 cm^{-1}, the Q_B^-/Q_B spectra of the native RC shows a single prominent band at 1728 cm^{-1}, which was assigned to substoichiometric proton uptake by Glu-L212 upon Q_B reduction because of its absence in RCs lacking Glu at L212 (*43*, *44*, *46*, *47*). Analogously, this band is absent in RCs in which Glu-L212 was replaced with Asp or Asn (parts b−d of Figure 2). However, several new bands appear in the mutants; in particular, there are a large differential signal at

[2] A model of the swap mutation generated with Xfit using the RC crystal structure in ref *64* shows that the new side chains of Glu-L213 and Asp-L212 can be accommodated in the Q_B-binding site without any clashes of the side chains with the protein or the quinone and with only minor repositioning of water molecules (Fyfe, P. K., Jones, M. R., and Breton, J., unpublished results).

Table 1: Summary of Carboxylic Acid IR Signals[a] in the Mutant RCs

reaction center	Glu-L213	Asp-L212
Asp-L212/Glu-L213	1752 cm^{-1}	1747 cm^{-1}
Asp-L212/**Gln**-L213	absent	1747 cm^{-1}
Asn-L212/Glu-L213	absent	absent

[a] Assignment of a signal to a carboxylic acid is based on two criteria: its observation in the carboxylic acid region from 1770 to 1700 cm^{-1} of the FTIR spectrum and a characteristic frequency downshift in ^2H_2O. Other unidentified signals at 1731 (+) and 1765 (−) cm^{-1} are discussed in the text.

1753/1743 cm^{-1} and a smaller one at 1733/1724 cm^{-1} that are observed in the $^1H_2O - {}^2H_2O$ double-difference spectrum of the swap mutant (part b of Figure 3C) and that are possible signals resulting from protonation of Asp-L212 and/or Glu-L213. We discuss possible assignments below (see also Table 1).

Assigning Glu-L213. To identify the contribution of Glu-L213 in the Asp-L212/Glu-L213 RC (Figure 3b), we measured the Q_B^-/Q_B FTIR spectrum of the Asp-L212/**Gln**-L213 RC (Figure 3c). This latter mutant showed the absence of the highest frequency peak at 1752 cm^{-1} but the presence of peaks at 1747 and 1731 cm^{-1}, which are sensitive to $^1H/^2H$ exchange (Figure 3c). Thus, we can assign the 1752 cm^{-1} peak to the partial protonation of Glu-L213 upon Q_B^- formation (Table 1).

Assigning Asp-L212. Analogously, the contribution of Asp-L212 to the carboxylic acid signals observed in the Asp-L212/Glu-L213 RC (Figure 3b) can be obtained from a comparison to the FTIR spectrum of the **Asn**-L212/Glu-L213 RC (Figure 3d). The Q_B^-/Q_B spectrum in ^1H_2O of the **Asn**-L212/Glu-L213 RC displays a negative signal at 1739 cm^{-1} and a positive one at 1731 cm^{-1}, neither of which are sensitive to $^1H/^2H$ isotopic exchange (Figure 3d). Note that both the 1752 and 1747 cm^{-1} peaks are absent. Since the 1752 cm^{-1} was previously assigned to Glu-L213, we assign the 1747 cm^{-1} signal observed in both Asp-L212/Glu-L213 and Asp-L212/**Gln**-L213 to partial protonation of Asp-L212 upon Q_B reduction (Table 1). The absence of the Glu-L213 signal in the **Asn**-L212/Glu-L213 mutant indicates a strong coupling between L212 and L213 in the swap mutant, which upon replacing Asp-L212 with Asn affects the ionization state of Glu-L213 and thus quenches its protonation upon Q_B^- formation in the **Asn**-L212/Glu-L213 RC. Similarly, no spectral changes can be attributed to Glu-L213 in the Q_B^-/Q_B spectrum of the single Glu-L213 mutant RC at pH 7. From all of these observations, it can be concluded that the state of ionization of Glu-L213, i.e., its pK_a, is comparable in the single Glu-L213 and double **Asn**-L212/Glu-L213 mutants, while it is different in the swap mutant.

Environment of Asp-L212 and Glu-L213 in the Swap Mutant. The IR absorption frequencies for the two carboxylic acids near Q_B, 1747 cm^{-1} for Asp-L212 and 1752 cm^{-1} for Glu-L213, occur at higher energy than that of 1728 cm^{-1} for Glu-L212 in the native RCs. The frequency of the $\nu_{C=O}$ mode of the COOH group is very sensitive to the local environment, such as solvent polarity, hydrogen bonding, and other electrostatic interactions (*61*). Thus, the frequency shifts observed for Asp-L212 and Glu-L213 indicate differences in the polarity of the environment or in the interactions of the modified carboxylic acids with the protein in the mutants compared to that of Glu-L212 in the native protein.

Other Carboxylic Acid Bands. Other unanticipated signals appear in the FTIR spectra of the mutant RCs. The FTIR spectra of the Asp-L212/Glu-L213 and Asp-L212/**Gln**-L213 mutant RCs show bands at 1731 cm^{-1} (+) and near 1765 cm^{-1} (−) that are in part sensitive to $^1H/^2H$ exchange (Figure 3), leading to very small signals in the $^1H_2O - {}^2H_2O$ spectrum (part b and c of Figure 3C). These signals are likely from changes of a carboxylic acid upon Q_B reduction. Because the changes observed in ^2H_2O are very small, they could arise from protonation/deprotonation or environmental changes of acids located further from Q_B and which are hence less-perturbed by its reduction. Alternatively, the negative signal at 1765 cm^{-1} could be a proton donor to Asp-L212 in the swap mutant. In contrast, the 1739(−)/1731(+) cm^{-1} signal of the **Asn**-L212/Glu-L213 RC (Figure 3d), which is not sensitive to $^1H/^2H$ exchange, is not assigned to a change of a carboxylic acid (for a possible assignment of this signal, see refs *43* and *62*).

Why Are the Native RC and the Swap Mutant Different? In contrast to the native RC, the FTIR spectra of the swap mutant show a rich structure in the carboxylic acid region from 1770 to 1700 cm^{-1}. Whereas only one predominant protonation signal, assigned to Glu-L212, is observed in the native RC, several signals are observed in the mutant RC. On the basis of the most logical assignments of these signals, Asp-L212 and Glu-L213 contribute to the FTIR spectrum at 1747 and 1752 cm^{-1}, respectively. Why are the native RC and the swap mutant different? There are two most likely explanations. One explanation is that only Glu-L212 protonates in the native RC with the other carboxylic acids remaining in a fixed ionization state following Q_B^- formation. One should note, however, that this is not in agreement with most of the electrostatic calculations that show protonation (or internal proton movement) among several carboxylic acids upon Q_B^- formation (*31*−*39*). Although the details of the proton location are dependent on the starting parameters and algorithms, the idea of proton movement toward Q_B^- comes from basic electrostatic interactions. The observation of several bands in the swap mutant is consistent with this view; i.e., the proton is shared between several carboxylic acids, mainly those at the L212 and L213 sites (see Figure 1). Unless offset by other interactions that energetically compensate for the electrostatic term, proton movement would be expected in the native RC as well.[3]

A second explanation is that internal proton movement occurs but is not observed in the carboxylic acid region of the spectrum of the native RC. This would be possible if a proton donor and acceptor absorb at the same frequency

[3] It should be noted that electrostatic calculations performed on *Rhodopseudomonas* (*Rp.*) *viridis* RCs have also predicted proton uptake upon Q_B^- formation by a cluster of acids near Q_B (Glu-H177, Glu-M234, and possibly Glu-L212; see refs *65* and *66*). In contrast, the Q_B^-/Q_B spectrum of *Rp. viridis* RCs in the 1770 to 1700 cm^{-1} range does not show spectral changes attributable to proton uptake by carboxylic acids in response to Q_B reduction (*67*). Although Glu-L212 is present in *Rp. viridis*, this residue appears to protonate only in *Rb. sphaeroides*. Moreover, the small differential signal observed at 1727(−)/(+)1715 cm^{-1} in the calculated double-difference spectrum $^1H_2O - {}^2H_2O$ of *Rp. viridis* RCs indicates instead an environmental shift or even a slight deprotonation of Glu-L212 upon Q_B^- formation (*67*).

(signals would overlap) or the absorption peak is shifted to a different part of the normal IR spectral range. However, to our knowledge, there is no precedence for the latter hypothesis in a protein, and the native RC would represent the first such example. On the other hand, a broad positive continuum IR band around 2600 cm^{-1} in the Q_B^-/Q_B spectrum of native RCs suggested the presence of delocalized proton(s) in a highly polarizable hydrogen-bonded network; this IR continuum could reflect internal proton motion within the network (*63*).

CONCLUSIONS

The strategy to interchanging Asp and Glu at the L212 and L213 sites has permitted the identification of new protonation patterns of carboxylic acids upon Q_B^- formation in bacterial photosynthetic RCs from *Rb. sphaeroides*. The results observed in this study of the Asp-L212/Glu-L213 mutant RC show that the FTIR spectrum of the carboxylic acid region is very sensitive to the environment and can be quite different with what is often considered fairly conservative amino acid replacements (Asp with Glu and Glu with Asp). These data indicate strong interactions between several carboxylic acids in the Q_B site, in particular, between those at L212 and L213 sites, as previously suggested for native *Rb. sphaeroides* RCs (*31*−*39*). In addition to the observation of protonation of Glu-L213 (at 1752 cm^{-1}) and Asp-L212 (at 1747 cm^{-1}) upon Q_B^- formation in the swap mutant, the Q_B^-/Q_B spectrum also shows altered behavior of several other unassigned carboxylic acids. These results show that there is proton sharing among carboxylic acids in a system very similar to the native RC, which differs in the absorption frequency of protonating carboxylic acids and in the extent of proton sharing. Understanding this difference is of ultimate importance for elucidating proton-transfer reactions within the bacterial RC.

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NOTE ADDED AFTER ASAP POSTING

An earlier version of this paper posted to the ASAP website on May 15, 2004, had an incorrect symbol in the last paragraph, line 2 of Experimental Procedures. The symbol has been corrected in this new version posted May 24, 2004.

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