Fourier Transform Infrared Difference Spectroscopy of Secondary Quinone Acceptor Photoreduction in Proton Transfer Mutants of *Rhodobacter sphaeroides*†

E. Nabedryk,*,‡ J. Breton,‡ R. Hienerwadel,‡ C. Fogel,§ W. Mäntele,§ M. L. Paddock,|| and M. Y. Okamura||

Section de Bioénergétique, Département de Biologie Cellulaire et Moléculaire, CEA-Saclay, 91191 Gif-sur-Yvette Cédex, France, Institut für Physikalische und Theoretische Chemie, Universität Erlangen-Nürnberg, 91058 Erlangen, Germany, and Department of Physics, University of California, San Diego, La Jolla, California 92093-0319

Received July 20, 1995; Revised Manuscript Received September 18, 1995®

ABSTRACT: In order to investigate the changes of protonation or environment of carboxylic residues occurring upon photoreduction of the secondary quinone acceptor (Q_B) in the reaction center (RC) of the photosynthetic bacteria *Rhodobacter sphaeroides* 2.4.1., we have performed light-induced Fourier transform infrared (FTIR) spectroscopy on RCs from wild-type (Wt) and several site-directed mutants. The FTIR Q_B^-/Q_B spectra have been obtained at pH 7 upon single-saturating flash excitation for native RCs and RC mutants containing either a single-site mutation, with Gln at L212 (EQ L212), Asn at L213 (DN L213), or Asn at L210 (DN L210), or a double-site mutation with both Gln at L212 and Asn at L213 (EQ L212 + DN L213). The assignment of an IR band to the protonation/deprotonation of a particular carboxylic side chain was analyzed by combining the effects of site-directed mutagenesis and $^1H/^2H$ isotope exchange. A positive band at 1728 cm^{-1} in the Q_B^-/Q_B spectra was observed in Wt, DN L213, and DN L210 and was absent in the mutants EQ L212 and EQ L212 + DN L213. The intensity of the 1728 cm^{-1} band was significantly reduced in ^2H_2O, and a new feature appears at 1717 ± 1 cm^{-1}. Furthermore, the amplitude of the 1728 cm^{-1} band was similar in native and DN L210 RCs but was increased in DN L213. This band is attributed to partial proton uptake by Glu L212 estimated to be 0.3−0.4 H^+/Q_B^- in native and DN L210 RCs and 0.5−0.6 H^+/Q_B^- in DN L213 RCs. In contrast, the FTIR Q_B^-/Q_B spectra show no evidence for change of protonation or environment of Asp L213 upon Q_B^- formation. The increased protonation of Glu L212 in DN L213 RCs is explained by a decreased Glu L212 pK_a value due to the loss of a negatively charged Asp L213. Part of a small differential signal at 1732 (+)/1740 (−) cm^{-1} that is affected by $^1H/^2H$ exchange is tentatively assigned to an environmental shift of the protonated Asp L210. A negative signal at 1685 cm^{-1} is proposed to arise from the absorption change of the amide I carbonyl mode of Glu L212. The most important conclusions from these FTIR studies are that, after the first electron transfer step which forms Q_B^-, (1) Glu L212 increases its protonation state, consistent with its proposed function as a proton donor to the fully reduced quinone, and (2) Asp L213 is proposed to be in the ionized state, consistent with the increased proton uptake of Glu L212 in DN L213 RCs and with its function to facilitate proton transfer by creating a negative electrostatic potential near Q_B.

The photosynthetic bacterial reaction center (RC)[1] is a membrane spanning pigment−protein complex that performs the photochemical electron transfer from the primary donor (a dimer of bacteriochlorophyll) through a series of electron donor and acceptor molecules to Q_B. Q_B accepts electrons from Q_A in two sequential electron transfer reactions. The second electron transfer to Q_B is coupled to the uptake of two protons from the cytoplasmic phase, leading to the formation of the dihydroquinone Q_BH_2 that diffuses out of the RC [for reviews, see Feher et al. (1989) and Okamura and Feher (1992)].

The X-ray structure of the RC from *Rb. sphaeroides* shows that Q_B is deeply buried within the protein RC complex (Allen et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1994; Lancaster et al., 1995). The binding site of Q_B is formed by a loop of amino acid residues from the L subunit. In addition to the amino acids proposed to ligate the carbonyls of Q_B (His L190 for the proximal carbonyl oxygen and possibly Ser L223 or Ile L224 and Gly L225 for the distal carbonyl oxygen), the acidic residues Glu L212, Asp L213, and Asp L210 and the basic residue Arg L217 are also nearby (≤6 Å). The possibility that these amino acid residues could participate in the proton transfer pathway to Q_B within the protein matrix has been investigated by proton uptake and electron transfer measurements in native RCs and RC mutants containing site-directed modifications, leading to the replacement of protonatable groups with nonprotonatable ones (Okamura & Feher, 1992, 1995; Takahashi & Wraight, 1994). In particular, it has been shown that

† Part of this work was supported by an EEC grant (SCI* 0335-C) to J. Breton and W. Mäntele and a NIH grant (GM41637) to M. Y. Okamura.

* Address correspondence to Eliane Nabedryk, SBE/DBCM, Bât 532, CEA-Saclay, 91191 Gif-sur-Yvette Cédex, France. Fax: (331) 6908 8717. Telephone: (331) 6908 7112.

‡CEA-Saclay.

§ Universität Erlangen-Nürnberg.

|| University of California, San Diego.

® Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: RC, reaction center; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; *Rb.*, *Rhodobacter*; FTIR, Fourier transform infrared; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); $k_{AB}^{(1)}$ and $k_{AB}^{(2)}$, first and second electron transfer rate constants, respectively; k_{BD}, charge recombination rate constant from Q_B to the primary donor.

0006-2960/95/0434-14722$09.00/0

mutations of Glu L212 to Gln (Paddock et al., 1989; Takahashi & Wraight, 1992; Shinkarev et al., 1993), Asp L213 to Asn (Takahashi & Wraight, 1990, 1992; Paddock et al., 1994), and Ser L223 to Ala (Paddock et al., 1990) greatly reduced the rates of proton transfer associated with Q_B reduction, demonstrating that the protein plays an important role in proton transport. It has been suggested that the first proton to Q_B is transferred via Asp L213 and Ser L223, the second via Asp L213 and Glu L212 [reviewed in Okamura and Feher (1992)]. Bound water molecules may also be important. A high density of water has been predicted to be near the methoxy groups of Q_B (Beroza et al., 1992) in the vicinity of Glu L212, Asp L213, and Asp L210. In addition, in the most recent RC structure of *Rb. sphaeroides* (Ermler et al., 1994) and *Rhodopseudomonas viridis* (Deisenhofer et al., 1995; Lancaster et al., 1995), a chain of ordered water molecules (up to 14 fixed water molecules) extending from the cytoplasm surface through the protein to near Q_B was described.

Certain aspects of the role of the protein in proton transfer to Q_B can be ascertained by infrared spectroscopy. Vibrational spectroscopy has previously permitted the direct detection of transient light-induced proton binding and release at side chains of specific residues in the proton transport protein bacteriorhodopsin [for reviews, see Rothschild (1992) and Siebert (1993)]. Analyzing proton binding sites with IR spectroscopy is especially suited because protonation/deprotonation reactions result in changes of molecular vibrational frequencies in the 1760−1700 cm^{-1} spectral range where the C=O stretching mode from the COOH side chain group of protonated carboxylic acid residues (Asp and Glu) is the main IR contributor (Siebert et al., 1982; Eisenstein et al., 1987; Rothschild, 1992). Changes of protonation or of environment of Asp and Glu residues in the photoreaction can be revealed by either steady-state FTIR difference spectroscopy or time-resolved IR spectroscopy. Transient IR signals associated with the electron transfer from $Q_A^-Q_B$ to $Q_AQ_B^-$ and with the concomitant proton uptake have been monitored in the microsecond to millisecond time range in native *Rb. sphaeroides* RCs and in EQ L212 RCs where Glu L212 is changed to Gln, in the spectral range from 1780 to 1695 cm^{-1} (Hienerwadel et al., 1992a,b, 1995). A transient absorbance change at 1725 cm^{-1} present in Wt and absent in Glu L212 was proposed to arise from the protonation of a carboxylate group, most probably that of Glu L212, corresponding to a proton uptake of 0.3−0.6 H^+/RC. In the present study, light-induced FTIR absorption changes associated with the photoreduction of Q_B in ^1H_2O and ^2H_2O are compared in the 1800−1200 cm^{-1} spectral region for native (2.4.1) RCs and RCs from mutants EQ L212, DN L213 (Asp L213 to Asn), and DN L210 (Asp L210 to Asn) and the double mutant EQ L212 + DN L213. This allows us to elucidate whether the Q_B to Q_B^- transition involves changes of protonation states or environment of these carboxylic acid residues.

MATERIAL AND METHODS

The construction of *Rb. sphaeroides* mutants (from 2.4.1 strain), bacterial growth, and reaction center isolation were performed as described in Paddock et al. (1994). In order to generate the Q_B^- state under single-saturating flash excitation, the RC samples were prepared essentially as reported previously (Breton et al., 1991a, 1995). RC samples (≈1 mM) contained a 10-fold excess of ubiquinone-10 at pH 7.0 (Tris-HCl, 50 mM), 10 mM ascorbate, and 20 mM diaminodurene (DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine). The IR absorbance at ≈1655 cm^{-1} was typically 0.6−0.8 absorbance units (au). $^1H/^2H$ exchange was performed by incubation of RCs in ^2H_2O for 24 h at 20 °C (Nabedryk et al., 1988). The corresponding buffer and mediator solutions were made in ^2H_2O.

IR data were acquired with a Nicolet FTIR spectrometer equipped with a MCT-A detector. Typically, the Q_B^-/Q_B spectra were recorded at 15 °C by measuring a series of three consecutive single-beam spectra (128 interferograms each, 23 s measurement time) with an interval of 2 s in between. The laser flash (Nd YAG laser, 7 ns, 530 nm) was fired in the interval between the recording of the first and second single-beam spectra. From the three single-beam spectra, two difference spectra were calculated. This procedure which allows for an estimate of the decay of the Q_B^- state after the flash was used to set a conservative delay between the successive series of single-beam measurements in order to prevent any contribution from the Q_BH_2 state. Furthermore, the identical shape of all the signals in the two successive difference spectra shows that only one decaying state is involved. The sample was then allowed to stand in the dark for complete recovery of the ground state (≈0.5−5 min) before another cycle was started. These measurements were repeated over ≈15 h with the same sample, and the results were averaged. The data presented are the average of several measurements for RC samples prepared from two to three independent RC preparations. On the basis of the comparison of the light minus dark difference spectra with those corresponding to the well-characterized Q_A^-/Q_A (Breton et al., 1991b, 1992, 1994; Brudler et al., 1994) and $P^+Q_A^-(Q_B^-)/PQ_A(Q_B)$ (Nabedryk et al., 1990) states, possible contribution of these states to the Q_B^-/Q_B spectra can be ruled out, as also discussed in Breton et al. (1995).

RESULTS

(1) Q_B^-/Q_B Spectra in ^1H_2O

Figure 1 depicts the light-induced FTIR difference spectra corresponding to the Q_B to Q_B^- transition in native 2.4.1 *Rb. sphaeroides* RCs and in the mutants DN L210, DN L213, EQ L212, and EQ L212 + DN L213. In these Q_B^-/Q_B spectra, negative bands originate from the neutral Q_B state while positive bands arise from the Q_B^- state.

(1.1) Native RCs. The Q_B^-/Q_B spectrum for 2.4.1 RCs obtained after a saturating flash (Figure 1a) is very similar to that previously reported for R26 RCs using either low-light intensity continuous illumination or single flash (Breton et al., 1991a, 1995; Brudler et al., 1995). It shows prominent positive peaks at 1728, 1651, and 1479 cm^{-1} and negative bands at 1685, 1640, 1617, 1581, 1290, and 1265 cm^{-1}. It has been shown that the 1640 and 1617 cm^{-1} negative bands arise at least partly from the C=O and C=C modes and that the 1265 and 1290 cm^{-1} negative signals come from the $COCH_3$ methoxy groups of the neutral ubiquinone (Breton et al., 1991a, 1995). On the basis of *in vitro* model quinone studies (Bauscher et al., 1990; Bauscher & Mäntele, 1992), the main positive band at 1479 cm^{-1} has been assigned to the C•••O/C•••C stretching modes of the semiquinone anion

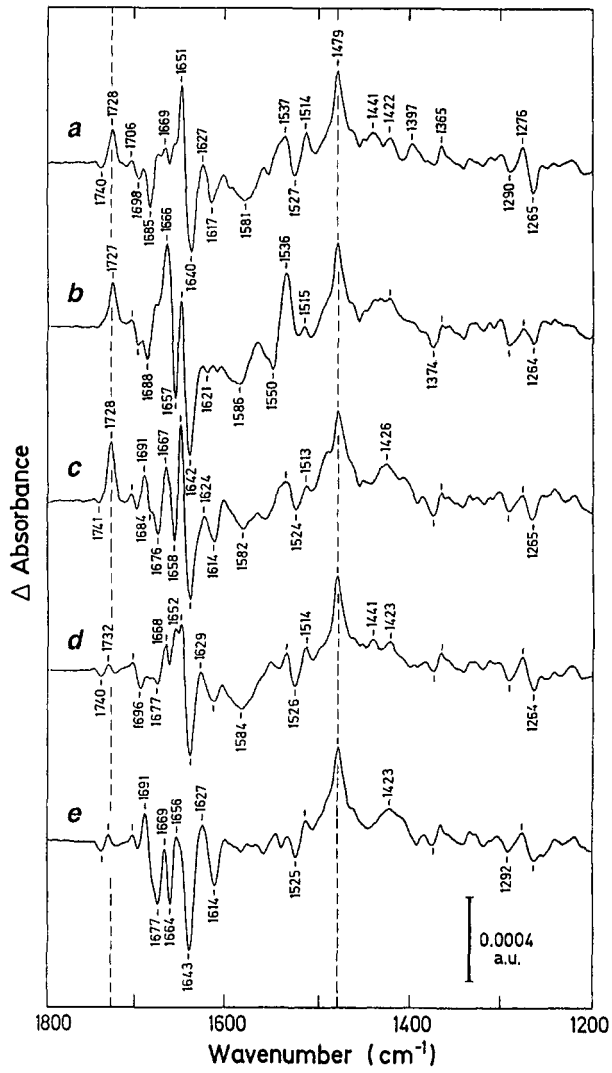


FIGURE 1: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of 2.4.1 *Rb. sphaeroides* RCs in ^1H_2O, with single-turnover flash excitation: (a) wild type, (b) DN L210, (c) DN L213, (d) EQ L212, and (e) EQ L212 + DN L213. Resolution, 4 cm^{-1}. About 40 000 interferograms coadded. The position of the bands is given at ±1 cm^{-1}; au is absorbance units.

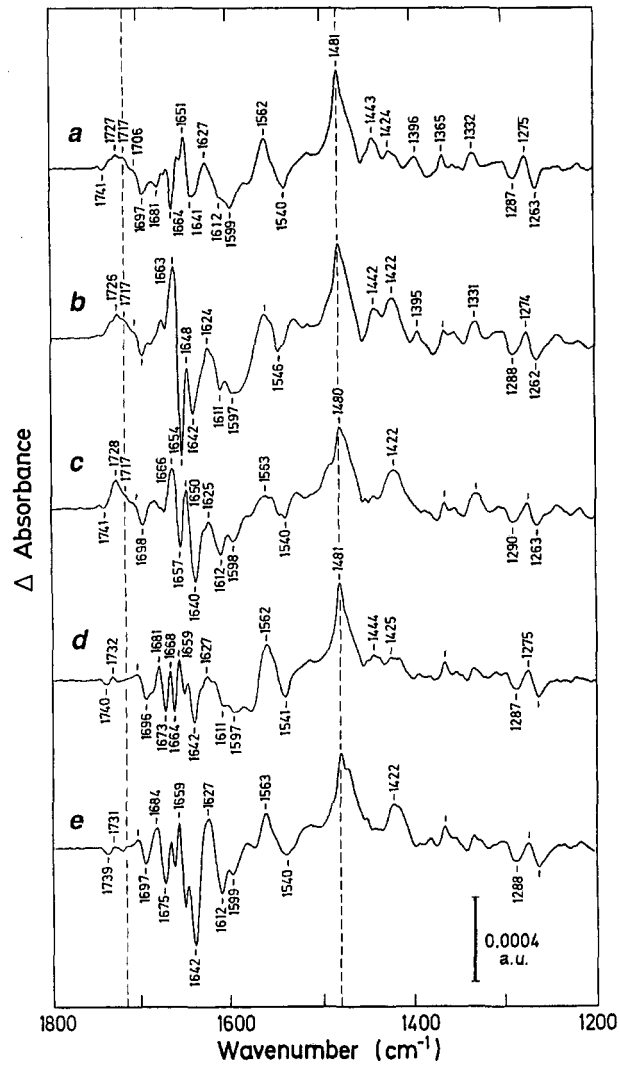


FIGURE 2: Light-induced Q_B^-/Q_B FTIR difference spectra at 15 °C of 2.4.1 *Rb. sphaeroides* RCs in ^2H_2O, with single-turnover flash excitation: (a) wild type, (b) DN L210, (c) DN L213, (d) EQ L212, and (e) EQ L212 + DN L213.

(Breton et al., 1991a, 1995; Bauscher et al., 1993). In addition to these intrinsic quinone contributions, a number of signals arise from the protein, in particular in the amide I (predominantly peptide C=O stretching) and amide II (60% peptide NH bending, 40% CN stretching) regions at 1651 and 1537 cm^{-1}, respectively. Of particular interest is the positive 1728 cm^{-1} peak lying in the typical absorption region of COOH groups from Asp and Glu carboxylic amino acid residues. The increase of absorption at 1728 cm^{-1} (without a negative counterpart) is indicative of an increase in protonation of an Asp or Glu residue concomitant with the reduction of Q_B. The small signals (hereafter, positive and negative bands are designated by + and −, respectively) at 1740 (−) and 1706 (+) cm^{-1} could be due to changes of environment and/or pK_a shifts of other carboxylic residues. The frequency of the negative signal at 1685 cm^{-1} is compatible with an amide I mode, but it is also within the spectral absorption range of side chains of Arg, Asn, and Gln residues (Venyaminov & Kalnin, 1990). This negative peak could also arise from a decrease of protonation of a carboxylic residue, although its frequency would be unusually low (see Discussion). The antisymmetric (ν_{as}) and symmetric (ν_s) COO^- stretching modes of carboxylate groups are expected in the 1610−1550 and 1420−1300 cm^{-1} regions (Bellamy, 1975), respectively. Although absorption changes are seen in these regions, the bands caused by the COO^- groups cannot unambiguously be assigned since they are located in congested spectral regions where additional modes overlap. The broad negative band at 1581 cm^{-1} probably contains contributions from the ν_{as} COO^- mode of Asp and/or Glu amino acid residues, although it is difficult to locate the corresponding symmetric mode.

(1.2) Mutant RCs. Q_B^-/Q_B spectra from native and mutant RCs (Figure 1) present several common features, in particular the main positive semiquinone anion band at 1479 cm^{-1} (this peak was normalized in all of the spectra), the main negative Q_B band at 1641 ± 1 cm^{-1} (1643 cm^{-1} in the double mutant) as well as bands at ≈1290 (−) and ≈1265 (−) cm^{-1}. With respect to Wt, the 1617 cm^{-1} negative band is downshifted by 3 cm^{-1} in DN L213, EQ L212, and EQ L212 + DN L213, and it is very weak in DN L210. These observations indicate that, in addition to the contribution of the C=C mode of the neutral Q_B at 1617 cm^{-1} (Breton et al., 1991a, 1995), other modes are involved, as also inferred from the sensitivity of this spectral region to $^1H/^2H$ exchange (see below). From

kinetic IR studies of Wt RCs, a transient signal at 1617 cm^{-1} exhibiting a 1 ms component has been tentatively assigned to a ν_{as} COO^- mode (Hienerwadel et al., 1992a).

Besides these common features, the spectra of the mutants also exhibit very distinct characteristics. In particular, large effects due to the mutations are observed, notably in the 1750−1655 cm^{-1} spectral range where modes of side chains of amino acid residues containing COOH carboxylic (Asp and Glu), CN_3H_4 guanidine (Arg), and $CONH_2$ (Asn and Gln) groups are expected (Venyaminov & Kalnin, 1990) in addition to backbone carbonyls. We will further discuss these distinct spectral features for the mutant RCs below.

EQ L212 and EQ L212 + DN L213. The Q_B^-/Q_B spectra of the EQ L212 and EQ L212 + DN L213 mutants (Figure 1d,e) are considerably modified compared to all the other spectra. In particular, in the spectral region of the C=O stretching mode of protonated carboxyl groups, there is the conspicuous absence of the 1728 cm^{-1} peak in the mutant spectra. Instead, a small differential signal at 1732 (+)/1740 (−) cm^{-1} remains. Several other bands are also affected by the mutations. The 1685 cm^{-1} band is detected neither in EQ L212 nor in the double mutant. The amplitude of the absorption changes in the amide I (at 1651 cm^{-1}) and amide II (at 1537 cm^{-1}) regions is reduced in EQ L212 with respect to Wt and is very small in EQ L212 + DN L213. In addition, the spectrum of the double mutant (Figure 1e) shows new bands at 1691 (+), 1677 (−) and 1664 (−) cm^{-1}. The broad negative band seen in Wt at 1581 cm^{-1} is nearly absent in the double mutant, and only a very small signal is observed at ≈1585 cm^{-1}.

DN L210 and DN L213. In contrast to the mutants containing the Glu L212 to Gln substitution, the 1728 cm^{-1} peak is observed in the Q_B^-/Q_B spectra of DN L210 and DN L213 (Figure 1b,c). In DN L213, the amplitude of the absorption increase at 1728 cm^{-1} is significantly larger than in native RCs, while in DN L210, it is comparable. The 1688 (−) cm^{-1} band in DN L210 and the shoulder at 1684 cm^{-1} on the 1676 (−) cm^{-1} band in DN L213 probably correspond to the 1685 (−) cm^{-1} band seen in Wt. Furthermore, the formation of Q_B^- in DN L213 is accompanied by a new absorption increase at 1691 (+) cm^{-1} and a decrease at 1676 (−) cm^{-1}, as also observed for the double mutant. With respect to Wt, the small negative signal at 1740 cm^{-1} is absent in DN L210. In Wt, this signal disappears at high pH (Nabedryk et al., 1993). It is worth noting that the spectrum of DN L210 displays a new differential signal at 1666 (+)/1657 (−) cm^{-1} and concomitantly a larger absorption change at 1536 (+)/1550 (−) cm^{-1}. In DN L213, a differential signal is also observed at 1667 (+)/1658 (−) cm^{-1}.

(2) Q_B^-/Q_B Spectra in ^2H_2O

Figure 2 shows the Q_B^-/Q_B spectra in ^2H_2O for native and mutant RCs. These spectra exhibit several similar features, in particular the main anion band of Q_B^- at 1481 cm^{-1}, the methoxy bands of Q_B at ≈1287 and 1263 cm^{-1}, and the Q_B mode at ≈1641 cm^{-1}. This last mode does not appear to be significantly shifted upon $^1H/^2H$ exchange; however, its amplitude is decreased for Wt and the mutants, except for EQ L212 + DN L213. In addition, large changes are observed between all of the spectra in Figure 2, especially

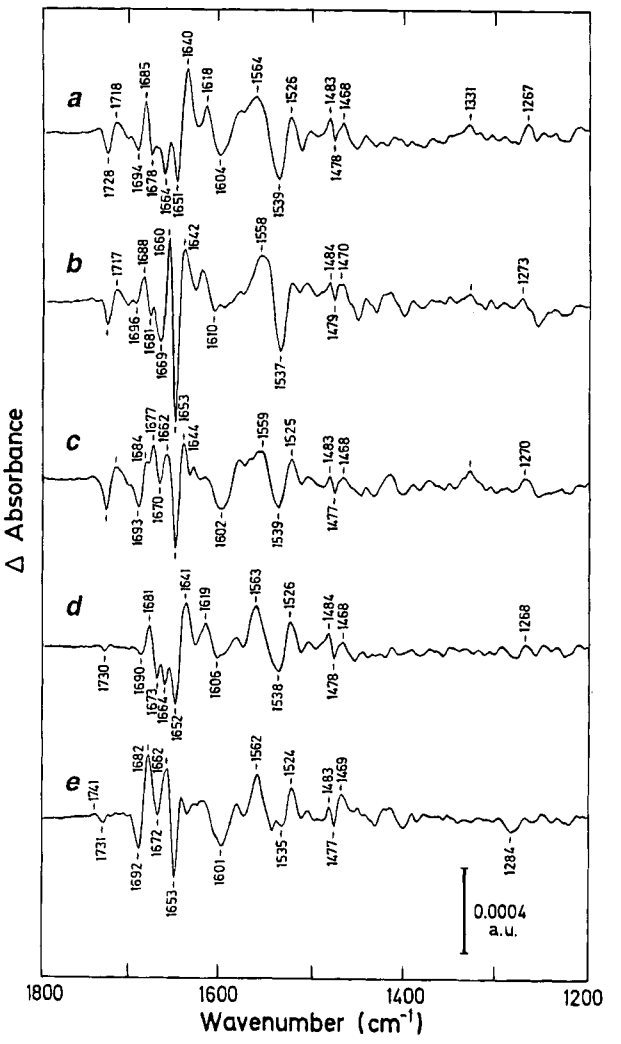


FIGURE 3: Calculated double-difference spectra in the 1800−1200 cm^{-1} region between Q_B^-/Q_B spectra of *Rb. sphaeroides* RCs obtained in ^2H_2O and ^1H_2O (i.e., ^2H_2O minus ^1H_2O) upon normalization on the 1479 cm^{-1} semiquinone anion band: (a) wild type, (b) DN L210, (c) DN L213, (d) EQ L212, and (e) EQ L212 + DN L213.

in the 1750−1650 cm^{-1} spectral region. These changes are even more pronounced than in ^1H_2O (Figure 1). They will be presented below.

(2.1) C=O Stretching Region of Carboxylic Amino Acids. Changes in the C=O stretching vibrations of protonated carboxylic groups are expected upon $^1H/^2H$ exchange and are indeed observed in the 1750−1700 cm^{-1} region (Figure 2). The positive 1727 ± 1 cm^{-1} peak in Wt, DN L210, and DN L213 has its amplitude significantly reduced in ^2H_2O, and a shoulder appears at 1717 cm^{-1} (Figure 2a−c). These changes in ^2H_2O are best seen in the double-difference spectra (Figure 3a−c) calculated between Q_B^-/Q_B spectra obtained for a given RC in ^2H_2O and ^1H_2O (^2H_2O minus ^1H_2O). In these double-difference spectra, only the residues affected by the photoreduction of Q_B and sensitive to $^1H/^2H$ exchange will give rise to differential and/or shifted bands. Note the negative peak at 1728 cm^{-1} and positive peak at 1717 ± 1 cm^{-1} in Figure 3a−c. This ≈10 cm^{-1} downshift is typical of a protonated Asp or Glu carboxylic group exchangeable with the solvent (Siebert et al., 1982; Eisenstein et al., 1987; Rothschild, 1992; Maeda et al., 1992). The smaller intensity of the peak at 1717 ± 1 (+) cm^{-1} in ^2H_2O

relative to that at 1728 (+) cm^{-1} in ^1H_2O suggests that either COO^2H has a smaller extinction coefficient than COO^1H or the extent of protonation of the carboxylic residue upon Q_B^- formation is smaller in ^2H_2O than in ^1H_2O. As already reported (Maeda et al., 1992), ^2H_2O could cause a subtle pK_a increase of Asp/Glu residues. However, the relative pH independence of the 1725 cm^{-1} signal in the kinetic IR study (Hienerwadel et al., 1995) suggests that the latter interpretation may not be favored. Nevertheless, the remarkable similarity between the ^2H_2O minus ^1H_2O double-difference spectra of Wt, DN L210, and DN L213 in the 1735−1710 cm^{-1} region (Figure 3a−c) provides compelling evidence that the same carboxylic residue is responsible for the signal in all three RCs. Note that the amplitude of the change at 1728 cm^{-1} (Figure 3a−c) is comparable in Wt and DN L210 and larger in DN L213, as already observed in the Q_B^-/Q_B spectra (Figure 1). On the other hand, the double-difference spectra ^2H_2O minus ^1H_2O for EQ L212 and the double mutant (Figure 3d,e) show only small signals at ≈1731 (−) and 1741 (+) cm^{-1}. This supports the proposal that Glu L212 is involved in the 1728 cm^{-1} peak seen in Wt, DN L210, and DN L213 RCs. It can be noticed that the 1706 (+) cm^{-1} signal (Figure 1) is not shifted in ^2H_2O (Figure 2) in Wt or the mutants.

(2.2) The 1700−1670 cm^{-1} Spectral Region. Differences in the 1700−1670 cm^{-1} spectral region of the Q_B^-/Q_B spectra in ^2H_2O for native and mutant RCs are revealed in the double-difference spectra ^2H_2O minus ^1H_2O for Wt and mutants RCs (Figure 3). In particular, the 1685 cm^{-1} positive band in Wt is flanked by two small troughs at 1694 and 1678 cm^{-1}. Similarly, in DN L210, the 1688 cm^{-1} band is flanked by two weak negative signals at 1696 and 1681 cm^{-1}. In DN L213, a shoulder is observed at 1684 cm^{-1} on the positive signal at 1677 cm^{-1} together with a negative signal at 1693 cm^{-1}. The spectrum of the double mutant also displays a negative signal at 1692 cm^{-1}. In addition, both EQ L212 and EQ L212 + DN L213 spectra show a positive signal at 1681−1682 cm^{-1} with a trough at 1672−1673 cm^{-1}. These distinct features will be analyzed in details in the Discussion.

(2.3) Amide I and Amide II Regions. The 1537 cm^{-1} positive band present in the amide II region of the Q_B^-/Q_B spectra in ^1H_2O of Wt and mutant RCs (Figure 1) is missing in ^2H_2O (Figure 2), consistent with $^1H/^2H$ exchange of peptide NH groups. From the amide II to amide I ratio in the absorption spectra of RCs in ^2H_2O (data not shown), the extent of deuteration of the peptide nitrogen atoms is estimated to be ≈70%. In the Q_B^-/Q_B spectra in ^2H_2O (Figure 2), the amide II′ could be located at ≈1465 cm^{-1} on the low-energy side of the semiquinone peak that appears more asymmetrical in ^2H_2O than in ^1H_2O. Note that the amplitude of the large differential signal at ≈1651 (+)/1641 (−) cm^{-1} is always reduced in ^2H_2O compared to ^1H_2O. For DN L210 and DN L213, the downshift by ≈1−3 cm^{-1} upon $^1H/^2H$ exchange (Figures 1b,c, and 2b,c) for the peaks at 1667−1666 (+) cm^{-1} and 1658−1657 (−) cm^{-1} is consistent with an assignment to peptide C=O group(s). All the double-difference spectra ^2H_2O minus ^1H_2O (Figure 3) display negative bands at 1652 ± 1 cm^{-1} and 1537 ± 2 cm^{-1} and a small positive band at 1469 ± 1 cm^{-1}, reflecting the above described amide I, amide II, and amide II′ absorption changes, respectively. Consistent with the Q_B^-/Q_B spectra in ^1H_2O (Figure 1), the largest changes of the protein backbone upon Q_B^- formation are observed for DN L210 (Figures 2b and 3b). However, the amplitude of these changes does not exceed a few (1−2) peptide C=O groups. The positive peak at 1514 cm^{-1}, which is consistently observed in the Q_B^-/Q_B spectra of Wt and mutants in ^1H_2O (Figure 1), disappears in ^2H_2O (Figure 2). Although this band is in a region corresponding to Tyr ring vibrations, its absence in ^2H_2O does not favor such an assignment.

DISCUSSION

We have performed light-induced FTIR studies of *Rb. sphaeroides* RCs from Wt and several site-directed mutants (Glu to Gln and/or Asp to Asn) to investigate protonation or environmental changes of carboxylic acid residues that can occur upon formation of Q_B^-. In particular, RCs modified at three carboxylic acid residues near the Q_B binding pocket (Glu L212, Asp L213, and Asp L210) were investigated. The assignment of an IR band to the protonation or deprotonation of a particular residue side chain is based on changes in the FTIR spectrum upon site-directed replacement and $^1H/^2H$ isotope exchange. Although in general any mutation can influence protein structure, crystallographic analysis of EQ L212 and DN L213 mutants at 3.3 and 3.0 Å resolution, respectively, shows no significant tertiary structure changes (Chirino et al., 1994). In agreement with these conclusions, the present FTIR data show that upon Q_B photoreduction the amplitude of the absorbance changes in the amide I and amide II regions is approximately comparable in Wt and in the mutants, demonstrating the absence of large structural changes induced by the mutations. These results also imply no mutation-induced structural changes in the RC for the Q_B^- state. These observations are taken to indicate that the differences found in the Q_B^-/Q_B spectra of Wt and of the mutants are localized to the direct site of mutation. Accordingly, the pronounced changes of intensity and of frequency of the IR bands observed when comparing the Q_B^-/Q_B spectra of Wt and mutants will be interpreted in terms of the vibrations of the chemical groups which have been modified by the mutation. It should however be kept in mind that subtle secondary effects may also contribute.

We will first discuss the contributions of Glu L212, Asp L210, and Asp L213 to the native RC spectrum based on the effects of site-specific replacements and $^1H/^2H$ isotope exchange on the IR spectrum. We will then discuss the assignment of the 1685 cm^{-1} band and possible contributions of Asn L213 to the mutant IR spectra. We end with a model for protonation change of carboxylic acid residues upon Q_B^- formation, in particular, protonation changes of Glu L212.

(1) Glu L212. The 1728 cm^{-1} band is assigned to increased protonation of Glu L212 upon Q_B photoreduction in Wt RCs. The evidence for this assignment follows.

(i) The 1728 cm^{-1} single absorption band lies in the typical absorption range for COOH side chain groups of Asp and Glu residues. From IR absorption studies of model compounds in ^1H_2O (Venyaminov & Kalnin, 1990) and in ^2H_2O (Chirgadze et al., 1975), the amino acid residue of Glu (Asp) in its protonated form exhibits a band at 1712 (1716) cm^{-1} in ^1H_2O and at 1706 (1712) cm^{-1} in ^2H_2O. In photoactive membrane proteins such as bacteriorhodopsin and rhodopsin, the IR signatures of Asp and Glu side chains are found in the 1770−1710 cm^{-1} range [for reviews, see Rothschild

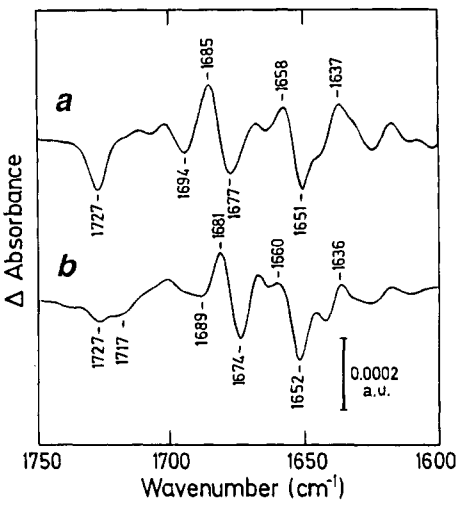


FIGURE 4: Calculated double-difference spectra in the 1750−1600 cm^{-1} region between Q_B^-/Q_B spectra of EQ L212 and Wt RCs (EQ L212 minus Wt) in ^1H_2O (a) and in ^2H_2O (b) upon normalization on the 1479/1481 cm^{-1} semiquinone anion band.

(1992) and Siebert (1993)], depending on the hydrogen-bonding strength of the C=O···H bond (Bellamy, 1975) or on the environment of this group (change of hydrophobicity or of dielectric constant).

(ii) Carboxylic acid residues can undergo several types of changes upon photoactivation, and each type has a typical FTIR difference spectrum. Protonation (deprotonation) of an Asp or Glu residue gives rise to a single positive (negative) absorption band. An internal proton transfer would result in a positive and negative signal of approximately equal amplitude, provided that the carboxylic groups exhibit well-separated COOH modes. A change of the environment or of the hydrogen bonding of a protonated carboxylic group appears as a differential (S-shaped) signal. A broadening (narrowing) of an absorption band with conserved integrated intensity would give rise to a signal with a second derivative shape.

The shape of the 1728 cm^{-1} peak in the Q_B^-/Q_B spectra of Wt, DN L210, and DN L213 RCs (Figure 1a−c) suggests proton binding of a carboxylic group as opposed to an environmental shift. This is further supported by the examination of the double-difference spectrum calculated between Q_B^-/Q_B spectra of EQ L212 and Wt RCs. In the EQ L212 minus Wt spectrum (Figure 4a) as well as in the EQ L212 + DN L213 minus Wt spectrum (data not shown), a negative band at 1727 (−) cm^{-1} corresponding to the loss of the positive 1728 (+) cm^{-1} signal of Wt is seen. The almost symmetrical shape of the 1727 (−) cm^{-1} band in Figure 4a is taken to show that protonation of an Asp or Glu residue occurs in Wt upon the Q_B to Q_B^- transition.

(iii) The 1728 cm^{-1} band in Wt is sensitive to $^1H/^2H$ exchange. The frequency of the new feature observed at 1717 ± 1 cm^{-1} is consistent with a $^1H/^2H$ isotope shift characteristic for a protonated carboxylic group accessible to solvent. Moreover, the same downshift of the 1728 cm^{-1} signal is observed in ^2H_2O for the DN L210 and DN L213 mutants. The comparison of the double-difference spectra between Q_B^-/Q_B spectra obtained in ^2H_2O and ^1H_2O for Wt, DN L210, and DN L213 (Figure 3a−c) demonstrates that a common residue gives rise to the 1728 cm^{-1} signal in the FTIR difference spectra and is similarly sensitive to $^1H/^2H$ isotope exchange. The remaining signal at 1727 ± 1 cm^{-1} in the ^2H_2O spectra of Wt, DN L210, and DN L213 RCs (Figure 2a−c) could be explained by an incomplete exchange of Glu L212. This could be due to some heterogeneity in the accessibility of this residue as well as to the presence of some residual $^1H^2HO$ in the final buffer. On the other hand, we cannot exclude that another carboxylic residue insensitive to $^1H/^2H$ exchange contributes to the remaining signal.

(iv) The persistence of a positive band at 1728 cm^{-1} in the Q_B^-/Q_B spectra of the DN L210 and DN L213 mutants (Figure 1b,c) excludes the possibility that it arises from the protonation of Asp L210 or Asp L213. In contrast, the EQ L212 spectrum exhibits a dramatic change in the carboxylic acid C=O stretching region with the disappearance of the 1728 cm^{-1} signal. A similar effect is also observed for the double mutant EQ L212 + DN L213. These results provide strong evidence that Glu L212 contributes to this signal in Wt RCs. The increased protonation could be of Glu L212 or some other nearby residue that interacts strongly with Glu L212. The latter explanation appears less likely since the 1728 cm^{-1} band is still present upon replacement of Asp L213 or Asp L210, the two most likely alternate proton binding candidates. Therefore, the 1728 cm^{-1} band is assigned to increased protonation of Glu L212. In agreement with these FTIR data, the $Q_A^-Q_B$ to $Q_AQ_B^-$ transition analyzed by kinetic IR spectroscopy with microsecond time resolution showed that a transient signal at 1725 cm^{-1} in Wt is absent in EQ L212 (Hienerwadel et al., 1995). This signal was assigned to the protonation of a carboxylate residue, most probably that of Glu L212.

An estimation of the proton uptake by Glu L212 in Wt, DN L210, and DN L213 can be performed on the basis of molar extinction coefficients and half-widths for COOH (Venyaminov & Kalnin, 1990) and semiquinone (Bauscher et al., 1990) bands. For Glu in ^1H_2O, the extinction coefficient of the C=O band from the COOH group has been measured to be 220 ± 10 $L \cdot mol^{-1} \cdot cm^{-1}$, and the full width at half-height of the band is 56 ± 2 cm^{-1}. The IR signal at 1728 cm^{-1} in the Q_B^-/Q_B spectrum of Wt RCs has a half-width of ≈10 cm^{-1}, considerably less than for Glu in solution. Such a narrowing of the carboxylic bands has been previously observed in spectra of bacteriorhodopsin and rhodopsin (Rothschild, 1992; Siebert, 1993). For the C⋯O/C⋯C anion band of the semiquinone of 2,3-dimethoxy-5-methyl-1,4-benzoquinone, the extinction coefficient is 350 $L \cdot mol^{-1} \cdot cm^{-1}$ in methanol and 600 $L \cdot mol^{-1} \cdot cm^{-1}$ in acetonitrile, with half-widths of ≈32.5 and ≈15 cm^{-1}, respectively (Bauscher et al., 1990). The half-width of Q_B^- at 1479 cm^{-1} is ≈15 cm^{-1}, while the amplitude of the semiquinone peak is about twice that of the 1728 cm^{-1} band (Figure 1a). On the basis of these data, the integrated absorption at 1728 cm^{-1} corresponds to a proton uptake by Glu L212 upon Q_B^- formation of 0.3−0.4 H^+ in native RCs and DN L210 and 0.5−0.6 H^+ in DN L213. From kinetic IR studies on Wt RCs, a value of 0.3−0.6 H^+/Q_B^- has been reported (Hienerwadel et al., 1995). The main sources of uncertainty are the values for the extinction coefficients and the determination of the base lines and half-widths of the IR bands. For the 1479 cm^{-1} Q_B^- FTIR band, the base line observed in the FTIR Q_B^-/Q_B spectra of RCs reconstituted with uniformly ^{13}C-labeled ubiquinone (Breton et al., 1995) was used.

(2) Asp L210 and Asp L213. Tentative assignment of part of the 1732 (+)/1740 (−) cm^{-1} signal to an environmental shift of protonated Asp L210 is presented below. In the Q_B^-/Q_B spectra of Wt, DN L213, EQ L212, and EQ L212 + DN L213 RCs (Figure 1), the small negative signal at 1740 cm^{-1} is consistently observed. In contrast, the Q_B^-/Q_B spectrum of DN L210 is flat at 1740 cm^{-1} (Figure 1b). Moreover, in the EQ L212 and in the double mutant spectra (Figure 1d,e), the 1740 cm^{-1} signal is part of a small differential signal at 1732 (+)/1740 (−) cm^{-1} that is sensitive to ^2H_2O (small amplitude decrease and ≈1 cm^{-1} frequency downshift upon $^1H/^2H$ isotope exchange, notably in the double mutant; see Figure 3d,e). This differential signal most probably arises from a carboxylic residue protonated in both the Q_B and Q_B^- states but located in different environments in the two states. An alternative interpretation of the 1732/1740 cm^{-1} signal is that it involves a shift of the 10a-ester C=O mode of the bacteriopheophytin on the M side, due to the presence of an electron on Q_B, as already proposed for a comparable signal in the Q_A^-/Q_A spectra of Wt RCs for the bacteriopheophytin on the L side (Mäntele et al., 1990; Breton et al., 1992). Since X-ray data do not show a hydrogen bond to the 10a-ester C=O of the bacteriopheophytin on the M side (Ermler et al., 1994), the corresponding IR mode is not expected to be sensitive to $^1H/^2H$ isotope exchange, and thus, the part of the 1732/1740 cm^{-1} signal that is affected in ^2H_2O can be assigned to a carboxylic residue. In the Q_B^-/Q_B spectra of Wt and DN L213 RCs (Figure 1a,c), the 1732 cm^{-1} signal would be buried under the 1728 cm^{-1} positive band. For DN L210, in agreement with the loss of the 1740 cm^{-1} signal directly seen in the Q_B^-/Q_B spectrum, the calculated double-difference spectrum DN L210 minus Wt (data not shown) reveals a weak differential signal at ≈1739 (+)/1731 (−) cm^{-1}. We therefore suggest that the 1732/1740 cm^{-1} signal is partly due to the protonated carboxylic group of Asp L210 undergoing a change of its environment or an increase of its hydrogen bonding between the Q_B and Q_B^- states (the extent of protonation of Asp L210 will be discussed in section 5). Further studies on RCs containing isotope-labeled Asp or Glu residues will be necessary to resolve this issue and to assign more definitively the contribution of carboxylic acid groups to the Q_B^-/Q_B spectra.

Evidence that Asp L213 does not change protonation upon Q_B^- formation comes from the analysis of the 1760−1700 cm^{-1} region of the Q_B^-/Q_B spectrum of DN L213 RCs (Figure 1c). Apart from the increase of the amplitude of the 1728 cm^{-1} band which has been assigned to increased proton uptake by Glu L212 in this mutant,[2] the other signals are not significantly influenced by the mutation of Asp L213 to Asn. This indicates that Asp L213 does not change its protonation or environment state between the Q_B and Q_B^- states. Since there are no other assigned bands at a frequency that could be due to a protonated carboxylic acid, we are left with the conclusion that Asp L213 is not observed in the 1760−1700 cm^{-1} region of the FTIR Q_B^-/Q_B spectrum of native RCs at pH 7. This is consistent with the attributed $pK_a < 5$ for Asp L213 (Takahashi & Wraight, 1992). Further studies at a lower pH (e.g., below pH 5) may reveal protonation events upon Q_B^- formation assignable to Asp L213 and/or Asp L210.

(3) Assignment of the 1685 cm^{-1} Band. Several possible assignments of the 1685 cm^{-1} negative band are presented below. Given that the 1685 cm^{-1} band is similar in amplitude and shape but opposite in sign to the 1728 cm^{-1} band and that both bands are sensitive to $^1H/^2H$ exchange, it is appealing to associate the 1685 cm^{-1} band in Wt with a protonated carboxyl group that becomes deprotonated upon Q_B^- formation. However, this band occurs at an unsually low-frequency for a protonated carboxyl group. Such a low frequency absorption would require a special environment such as a very strong hydogen bond to the carbonyl group and/or a highly polar environment (almost diacid), as it is observed for dimers of organic acids (Colthup et al., 1964; Haurie & Novak, 1965a,b; Maeda et al., 1992; Iliadis et al., 1994). If this were the case, the deprotonation of a carboxylic residue at 1685 cm^{-1} in Wt could be coupled to the protonation of Glu L212 at 1728 cm^{-1}. In this scheme of coupled residues, the 1685 cm^{-1} band in Wt RCs which can be due neither to Glu L212 nor to Asp L210 might originate from Asp L213. Inconsistent with this model, these two bands are indeed observed in DN L213 with large differences in their relative intensities. These observations are incompatible with coupled protonation/deprotonation events at 1728 and 1685 cm^{-1}. In addition, preliminary studies of mutant RCs containing other amino acid residues than Asn at L213 indicate that a small band at ≈1685 cm^{-1} is always present in the Q_B^-/Q_B spectra of these mutants (Nabedryk et al., 1995), and thus, the possibility that Asp L213 is the main contributor at ≈1685 cm^{-1} is not presently favored.

The second possible assignment of the 1685 cm^{-1} signal in Wt is to an amide I component or to a non-carboxylic acid side chain mode, e.g., the antisymmetric C−N stretch mode of an Arg residue (≈1673 cm^{-1} in model compound IR studies) or the C=O stretch of Asn (≈1678 cm^{-1}) or Gln (≈1670 cm^{-1}) [Venyaminov & Kalnin, 1990; see also Braiman et al. (1994)]. In order to test the possibility that an Arg side chain contributes to the 1685 cm^{-1} signal, we have undertaken preliminary experiments with ^{15}N-labeled RCs. Upon ^{15}N substitution, this signal is not significantly affected (data not shown) and appears at 1684 cm^{-1} (1 cm^{-1} downshift compared to the 26 cm^{-1} shift calculated for a C−N mode using the harmonic oscillator approximation), indicating that it cannot arise from a ^{15}N sensitive mode. This is taken to show that C−N modes from Arg are not responsible for the 1685 cm^{-1} band in native RCs.

In model compound IR studies, side chain modes of Asn and Gln are observed to downshift by 30−35 cm^{-1} in ^2H_2O (Venyaminov & Kalnin, 1990; Chirgadze et al., 1975). In contrast, amide I modes are expected to downshift by a few wavenumbers in ^2H_2O. It is thus essential to assign the shifted band in ^2H_2O corresponding to the 1685 cm^{-1} band in ^1H_2O. Upon $^1H/^2H$ exchange, the 1685 cm^{-1} band (Figure 1a) disappears and probably contributes to the small band at 1681 cm^{-1} (4 cm^{-1} downshift) in Figure 2a, giving rise to the positive signal at 1685 cm^{-1} and the two small negative signals at 1694 and 1678 cm^{-1} in the double-difference spectrum ^2H_2O minus ^1H_2O (Figure 3a). However, a shift of the 1685 cm^{-1} band to 1664 cm^{-1} cannot be excluded (21 cm^{-1} downshift) (Figures 2a and 3a). Further informa-

[2] In Wt RCs, the 1728 cm^{-1} band could be due to the superposition of signals arising from protonation of Glu L212 and deprotonation of Asp L213, thus explaining the increase of this band in DN L213. However, the identical shape of the 1727/1717 cm^{-1} signal in the double-difference spectra of ^2H_2O minus ^1H_2O for Wt and DN L213 (Figure 3a,c) makes this interpretation rather unlikely.

tion comes from the examination of the double-difference spectra EQ L212 minus Wt, in ^1H_2O and in ^2H_2O (Figure 4). The EQ L212 minus Wt spectrum in ^1H_2O (Figure 4a) shows a positive absorption at 1685 cm^{-1} flanked by two troughs at 1694 and 1677 cm^{-1} corresponding to the loss of the 1685 cm^{-1} peak in the Q_B^-/Q_B spectrum of EQ L212 with respect to Wt. These signals appear to be downshifted to 1689, 1681, and 1674 cm^{-1}, respectively, in ^2H_2O (Figure 4b). This small ^2H_2O effect ($\approx$4 cm^{-1} downshift) is not compatible with an assignment to a side chain mode from Asn or Gln, and we are led to conclude that the 1685 cm^{-1} band is due to a peptide C=O mode. The absence of the 1685 cm^{-1} band in the Q_B^-/Q_B spectra of EQ L212 and of the double mutant (Figure 1d,e) strongly suggests that this band is coupled to Glu L212. The simplest explanation is that the 1685 cm^{-1} band in Wt is due to the direct contribution of the Glu L212 backbone C=O vibration. Such a mode is also seen in DN L210. Note the similarity of the 1694 (−)/1685 (+)/1678 (−) and 1696 (−)/1688 (+)/1681 (−) cm^{-1} features in the double-difference spectra ^2H_2O minus ^1H_2O of Wt and DN L210, respectively (Figure 3a,b). In DN L210, the 1688 cm^{-1} band is thus assigned to the absorption change of the peptide C=O of Glu L212. In DN L213, new bands at 1692 (+) and 1676 (−) cm^{-1} appear in the Q_B^-/Q_B spectrum (Figure 1c), partially masking the 1685 cm^{-1} signal observed as a shoulder at 1684 cm^{-1}. However, the double-difference spectrum ^2H_2O minus ^1H_2O of DN L213 (Figure 3c) clearly displays a small positive peak at 1684 cm^{-1}, while the corresponding spectrum for the mutant DL L213 (Asp L213 to Leu) shows a pronounced peak at 1687 cm^{-1} (Nabedryk et al., 1995). The present FTIR data on RCs from Wt, DN L213, and DN L210 thus favor the assignment of the 1685 cm^{-1} signal in Wt to the amide I component of Glu L212. In DN L210 and DN L213, this mode would be upshifted (+3 cm^{-1}) or slightly downshifted (−1 cm^{-1}), respectively, in response to the mutation of the nearby residue.

The double-difference spectrum ^2H_2O minus ^1H_2O of EQ L212 (Figure 3d) reveals a positive signal at 1681 cm^{-1} flanked by a small trough at 1690 cm^{-1} and a negative signal at 1673 cm^{-1}. These features are downshifted by 4 cm^{-1} compared to the corresponding signals observed at 1694 (−)/1685 (+)/1678 (−) in Wt RCs (Figure 3a). We propose that, in EQ L212, the 1681 cm^{-1} band (Figure 3d) arises from the C=O amide I of Gln L212 (4 cm^{-1} downshift compared to the C=O amide I of Glu L212 in Wt). In the double mutant, the Gln C=O mode would contribute to the 1682 cm^{-1} (+) signal seen in the double-difference spectrum ^2H_2O minus ^1H_2O (Figure 3e). So, from the present IR data, we propose that the peptide C=O at L212 (1684 $\pm$ 4 cm^{-1}) is always affected by the photoreduction of Q_B in all RCs from Wt and mutants.

(4) Possible Contributions of the Asn L213 Side Chain. Do Asn or Gln residues in the mutants contribute to the Q_B^-/Q_B spectra? A new derivative signal at 1691 (+)/1677 (−) cm^{-1} is observed in both DN L213 and the double mutant (Figure 1c,e). Since the 1691/1677 cm^{-1} signal is very small in EQ L212, it can be inferred that it is associated with Asn L213 in the two mutants DN L213 and EQ L212 + DN L213. In Figure 1c,e, the 1691/1677 cm^{-1} differential signal could in part correspond to an absorption change of the C=O side chain mode of Asn L213, provided this residue or its microenvironment is affected by the presence of the electric charge on Q_B^-. In agreement with this proposed tentative assignment, only weak signals at $\approx$1675 and 1692 cm^{-1} are detected in the Q_B^-/Q_B spectra of the mutants DL L213, DS L213 (Asp L213 to Ser), and DH L213 (Asp L213 to His) (Nabedryk et al., 1995). Since a frequency downshift by $\approx$30 cm^{-1} is expected for the C=O stretch of an Asn side chain in ^2H_2O, the pair of differential signals observed at 1682(+) /1692 (−) and 1662 (+)/1653 (−) cm^{-1} for EQ L212 + DN L213 (Figure 3e) or at 1677 (+)/1693 (−) cm^{-1} and 1662 (+)/1653 (−) cm^{-1} for DN L213 (Figure 3c) may account for the $^1H/^2H$ shift of the C=O side chain mode of Asn L213. However, the Q_B^-/Q_B spectra in ^1H_2O (Figure 1) and ^2H_2O (Figure 2) indicate that amide I mode(s) also contribute to the low-frequency differential signal.

(5) Model for Protonation Changes. Several models for the protonation of acid residues in the interior of the RC near Q_B have been proposed. Early models accounted for the pH dependence proton uptake measurements and the recombination rate k_{BD} and electron transfer rate $k_{AB}^{(1)}$ in terms of ionized acid groups interacting with Q_B^-. The pH dependence of proton uptake, k_{BD}, and $k_{AB}^{(1)}$ suggested the involvement of an acid residue near Q_B having a pK_a near 9.5 in the Q_B state that is shifted to >10.5 in the Q_B^- state (Wraight, 1979; Kleinfeld et al., 1984; Mc Pherson et al., 1988; Maroti & Wraight, 1988). The role of Glu L212 in proton uptake was indicated by site-directed mutation of Glu L212 to Gln which eliminated an inflection in the pH dependence of electron transfer rates k_{BD} and $k_{AB}^{(1)}$ near pH 9.5. Consequently, the pK_a of Glu L212 was proposed to be $\approx$9.5 (Q_B state) (Paddock et al., 1989; Takahashi & Wraight, 1992). The protonation state of Asp L213 was inferred from the slower recombination rate k_{BD} observed in DN L213 RCs compared to that of native RCs by a mechanism in which the mutation of Asp L213 to Asn lowered the energy of the Q_B^- state due to the loss of a negatively charged Asp L213. Thus, Asp L213 was proposed to be ionized in native RCs (pK_a < 6) in both Q_B and Q_B^- states (Takahashi & Wraight, 1990; Paddock et al., 1994; Okamura & Feher, 1992). The mutation of Asp L210 to Asn had a smaller effect on k_{BD} and on the energy of the Q_B^- state. However, the pH dependence of the recombination state k_{BD} in the low-pH region was eliminated, leading to a tentative pK_a assignment $\approx$5 for Asp L210 in the Q_B state and $\approx$6 in the Q_B^- state (Okamura & Feher, 1992). In these early models, the electrostatic interactions between titrating groups were not considered.

The need to take these interactions into account is indicated by the proton uptake by Glu L212 at pH 7 reported here and in kinetic IR experiments in the pH range from 5.6 to 10 (Hienerwadel et al., 1995). This proton uptake is inconsistent with the assignment of a pK_a of 9.5 for Glu L212. According to the Henderson−Hasselbalch equation, an isolated acid group with a pK_a of 9.5 should be protonated at pH 7 and thus could not be further protonated upon Q_B^- formation. The proton uptake by Glu L212 is consistent with recent models based on electrostatic calculations by Gunner and Honig (1992) and Beroza et al. (1995) who showed that the cluster of acid residues near Q_B can have complex titration behavior due to electrostatic interactions within the cluster. Several titrating acid groups, including Glu L212, Asp L213, and Asp L210, had a significant fractional ionization over a wide pH range (>5 pH units), contrary to the classical Henderson−Hasselbalch equation. Both

calculations found that the major contribution to proton uptake was the protonation of Glu L212 due to the strong electrostatic coupling between the ionized Glu L212 and Q_B^-, although differences in the detailed titration behavior were obtained. Gunner and Honig (1992) calculated uptake of 1 H^+/Q_B^- at pH 7 with a decrease uptake at pH > 9. Beroza et al. (1995) calculated a fractional proton uptake by Glu L212 due to partially protonated Glu L212 in the Q_B state and fully protonated Glu L212 in the Q_B^- state. The fractional protonation of Glu L212 had a weak nonclassical pH dependence. The latter model is consistent with the stoichiometry of proton uptake (0.3−0.4 H^+/Q_B^-) in native RCs and the increased proton uptake (0.5−0.6 H^+/Q_B^-) in DN L213 RCs determined from the present FTIR data. It is also in agreement with the relatively weak dependence for proton uptake and the increase at high pH observed in kinetic IR measurements (Hienerwadel et al., 1995) and the earlier kinetic measurements that suggested a high pK_a of 9.5 for Glu L212. On the basis of these considerations and those from the preceding paragraph, we favor a model for native RCs (pH 7) in which Glu L212 is mostly protonated (60−70% COOH in the Q_B state), becoming fully protonated in the Q_B^- state (100% COOH).

Although the proton uptake by Asp L213 is not observed in the present study, information about its ionization state can be obtained from the FTIR measurements on native and DN L213 RCs. The lack of proton uptake by Asp L213 could be explained if Asp L213 were either fully ionized or fully protonated in both Q_B and Q_B^- states or if electrostatic interactions between Asp L213 and Q_B^- are weak or cancelled by changes in protonation of other residues such as Glu L212. Kinetic studies on DN L213 RCs (discussed above) suggested that Asp L213 is ionized in both Q_B and Q_B^- states. The increased proton uptake by Glu L212 (0.5−0.6 H^+/Q_B^-) in the DN L213 RCs is consistent with this assignment. The removal of a negatively charged Asp L213 would stabilize the ionized form of Glu L212 in DN L213 RCs. The larger ionized fraction of Glu L212 would lead to an increased proton uptake by this residue. Assuming Glu L212 is fully protonated in the Q_B^- state, the fraction of Glu L212 being ionized in the Q_B state would be 50−60 and 30−40% for DN L213 and native RCs, respectively. Thus, FTIR measurements are consistent with a large fraction of Asp L213 in the ionized state. The calculations of Gunner and Honig (1992) and Beroza et al. (1995) both predict that Asp L213 should be more highly protonated than Glu L212 and display an interesting inverse titration, i.e., an increase in protonation with increasing pH. Both studies predict that the proton uptake by Asp L213 is low but for different reasons. Beroza et al. (1995) predict that Asp L213 is almost fully (90%) protonated in the Q_B state; thus, the proton uptake upon formation of Q_B^- is small (<5% at pH 7), decreasing with increasing pH. This result would explain the FTIR data but is inconsistent with the kinetic studies indicating a negative charge on Asp L213. Gunner and Honig (1992) predict that Asp L213 is about 30% protonated in the Q_B state and 50% protonated in the Q_B^- state (at pH 7), i.e., $\Delta H^+ = 0.2$ H^+/Q_B^- (increasing with increasing pH). A partially protonated Asp L213 would be consistent with the FTIR data if the proton uptake were reduced due to screening of the charge on the Q_B^- produced largely by protonation of Glu L212. However, in that case, we would expect to see the protonation of Asp L213 in EQ L212 RCs,

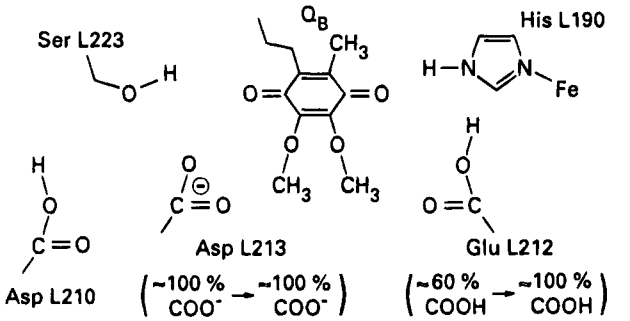


FIGURE 5: Model for the protonation of acidic residues near Q_B at pH 7. The protonation of the residue for the Q_B and Q_B^- states is indicated to the left and right of the arrows in the figure ($Q_B \rightarrow Q_B^-$), respectively. The model is based on FTIR measurements described above and on previous kinetic studies (see Discussion).

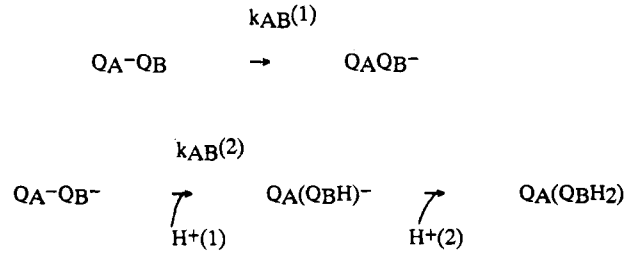
which was not observed. Thus, we favor a model in which Asp L213 is essentially ionized in both the Q_B and Q_B^- states. This model would account for the absence of FTIR bands in the COOH region due to Asp L213 and for the large increase in stability of Q_B^- in DN L213 RCs due to the loss of the charge on Asp L213 (Takahashi & Wraight, 1990, 1992; Paddock et al., 1994). However, we should note a discrepancy with theoretical results which indicate a higher protonation of Asp L213.

The FTIR indication for the protonation state of Asp L210 comes from the tentative assignment of part of the small differential signal at 1732 (+)/1740 (−) cm^{-1} to a band shift of the protonated Asp L210. The protonation of Asp L210 at pH 7 is inconsistent with the earlier assignment of a $pK_a \approx 5$ (Okamura & Feher, 1992). This discrepancy could be due to interactions between titrating groups (discussed above) leading to a sharing of the proton on Asp L210 with other residues, including Glu L212. Computational studies by Gunner and Honig (1992) and Beroza et al. (1995) show a partial protonation of Asp L210 in the Q_B state (≈50% protonated) with a weak nonclassical pH dependence and little proton uptake. Indeed, Gunner and Honig (1992) calculated a small (≈0.2 H^+/Q_B^-) proton release. Thus, the FTIR data are not inconsistent with a model in which Asp L210 is partially protonated but does not change protonation upon Q_B^- formation.

The FTIR results can be summarized with the following working model for the ionization states of residues near Q_B and Q_B^- in native RCs (Figure 5). Glu L212 is partially ionized (60−70% COOH) in the Q_B state, becoming fully protonated (≈100% COOH) in the Q_B^- state. Asp L213 is mostly ionized in both Q_B and Q_B^- states. Asp L210 is partially protonated. This model is tentative and should be examined by further experiments and computation. It should be noted that the major features of this model are unchanged from earlier models discussed above. The two most important aspects are that (1) Glu L212 has an abnormally high pK_a due to interaction with negatively charged residues and is fully protonated in the Q_B^- state and (2) Asp L213 is largely ionized in the Q_B and Q_B^- states, accounting for a lack of a proton uptake signal due to Asp L213 and for the increased proton uptake by Glu L212 in the DN L213 mutant. These two results have important consequenses for the mechanism of proton transfer.

(6) Mechanism of Proton Transfer. The FTIR measurements give important information about the mechanism of proton transfer to Q_B. Q_B becomes reduced to quinol during

two sequential electron transfer steps:



The first electron transfer step $k_{AB}^{(1)}$ does not involve direct protonation of Q_B, although protons are taken up by surrounding protein residues, particularly Glu L212 as shown by IR measurements. The second electron transfer $k_{AB}^{(2)}$ involves the uptake of two sequential protons, $H^+(1)$ and $H^+(2)$, taken up before and after the second electron transfer step, respectively. Site-directed mutagenesis experiments showed the involvement of Ser L223, Asp L213, and Glu H173 in the proton uptake of $H^+(1)$ and the involvement of Glu L212 in the proton uptake of $H^+(2)$ (Paddock et al., 1989, 1990; Takahashi & Wraight, 1992; Okamura & Feher, 1992).

Glu L212 has been proposed to serve as the donor of the second proton $H^+(2)$ taken up after electron transfer (Paddock et al., 1989; Takahashi & Wraight, 1992). The FTIR measurements demonstrate proton uptake by Glu L212 after formation of Q_B^-. Thus, Glu L212 is protonated before the second electron transfer step. This directly confirms the earlier models in which a pK_a of >10.5 for Glu L212 in the Q_B^- state was deduced. The proton on Glu L212 is thus available to rapidly protonate the fully reduced quinone following electron transfer.

Two possible roles for Asp L213 in proton transfer have been proposed. (1) An ionized Asp L213 could produce a negative potential near Q_B that facilitates proton transfer (Paddock et al., 1994; Takahashi & Wraight, 1990; Rongey et al., 1993; Okamura et al., 1992; Hanson et al., 1992; Maroti et al., 1994); (2) the protonated Asp L213 could serve as a direct proton donor to Ser L223 (Paddock et al., 1990; Okamura & Feher, 1992) or to reduced Q_B (Takahashi & Wraight, 1990).

The FTIR data indicate that Asp L213 does not change protonation state upon formation of Q_B^-. This result is consistent with previous arguments that suggest that Asp L213 is essentially ionized and that this electrostatic potential is important for favoring proton transfer into and near Q_B. The other role of Asp L213 in direct proton transfer, i.e., as a component of a proton transfer chain (Takahashi & Wraight, 1990, 1992; Rongey et al., 1993; Paddock et al., 1994), remains unproven. The ability of distant suppressor mutations to Asn or Ala replacements at L213 (by as much as 15 Å from Asp L213) to increase proton transfer in *Rb. capsulatus* (Hanson et al., 1992; Maroti et al., 1994) and *Rb. sphaeroides* (Rongey et al., 1993; Okamura et al., 1992) may argue against a direct role of Asp L213 in proton transfer; however, in native RCs, Asp L213 might still serve in a proton donor role, optimizing the rate. If Asp L213 is involved in proton transfer, the FTIR data require that it only transiently changes protonation state during proton transfer steps.

At least two remaining questions are as follows. (1) What are the protonation states of Glu L212 and Asp L213 after the second electron transfer $k_{AB}^{(2)}$? (2) Does the protonation state of Asp L213 transiently change during proton transfer reactions, and in this case, where is the Asp L213 COOH mode? Further FTIR and kinetic IR studies of the protonation of carboxylic acids in native and mutant RCs at different pHs and upon double-flash excitation can help answer these questions and give further information about the mechanism of proton transfer in bacterial RCs.

ACKNOWLEDGMENT

The authors thank Professor G. Feher for his sustained interest in this FTIR work, Professor W. Lubitz for a gift of ^{15}N-labeled RCs, and Ed Abresch for preparation of the reaction centers.

REFERENCES

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8487−8491.
Bauscher, M., & Mäntele, W. (1992) *J. Phys. Chem. 96*, 11101−11108.
Bauscher, M., Nabedryk, E., Bagley, K., Breton, J., & Mäntele, W. (1990) *FEBS Lett. 261*, 191−195.
Bauscher, M., Leonhard, M., Moss, D. A., & Mäntele, W. (1993) *Biochim. Biophys. Acta 1183*, 59−71.
Bellamy L. J. (1975) in *The infrared spectra of complexed molecules*, Chapman & Hall, London.
Beroza, P., Fredkin, D. R., Okamura, M. Y., & Feher, G. (1992) in *The Photosynthetic Bacterial Reaction Center II* (Breton, J., & Verméglio, A., Eds.) pp 363−374, Plenum Press, New York.
Beroza, P., Fredkin, D. R., Okamura, M. Y., & Feher, G. (1995) *Biophys. J. 68*, 2233−2250.
Braiman, M. S., Walter, T. J., & Briercheck, D. (1994) *Biochemistry 33*, 1629−1635.
Breton, J., Berthomieu, C., Thibodeau, D. L., & Nabedryk, E. (1991a) *FEBS Lett. 288*, 109−113.
Breton, J., Thibodeau, D. L., Berthomieu, C., Mäntele, W., Verméglio, A., & Nabedryk, E. (1991b) *FEBS Lett. 278*, 257−260.
Breton, J., Burie, J.-R., Berthomieu, C., Thibodeau, D. T., Andrianambinintsoa, S., Dejonghe, D., Berger, G., & Nabedryk, E. (1992) in *The Photosynthetic Bacterial Reaction Center II* (Breton, J., & Verméglio, A., Eds.) pp 155−162, Plenum Press, New York.
Breton, J., Boullais, C., Burie, J.-R., Nabedryk, E., & Mioskowski, C. (1994) *Biochemistry 33*, 14378−14386.
Breton, J., Boullais, C., Berger, G., Mioskowski, C., & Nabedryk, E. (1995) *Biochemistry 34*, 11606−11616.
Brudler, R., de Groot, H. J. M., van Liemt, W. B. S., Steggerda, W. F., Esmeijer, R., Gast, P., Hoff., A. J., Lugtenburg, J., & Gerwert, K. (1994) *EMBO J. 13*, 5523−5530.
Brudler, R., de Groot, H. J. M., van Liemt, W. B. S., Gast, P., Hoff, A. J., Lugtenburg, J., & Gerwert, K. (1995) *FEBS Lett. 370*, 88−92.
Chirgadze, Yu. N., Fedorov, O. V., & Trushina, N. P. (1975) *Biopolymers 14*, 679−694.
Chirino, A. J., Lous, E. J., Huber, M., Allen, J. P., Schenck, C. C., Paddock, M. L., Feher, G., & Rees, D. C. (1994) *Biochemistry 33*, 4584−4593.
Colthup, N. B., Daly, L. H., & Wiberley, S. E. (1964) in *Introduction to infrared and Raman spectroscopy*, Academic Press, New York.
Deisenhofer, J., Epp, O., Sinning, I., & Michel, H. (1995) *J. Mol. Biol. 246*, 429−457.
Eisenstein, L., Lin, S.-L., Dollinger, G., Odashima, K., Termini, J., Konno, K., Ding, W.-D., & Nakanishi, K. (1987) *J. Am. Chem. Soc. 109*, 6860−6862.
El-Kabbani, O., Chang, C.-H., Tiede, D., Norris, J., & Schiffer, M. (1991) *Biochemistry 30*, 5361−5369.
Ermler, U., Fritzsch, G., Buchanan, S. K., & Michel, H. (1994) *Structure 2*, 925−936.
Feher, G., Allen, J. P., Okamura, M. Y., & Rees, D. C. (1989) *Nature 339*, 111−116.

Gunner, M. R., & Honig, B. (1992) in *The Photosynthetic Bacterial Reaction Center II* (Breton, J., & Verméglio, A., Eds.) pp 403−410, Plenum Press, New York.

Hanson, D. K., Nance, S. L., & Schiffer, M. (1992) *Photosynth. Res. 32*, 147−153.

Haurie, M., & Novak, A. (1965a) *J. Chim. Phys. 62*, 137−145.

Haurie, M., & Novak, A. (1965b) *J. Chim. Phys. 62*, 146−157.

Hienerwadel, R., Thibodeau, D., Lenz, F., Nabedryk, E., Breton, J., Kreutz, W., & Mäntele, W. (1992a) *Biochemistry 31*, 5799−5808.

Hienerwadel, R., Nabedryk, E., Paddock, M. L., Rongey, S., Okamura, M. Y., Mäntele, W., & Breton, J. (1992b) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. I, pp 437−440, Kluwer Academic Publishers, Dordrecht.

Hienerwadel, R., Grzybek, S., Fogel, C., Kreutz, W., Okamura, M. Y., Paddock, M. L., Breton, J., Nabedryk, E., & Mäntele, W. (1995) *Biochemistry 34*, 2832−2843.

Iliadis, G., Zundel, G., & Brzezinski, B. (1994) *FEBS Lett. 352*, 315−317.

Kleinfeld, D., Okamura, M. Y., & Feher, G. (1984) *Biochemistry 23*, 5780−5786.

Lancaster, C. R. D., Ermler, U., & Michel, H. (1995) in *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., & Bauer, C. E., Eds.) pp 503−526, Kluwer Academic Publishers, The Netherlands.

Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992) *Biochemistry* 31, 4684−4690.

Mäntele, W., Leonhard, M., Bauscher, M., Nabedryk, E., Breton, J., & Moss, D. A. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel-Beyerle, M.-E., Ed.) pp 31−44, Springer-Verlag, Berlin.

Maroti, P., & Wraight, C. A. (1988) *Biochim. Biophys. Acta 934*, 329−347.

Maroti, P., Hanson, D. K., Baciou, L., Schiffer, M., & Sebban, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5617−5621.

McPherson, P. H., Okamura, M. Y., & Feher, G. (1988) *Biochim. Biophys. Acta 934*, 348−368.

Nabedryk, E., Andrianambinintsoa, S., Mäntele, W., & Breton, J. (1988) in *The Photosynthetic Bacterial Reaction Center, Structure and Dynamics* (Breton, J., & Verméglio, A., Eds.) pp 237−250, Plenum Press, New York.

Nabedryk, E., Bagley, K., Thibodeau, D. L., Bauscher, M., Mäntele, W., & Breton, J. (1990) *FEBS Lett. 266*, 59−62.

Nabedryk, E., Breton, J., Hienerwadel, R., Mäntele, W., Paddock, M. L., Rongey, S. H., Feher, G., & Okamura, M. Y. (1993) *Biophys. J. 64*, A214, 468.

Nabedryk, E., Breton, J., Hienerwadel, R., Fogel, C., Mäntele, W., Paddock, M. L., & Okamura, M. Y. (1995) *Proceedings of the Xth International Photosynthesis Congress* (Mathis, P., Ed.) (in press).

Okamura, M. Y., & Feher, G. (1992) *Annu. Rev. Biochem. 61*, 861−896.

Okamura, M. Y., & Feher, G. (1995) in *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., & Bauer, C. E., Eds.) pp 577−594, Kluwer Academic Publishers, The Netherlands.

Okamura, M. Y., Paddock, M. L., McPherson, P. H., Rongey, S., & Feher, G. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. I, pp 349−356, Kluwer Academic Publishers, Dordrecht.

Paddock, M. L., Rongey, S. H., Feher, G., & Okamura, M. Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6602−6606.

Paddock, M. L., McPherson, P. H., Feher, G., & Okamura, M. Y. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6803−6807.

Paddock, M. L., Rongey, S. H., McPherson, P. H., Juth, A., Feher, G., & Okamura, M. Y. (1994) *Biochemistry 33*, 734−745.

Rongey, S. H., Paddock, M. L., Feher, G., & Okamura, M. Y. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1325−1329.

Rothschild, K. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.

Shinkarev, V. P., Takahashi, E., & Wraight, C. A. (1993) *Biochim. Biophys. Acta 1142*, 214−216.

Siebert, F. (1993) in *Methods in Enzymology, Biochemical Spectroscopy* (Sauer, K., Ed.) Vol. 246, pp 501−526, Academic Press, San Diego.

Siebert, F., Mäntele, W., & Kreutz, W. (1982) *FEBS Lett. 141*, 82−87.

Takahashi, E., & Wraight, C. A. (1990) *Biochim. Biophys. Acta 1020*, 107−111.

Takahashi, E., & Wraight C. A. (1992) *Biochemistry 31*, 855−866.

Takahashi, E., & Wraight C. A. (1994) in *Advances in Molecular and Cell Biology* (Barber, J., Ed.) Vol. 10, pp 197−251, JAI Press, Greenwich, CT.

Venyaminov, S. Yu., & Kalnin, N. N. (1990) *Biopolymers 30*, 1243−1257.

Wraight, C. (1979) *Biochim. Biophys. Acta 548*, 309−327.

BI951673A