Fourier Transform Infrared Study of the Primary Electron Donor in Chromatophores of *Rhodobacter sphaeroides* with Reaction Centers Genetically Modified at Residues M160 and L131†

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ABSTRACT: Structural changes in chromatophores of *Rhodobacter sphaeroides* reaction center mutants associated with the substitution of amino acid residues near the primary electron donor P have been investigated by light-induced FTIR difference spectroscopy. The single-site mutations Leu-L131 to His and Leu-M160 to His and the corresponding double mutation were designed to introduce a proton-donating residue that could form a hydrogen bond with the keto carbonyl of ring V of each bacteriochlorophyll (P_L and P_M) of the dimer. The presence of large positive bands at ≈1550, 1480, and 1295 cm^{-1}, as well as at 2600–2800 cm^{-1} in the light-induced $P^+Q_A^-/PQ_A$ FTIR difference spectra, corresponding to the photooxidation of P and the photoreduction of the primary quinone Q_A, demonstrates that the BChl dimer state of P^+ is preserved in the LH(L131), LH(M160), and LH(M160)+LH(L131) mutants, although frequency shifts and amplitude changes can be observed, notably for LH(M160). Compared to wild type, these changes are thought to reflect a different charge repartition over the two BChls in P^+. Large frequency downshifts in the 9-keto C=O stretching region of the $P^+Q_A^-/PQ_A$ FTIR difference spectra of chromatophores are observed in the mutant samples relative to wild type. For the LH(M160) mutant, a large differential signal at 1678/1664 cm^{-1} is assigned to a shift, upon photooxidation, of the 9-keto C=O of P_M hydrogen-bonded to His-M160, while that at 1718/1696 cm^{-1} corresponds to the free 9-keto C=O of P_L. For the LH(L131) mutant, a signal at 1657 cm^{-1} is assigned to the 9-keto C=O of P_L hydrogen-bonded to His-L131 while two signals at 1692 and 1682 cm^{-1} are possible candidates for P_L^+. For the double mutant, the main differential signal at 1685/1662 cm^{-1}, which is downshifted by ≈20 cm^{-1} with respect to wild type, is interpreted in terms of the superposition of the contributions from the 9-keto C=O of both P_L and P_M hydrogen-bonded to His-L131 and His-M160, respectively. The changes observed in the IR spectra of the mutants support the conclusion that a hydrogen bond has been introduced to the dimer at the 9-keto C=O of P_L and/or P_M, and they suggest a stronger hydrogen bond in LH(L131) than in LH(M160). From the present data and those previously reported for heterodimer mutants where one bacteriochlorophyll P_L or P_M is replaced by bacteriopheophytin [Nabedryk, E., Robles, S. J., Goldman, E., Youvan, D. C., & Breton, J. (1992) *Biochemistry 31*, 10852–10858], a clear identification of the vibrational modes of P_L and P_M in the wild-type reaction center can be made. The band at 1683 cm^{-1} and the shoulder at 1692 cm^{-1} are assigned to the 9-keto C=O vibrations of P_M and P_L, respectively, their counterparts appearing at 1705 and 1713 cm^{-1} in the photooxidized state.

In the photosynthetic reaction center (RC)[1] from purple bacteria, for which the three-dimensional structure has been determined by X-ray diffraction (Michel et al., 1986; Allen et al., 1987; El-Kabbani et al., 1991), two symmetrically arranged protein subunits, L and M, form the basic structure to which the cofactors are noncovalently bound. These cofactors consist of four bacteriochlorophylls (BChl), a pair of them forming the primary electron donor P, two bacteriopheophytins (BPhe), two quinones (Q_A and Q_B), a carotenoid, and a non-heme iron. The detailed X-ray model has revealed the local pseudo-2-fold symmetry axis leading to the division of the cofactors into two apparently symmetrical branches (labeled L and M). However, kinetic and spectroscopic data indicate that the initial electron transfer from P to the first quinone acceptor Q_A proceeds along the L-branch (Kirmaier & Holten, 1987; Michel-Beyerle et al., 1988). Indeed, the RC crystal structure highlights a key role for some amino acid residues which break at several positions the 2-fold symmetry. Several of these residues appear to interact with specific groups of the cofactors. Using site-directed mutagenesis, RCs from *Rhodobacter* (*Rb.*) *capsulatus* (Coleman & Youvan, 1990; Stocker et al., 1992) and *Rb. sphaeroides* (Gray et al., 1990; Nagarajan et al., 1990; Williams et al., 1992a) have been genetically modified in order to perturb the binding sites or to symmetrize the amino acid residues forming the binding contours of P. The importance of these symmetry-breaking residues is underscored by the

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[1] Abbreviations: RC, reaction center; P, primary electron donor; P_L and P_M, primary donor bacteriochlorophylls associated with the L and M subunits, respectively; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Q_A, primary quinone acceptor; *Rb.*, *Rhodobacter*; Wt, wild type; FTIR, Fourier transform infrared; ENDOR, electron nuclear double resonance.

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alteration of the initial electron-transfer properties in these mutant RCs (Finkele et al., 1990; Nagarajan et al., 1990; Chan et al., 1991; Stocker et al., 1992; Woodbury et al., 1993).

The X-ray model of *Rb. sphaeroides* RC indicates that the 9-keto carbonyl of each BChl (P_L and P_M) constituting the dimer has no specific interaction with the protein (Yeates et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1992). The potential effect of the addition of a hydrogen bond to one or both BChls suggested the design of mutations involving amino acid substitutions at the Leu-L131 and Leu-M160 positions (Williams et al., 1992a). These symmetry-related amino acid residues are near the ring V keto carbonyls of P_L and P_M, respectively. The two single-site mutants Leu-M160 to His, LH(M160), and Leu-L131 to His, LH(L131), as well as the corresponding double mutant, LH(M160)+LH(L131), have been constructed and characterized with respect to their primary donor oxidation potential, initial electron-transfer rates, charge recombination kinetics, early-time thermodynamics (Williams et al., 1992a,b; Woodbury et al., 1993), and spin density distribution between the two halves P_L and P_M in P^+ (Rautter et al., 1992). Here, the putative hydrogen bonding of the 9-keto C=O of P_L and/or P_M to the proton-donating His residue is investigated by FTIR spectroscopy.

Vibrational IR spectroscopy is highly sensitive to changes in the interaction pattern of carbonyl groups within a molecule. In particular, light-induced FTIR difference spectroscopy appears to be a valuable approach for studying molecular changes in protein–cofactor interactions within the photosynthetic systems [reviewed by Hoff in Bixon et al. (1992) and by Mäntele (1993)]. In the present contribution, FTIR difference spectroscopy is used to investigate the effect of positioning a hydrogen bond donor group near the keto carbonyl of P_L and/or P_M on the light-induced PQ_A to $P^+Q_A^-$ transition for *Rb. sphaeroides* purified chromatophores of LH(L131), LH(M160), and LH(M160)+LH(L131) mutants, compared to wild type (Wt). IR data obtained with these mutants together with those previously obtained for the heterodimer mutants of *Rb. capsulatus* (Nabedryk et al., 1992a, 1993) are compared in order to discriminate the vibrational modes of P_L and P_M in Wt RCs.

MATERIALS AND METHODS

The construction and initial characterization of the mutants from *Rb. sphaeroides* are described in Williams et al. (1992a,b). Purified chromatophores were deposited on a CaF_2 window. Light-induced FTIR difference spectra of hydrated films were performed as described previously (Nabedryk et al., 1990) on a Nicolet 60SX FTIR spectrometer equipped with an MCT-A detector, a KBr beam-splitter, and a cryostat. Steady-state continuous illumination was provided by a tungsten lamp. The intensity of the actinic light was adjusted with neutral density filters in order to achieve saturation of the $P^+Q_A^-/PQ_A$ signals. Cycles of illumination were repeated several hundred times.

RESULTS

Figure 1 shows the light-induced FTIR difference spectra obtained at 100 K for *Rb. sphaeroides* chromatophores of Wt and of the LH(M160), LH(L131), and LH(M160)+LH(L131) mutants. At 100 K, the electron is no longer transferred from Q_A to Q_B (Parson, 1978), and the spectra referred to as $P^+Q_A^-/PQ_A$ spectra correspond to the difference between the charge-separated state $P^+Q_A^-$ and the neutral state PQ_A. In the 1650–1200-cm^{-1} region, several spectral features are common to all spectra of Figure 1, notably the

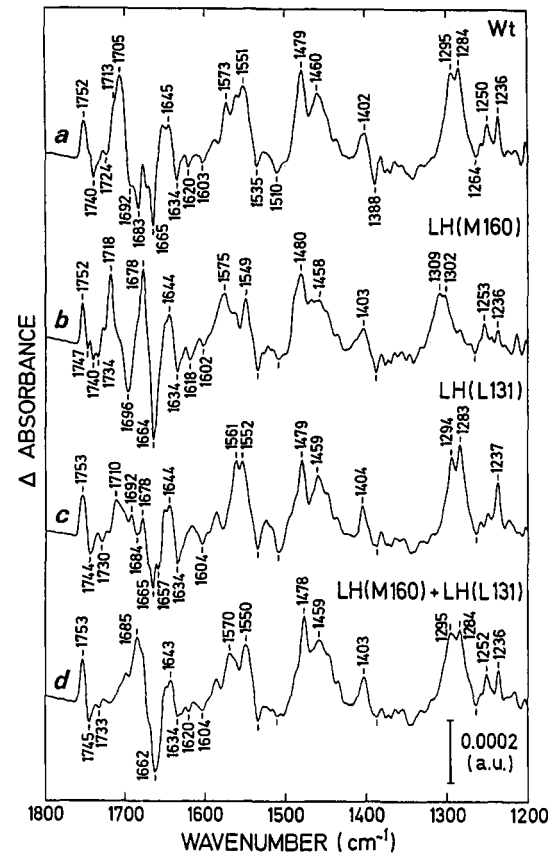

FIGURE 1: Light-induced FTIR difference spectra at 100 K of *Rb. sphaeroides* chromatophores from wild type (a) and from the mutants LH(M160) (b), LH(L131) (c), and LH(M160)+LH(L131) (d). 4-cm^{-1} resolution. Average of ≈70 000 interferograms.

large positive bands at around 1550, 1480, and 1295 cm^{-1} as well as the small negative signals at 1634, 1603, 1535, 1510, 1388, and 1264 cm^{-1}. The amplitude of these bands and more specifically of the 1480-cm^{-1} band correlates well with the amide I absorption of the samples. This is taken to indicate a comparable extent of $P^+Q_A^-$ formation under saturating illumination conditions for all the samples investigated. The main differences are observed in the 1720–1650-cm^{-1} carbonyl stretching region, with drastic changes in both the amplitudes and frequencies of the bands. In LH(M160), two large differential signals at 1718/1696 and 1678/1664 cm^{-1} are clearly separated while in LH(L131) a complex-structured positive signal is observed between 1710 and 1678 cm^{-1}. On the other hand, the double mutant shows a differential signal at 1685/1662 cm^{-1} with several shoulders.

The photooxidation of P has been previously characterized by light-induced FTIR difference spectroscopy for RCs and chromatophores of *Rb. sphaeroides* and *Rb. capsulatus* (Mäntele et al., 1985, 1988; Nabedryk et al., 1987, 1990, 1992a,b,c, 1993; Hayashi et al., 1990; Morita et al., 1991, 1993). In Figure 1a, the differential bands evident at 1752/1740 and 1705/1683 cm^{-1} (with shoulders at 1713 and 1692 cm^{-1}) are assigned to a predominant contribution of the 10a-ester and 9-keto carbonyls of P^+/P, respectively (Mäntele et al., 1988; Nabedryk et al., 1992a). A detailed interpretation of these C=O bands in terms of contributions from P_L and P_M will be presented in the following, in comparison with data obtained from various sets of mutants. The small negative band at 1620 cm^{-1} (Figure 1a) has been proposed to arise from the hydrogen-bonded 2a-acetyl C=O of one BChl (P_L) of the dimer [Nabedryk et al., 1992b; see also Mattioli et al. (1991)], on the basis of study of the HF(L168) mutant

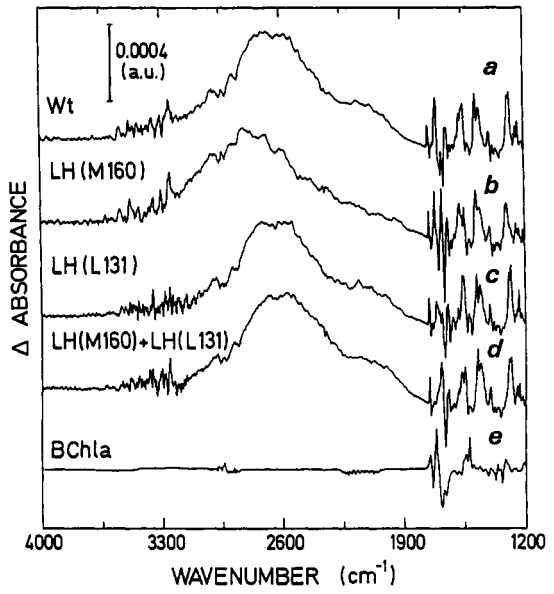

FIGURE 2: Comparison in the 4000–1200-cm^{-1} spectral range of $P^+Q_A^-/PQ_A$ spectra of *Rb. sphaeroides* chromatophores from wild type (a), LH(M160) (b), LH(L131) (c), and LH(M160)+LH(L131) (d) with (e) the cation-minus-neutral difference spectrum of BChl *a* in tetrahydrofuran (Mäntele et al., 1988). The spectra of Wt and mutants have been normalized on the 1480-cm^{-1} band. The ΔA bar applies to $P^+Q_A^-/PQ_A$ spectra.

containing the substitution His-L168 to Phe designed to remove the hydrogen bond between the 2a C=O of P_L and His-L168 (Yeates et al., 1988; El-Kabbani et al., 1991). The free acetyl C=O from P_M found at 1648 cm^{-1} in the $^3P/P$ FTIR spectrum of the triplet state of *Rb. sphaeroides* RCs at 85 K (Breton & Nabedryk, 1993) could account in Figure 1 for the small dip at 1648 cm^{-1} although a peptide C=O may contribute as well. The 1665-cm^{-1} signal has been assigned to a conformational change of a peptide C=O (Nabedryk et al., 1992a; Maiti et al., 1993) while in electrochemically generated P^+/P difference spectra of *Rb. sphaeroides* RCs, a negative band at 1662 cm^{-1} has been interpreted in terms of a His NH_2^+ bending mode (Leonhard & Mäntele, 1993). Contributions from Q_A and Q_A^- modes are also expected in Figure 1, although in a $P^+Q_A^-/PQ_A$ spectrum the large P^+/P signals dominate the quinone bands. At 290 K, the Q_A^-/Q_A spectrum obtained photochemically with *Rb. sphaeroides* RCs (Breton et al., 1991a) shows negative peaks notably at 1735, 1670, 1601, and 1263 cm^{-1} with a broad and structured positive band centered at 1466 cm^{-1} corresponding to C⋯O and C⋯C modes of the ubiquinone anion. In Figure 1, the modes of Q_A^- are thus expected to contribute notably in the cluster of positive bands peaking at ≈1480 cm^{-1} while the small negative signals at 1670, 1603, and 1264 cm^{-1} present in all spectra are assigned to vibrations from the neutral quinone state (Bagley et al., 1990; Breton et al., 1992a). The 1752/1740-cm^{-1} signal (Figure 1a) arises from at least one 10a-ester C=O group of the special pair, with the small shoulder at 1745 cm^{-1} possibly accounting for the second ester C=O of P in the neutral state. In the $^3P/P$ FTIR spectrum of *Rb. sphaeroides* RCs at 85 K, negative bands at 1740 and 1749 cm^{-1} have been identified to the 10a-ester carbonyls of the two BChl molecules of P (Breton & Nabedryk, 1993).

In Wt, the major part of the large positive signals at 1580–1540, 1480–1450, and 1295–1284 cm^{-1} (Figure 1a) and a broad band centered around 2600 cm^{-1} (Figure 2a) appear characteristic of the P^+ state of the BChl dimer (Nabedryk et al., 1992a; Breton et al., 1992b). In contrast, the P^+Q^-/PQ spectra of heterodimer mutants (Nabedryk et al., 1992a, 1993; Breton et al., 1992b) where one BChl, P_L or P_M, is replaced by BPhe, as well as the cation IR difference spectrum of BChla monomer *in vitro* (Mäntele et al., 1988), show only small signals in the 1600–1200-cm^{-1} range, with no evidence of the 2600-cm^{-1} band (see Figure 2e for the BChl a^+/BChl *a* spectrum). The data presented in Figure 1b,c,d and Figure 2b,c,d demonstrate that the positive bands characteristic of the dimeric state of P^+ are also present in the spectra of the three mutants with, however, frequency shifts and/or amplitude changes. In particular, perturbations are detected in the 1570–1550-cm^{-1} and ≈1300-cm^{-1} spectral ranges where C–C, C–N, and C–H porphyrin ring modes are expected (Lutz & Mäntele, 1991). Strong IR modes specific of ring oxidation of substituted metalloporphyrins have been reported to absorb at ≈1550 and ≈1280 cm^{-1} and to depend on the nature of the substituents (Shimomura et al., 1981). More specifically, in the LH(M160) spectrum (Figure 1b), the low-frequency band is clearly upshifted to 1309–1302 cm^{-1} (compared to 1295–1284 cm^{-1} in Wt) and exhibits a slightly decreased amplitude while the 1573-cm^{-1} band seen in Wt disappears in LH(L131) (Figure 1c). In addition, for LH(M160), the high-frequency band (Figure 2b) attributed to an electronic transition within the dimeric P^+ state (Breton et al., 1992b; Parson et al., 1992) is also upshifted (to ≈2800 cm^{-1}), with its amplitude slightly reduced compared to Wt. LH(L131) and the double mutant exhibit a broad absorption band at ≈2600 cm^{-1} (Figure 2c,d) comparable to that observed in Wt (Figure 2a). The data presented in Figures 1 and 2 are taken to indicate that the BChl dimer state of P^+ is essentially preserved in the LH(M160), LH(L131), and LH(M160)+LH(L131) mutants. However, the changes observed in the 1300- and 2800-cm^{-1} bands for the LH(M160) mutant probably reflect some modifications in the structure of the dimer and/or changes in interaction induced by the newly designed protein environment, leading to a new distribution of the positive charge over the two BChls in the P^+ state.

Hydrogen bonding must result in specific IR shifts of carbonyl bands. Very pronounced changes are observed in the 1720–1650-cm^{-1} carbonyl spectral region of the $P^+Q_A^-/PQ_A$ spectra of the mutants (Figure 1b,c,d), compared to Wt. On the basis of *in vitro* FTIR studies of the electrochemically generated BChl *a* radical cation (in tetrahydrofuran, the largest differential signal observed at 1716/1684 cm^{-1} corresponds to the 9-keto C=O of BChl; Mäntele et al., 1988; Hayashi et al., 1990), the large differential signal observed in Wt at 1705/1683 cm^{-1} was assigned to a free 9-keto C=O of P. The pronounced shoulder at 1692 cm^{-1} (Figure 1a) which is present but barely resolved in spectra of *Rb. sphaeroides* and *Rb. capsulatus* chromatophores (and RCs) at 275 K [Nabedryk et al., 1992a; see also also Morita et al. (1993)] was tentatively assigned to the keto group of the second BChl of P (Nabedryk et al., 1992a, 1993). This proposal is reinforced by the recent IR study of the triplet state of P in RCs from *Rb. sphaeroides* R26. The FTIR $^3P/P$ difference spectrum obtained at 85 K is characterized by two modes at 1695 and 1682 cm^{-1} attributed to the 9-keto C=O of the two BChls constituting P (Breton & Nabedryk, 1993). The 9-keto C=O vibrations of P have been also investigated by Raman spectroscopy (Zhou et al., 1989; Mattioli et al., 1991; Palaniappan et al., 1992), and, in particular, FT Raman spectra at room temperature of isolated *Rb. sphaeroides* RCs (Mattioli et al., 1991) exhibit bands at 1691 and 1680 cm^{-1} which have been identified as 9-keto C=O modes of P. In native RCs, both keto carbonyls of P thus appear non-hydrogen-bonded, as also inferred from the

X-ray data from *Rb. sphaeroides* RC (Yeates et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1992).

For the LH(M160) mutant (Figure 1b), the large differential signal observed at 1678/1664 cm^{-1} is assigned mostly to a shift, upon photooxidation, of the 9-keto C=O of P_M hydrogen-bonded to the side chain of His-M160, although some protein contribution can be expected at 1665 cm^{-1}, as a signal is also found at this frequency in Wt. The 1718/1696-cm^{-1} signal is assigned to the free keto C=O of P_L. In the heterodimer mutants from *Rb. capsulatus* where the His-M200 ligand to the Mg atom of P_M has been replaced with Leu or Phe (which results in P_M being a BPhe and P_L a BChl), a single negative signal at 1696 cm^{-1} was assigned to the non-hydrogen-bonded 9-keto C=O of P_L, with a split positive signal at 1722–1710 cm^{-1} corresponding to this group in P^+ (Nabedryk et al., 1992a, 1993). It therefore appears that there is a close similarity between the frequency of the free 9-keto carbonyl of P_L in the LH(M160) mutant and in the two HL(M200) and HF(M200) heterodimers.

In the FTIR spectrum of LH(L131) (Figure 1c), the C=O stretching region is also drastically perturbed. When compared to Wt and other mutants, the amplitude of the differential signals in the keto C=O region of LH(L131) is significantly reduced relative to the signals in the other regions. A broad positive peak is observed at 1710 cm^{-1} with satellite peaks at 1692 and 1678 cm^{-1}. A main negative signal is observed at 1665 cm^{-1} with shoulders at ≈1669 and 1657 cm^{-1}. The 1665- and 1669-cm^{-1} signals being also present in Wt, the only new band that could correspond to a hydrogen-bonded carbonyl is the one at 1657 cm^{-1}. The question then arises where to locate the positive peak corresponding to this group in the state P^+. The most likely candidates are the peaks at 1692 and 1678 cm^{-1}. These two peaks and the dip at 1684 cm^{-1} are interpreted as resulting from overlap of the positive contribution from the hydrogen-bonded 9-keto C=O in P^+ with the negative band from the free 9-keto C=O group which is observed at 1683 cm^{-1} in Wt. In this frame, the 1684-cm^{-1} signal in LH(L131) is assigned to the non-hydrogen-bonded 9-keto C=O of P_M shifting to 1710 cm^{-1} in the cation state. It can be noted that in both LH(L131) and LH(M160) mutants, a small but consistent frequency upshift (1–5 cm^{-1}) of the 9-keto C=O modes of the non-hydrogen-bonded P is observed for both neutral and cation states, compared to Wt, probably reflecting secondary effects of the single-site mutation on the whole binding pocket. While the interpretation of the LH(M160) spectrum in the 9-keto C=O region is rather straightforward, this is not the case for LH(L131). In order to help with the interpretation, the double difference spectrum between the $P^+Q_A^-/PQ_A$ spectra of LH(L131) and Wt has been constructed, i.e., LH(L131) minus Wt (Figure 3). This new spectrum was obtained by using interactive substraction of spectra c and a in Figure 1, in order to minimize the dimer bands and more specifically the 1480-cm^{-1} band which appears the most comparable in all the spectra of Figure 1. In the 9-keto carbonyl region, the double difference spectrum shows a negative signal at 1705 cm^{-1}, a split positive peak at 1692 and 1682 cm^{-1}, and a small negative signal at 1669 cm^{-1} and, in addition, clearly reveals a main negative peak at 1657 cm^{-1} which can be attributed to the formation of a strong hydrogen bond between the 9-keto of P_L and His-L131. However, the presence of negative signals at 1692 and 1683 cm^{-1} in the $P^+Q_A^-/PQ_A$ spectrum of Wt and of positive signals at 1692 and 1678 cm^{-1} in LH(L131) precludes a decisive assignment in terms of the P_L^+ contribution to the positive signals at 1692 and 1682 cm^{-1} found in the double difference spectrum (Figure 3). Furthermore, in the LH(L131) mutant, significant perturbations not only occur for the keto modes of P but also occur for the 10a-ester C=O (see Figure 3) as well as in the 2a-acetyl C=O absorption range, where a band at 1620 cm^{-1} (Figure 1a) assigned to the 2a-acetyl C=O of P_L in Wt (Mattioli et al., 1991; Nabedryk et al., 1992b) is missing (Figure 1c). In the double difference spectrum (Figure 3), a negative band at 1630 cm^{-1} and a corresponding positive one at 1619 cm^{-1} suggest a frequency upshift of the 2a-acetyl C=O of P_L in LH(L131), compared to Wt. In addition, a negative band at 1574 cm^{-1} (Figure 3) corresponds to the band missing in the LH(L131) spectrum (Figure 1c), as described above. All these observations support a strong perturbation of the whole macrocycle of P_L and its substituents in both P and P^+ states of LH(L131).

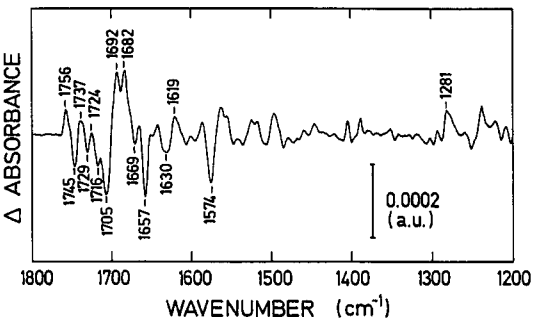

FIGURE 3: Calculated double difference spectrum between $P^+Q_A^-/PQ_A$ spectra of *Rb. sphaeroides* chromatophores from LH(L131) (Figure 1c) and wild type (Figure 1a), i.e., LH(L131) minus Wt. See the text for the normalization procedure. All the signals are reproducibly observed in double difference spectra calculated from three different sets of LH(L131) and Wt $P^+Q_A^-/PQ_A$ spectra measured on distinct samples.

The overall shape of the spectrum of the double mutant (Figure 1d) in the keto C=O range is most comparable to that of Wt as it also shows a main differential signal (peaking at 1685/1662 cm^{-1}) with several shoulders. However, it differs from Wt by a ≈20-cm^{-1} frequency downshift. The whole differential signal at 1685/1662 cm^{-1} is interpreted as the superposition of the two contributions from the 9-keto C=O of both P_L and P_M hydrogen-bonded to His-L131 and His-M160, respectively. Double difference spectra between the $P^+Q_A^-/PQ_A$ spectra of mutants and Wt have also been calculated for LH(M160)+LH(L131) and LH(M160) (data not shown). In both cases, the double difference spectrum clearly shows a differential signal corresponding to the downshift of the 9-keto C=O group, at 1683/1658 cm^{-1} in the double mutant minus Wt spectrum and at 1678/1661 cm^{-1} in LH(M160) minus Wt, and thus confirms the formation of new hydrogen bonds in these mutants.

DISCUSSION

The changes observed in the spectra of the three investigated mutants support the conclusion that a hydrogen bond has been introduced to the dimer at the 9-keto C=O of P_M and/or P_L due to the mutations Leu-M160 to His and/or Leu-L131 to His. A similar statement is also inferred from FT Raman spectra of isolated mutant RCs although some differences appear between IR and Raman frequencies, in particular for the 9-keto C=O of P and P^+ in LH(L131) and LH(M160)+LH(L131) mutants (T. Mattioli and B. Robert, personal communication). Differences of a few wavenumbers can be explained by the different experimental approaches of Raman and IR spectroscopies, i.e., recording of *absolute* FT Raman spectra and of light-induced *difference* FTIR spectra,

Table I: IR Frequencies (cm^{-1}) of 9-Keto Carbonyl Vibrations of Primary Donor Bacteriochlorophylls, P_L and P_M, in Chromatophores from Wild Type and Mutants[a]

wild type			LH(M160)			LH(L131)			LH(M160)+LH(L131)			BChl *a*	BChl a^+	
P_L	P_L^+	δ	P_L	P_L^+	δ	P_L	P_L^+	δ	P	P^+	δ	THF	THF	δ
1692	1713	21	1696	1718	22	1657			1662	1685	23	1684	1716	32
wild type			LH(M160)			LH(L131)						BChl *a*	BChl a^+	
P_M	P_M^+	δ	P_M	P_M^+	δ	P_M	P_M^+	δ				MeOH	MeOH	δ
1683	1705	22	1664	1678	14	1684	1710	26				1652	1708	56

[a] Comparison with bacteriochlorophyll *a* in tetrahydrofuran and methanol [from Mäntele et al. (1988) and Hayashi et al. (1990)]. δ is the frequency shift observed upon cation formation.

in addition to possible differences in temperature and sample preparation conditions. Small changes in the relative intensities of bands, notably for the positive signals in the 9-keto C=O region, have been previously observed between P^+Q^-/PQ FTIR difference spectra of chromatophores and RCs (Nabedryk et al., 1987; Robert et al., 1989; Morita et al., 1991, 1993), and it cannot be excluded that some perturbations in protein–cofactor interactions occurring upon solubilization and purification of RCs can be demasked by FTIR difference spectroscopy. For mutants, the probability of structural destabilization leading to a loss of integrity upon RC extraction is, in principle, increased. Thus, a serious risk exists that for genetically modified RCs, the observed spectroscopic properties reflect not only the direct effect of the mutation but also structural rearrangements brought about by the isolation and purification of these possibly destabilized RCs. This is the rationale for using chromatophores rather than isolated RCs in our FTIR studies of mutants (Breton et al., 1991b, 1992b; Nabedryk et al., 1992a,b,c, 1993), although it necessitates longer accumulation times for a comparable signal-to-noise ratio.

In view of the present data on the LH(M160), LH(L131), and LH(M160)+LH(L131) mutants and the FTIR spectra previously reported for the heterodimer mutants of *Rb. capsulatus* (Nabedryk et al., 1992a), tentative identifications of the 9-keto C=O vibrational modes of P_L and P_M in Wt chromatophores can be proposed. There is a close similarity between the frequency of the 9-keto C=O of P_L in LH(M160) and in the two heterodimers HL(M200) and HF(M200), for both the neutral and photooxidized states. It is found at 1696 cm^{-1} for P in all three mutants, and at 1718 cm^{-1} for P^+ in LH(M160) and between 1722 and 1710 cm^{-1} for P^+ in the heterodimers. The 9-keto C=O of P_M is observed at 1686 cm^{-1} in the heterodimer HL(L173) and at 1684 cm^{-1} in LH(L131). It therefore appears reasonable to relate, on the one hand, the 1696-cm^{-1} peak of LH(M160), HL(M200), and HF(M200) to the small peak observed at 1692 cm^{-1} in Wt and, on the other hand, the 1686- and 1684-cm^{-1} peaks of HL(L173) and LH(L131), respectively, to the 1683-cm^{-1} signal of Wt. It is proposed that in Wt, the 9-keto C=O vibrations of P_L and P_M contribute at 1692 and 1683 cm^{-1}, respectively (Table I). This assignment is also in agreement with FT Raman spectra of *Rb. sphaeroides* RCs at room temperature (Mattioli et al., 1991), where the bands at 1691 and 1680 cm^{-1} have been attributed to the 9-keto carbonyl modes of P_L and P_M, respectively. However, from an earlier study in which only the two heterodimer mutants HL(M200) and HL(L173) were investigated at 275 K (Nabedryk et al., 1992a), we had previously suggested that the differential band observed in Wt at 1704/1683 cm^{-1} was due to an absorption change upon photooxidation of the 9-keto C=O of P_L. The basis for this tentative assignemnt mainly relied on the near-identity in the 10a-ester C=O region of the Wt and HL(M200) spectra, thus favoring a predominant contribution of the P_L moiety in the C=O frequency stretching range of the light-induced FTIR spectrum of Wt. However, the ester C=O region has since been observed to vary with temperature, with species, and with mutations not supposed to directly modify the environment of the ester of P_L (Nabedryk et al., 1992b,c, 1993; Morita et al., 1993). In addition, the comparison between Wt and heterodimers has recently been extended to another heterodimer mutant, HF(M200), for which His-M200 is replaced by Phe. Although both HL(M200) and HF(M200) heterodimer spectra are very similar in the whole 1730–1200-cm^{-1} spectral range, they differ significantly in the 10a-ester C=O absorption region (Nabedryk et al., 1993). It therefore appears premature to make assignments in terms of ester C=O contributions from P_L and P_M in Wt.

The large differential signal at 1705/1683 cm^{-1} in Wt with its shoulders at 1713 and 1692 cm^{-1} can thus be considered as the superposition of the two individual keto carbonyl modes corresponding to P_L^+/P_L for the high-frequency one (at 1713/1692 cm^{-1}) and to P_M^+/P_M for the low-frequency one (at 1705/1683 cm^{-1}). In LH(M160), the downshift of the 9-keto C=O of P_M leads to a clear separation of the contributions from P_L^+/P_L at 1718/1696 cm^{-1} and from P_M^+/P_M at 1678/1664 cm^{-1} (Table I). On the other hand, in LH(L131), the frequency downshift induced by the bonding of the keto C=O of P_L leads to the overlap of the positive band of P_L^+ and of the negative band of P_M, resulting in a complex 9-keto C=O region for this mutant. Compared to Wt, the magnitude of the downshift of the 9-keto C=O in the neutral state being larger for LH(L131) than for LH(M160), i.e., 35 versus 19 cm^{-1}, it therefore appears that the effects of positioning a His residue near P_L or P_M are not equivalent. The present IR observations are taken to indicate differences in His–BChl interactions and to suggest a stronger hydrogen bond between His-L131 and the 9-keto C=O of P_L in LH(L131) than in the case of LH(M160). Interestingly, the 35-cm^{-1} decrease of the P_L frequency in LH(L131) resembles closely that observed for the 9-keto C=O mode of BChl *a in vitro* (Mäntele et al., 1988; Hayashi et al., 1990), changing the solvent from tetrahydrofuran (C=O at 1684 cm^{-1}) to methanol (C=O at 1652 cm^{-1}) (see also Table I), and thus reinforces the proposal of a strong hydrogen bond in LH(L131).

The upshift of the 9-keto C=O bands upon photooxidation of P is observed to be comparable in Wt for P_L (21 cm^{-1}) and P_M (22 cm^{-1}) as well as in the mutants (Table I), i.e., 22 cm^{-1} for P_L in LH(M160), 26 cm^{-1} for P_M in LH(L131), and 23 cm^{-1} for the double mutant. However, this upshift is clearly smaller for P_M (14 cm^{-1}) in LH(M160). This observation qualitatively suggests a more asymmetric charge distribution between P_L and P_M in the photooxidized state of LH(M160), compared to Wt, and is consistent with the increase in frequency and the slight decrease in intensity of the high-wavenumber band at $\approx$2800 cm^{-1} in this mutant (Figure 2b).

This proposal is also in good agreement with ENDOR spectroscopy of P^+ on isolated RCs indicating that 84% of the spin density resides on P_L^+ in LH(M160), compared to 68% in Wt (Huber et al., 1992; Rautter et al., 1992; Feher, 1992). For LH(L131), it has been proposed that the spin density is shifted toward the M half of the dimer, with 53% present on P_M^+ (Rautter et al., 1992). The lack of a detectable effect on the 2600-cm^{-1} band in LH(L131) (Figure 2c) can thus be rationalized by assuming that the charge asymmetry is reversed in this mutant with respect to Wt, but is similar in magnitude. The larger difference between the charge density in Wt and LH(L131) than between Wt and LH(M160) would be in accord with the idea that the new hydrogen bond is stronger in LH(L131) than in LH(M160).

In the double mutant, the 9-keto C=O modes are observed at 1662 cm^{-1} for the neutral state. This frequency is comparable to the value (1660.5 cm^{-1}) calculated from the average of the individual frequencies of P_L in LH(L131) at 1657 cm^{-1} and P_M in LH(M160) at 1664 cm^{-1}. The effect of addition of hydrogen bonds to C=O groups of one or both BChls of the dimer thus appears roughly additive. For these three mutants, a correlation has been observed between the increase in donor oxidation potential, the decrease in the initial electron-transfer rate, and the increase in the charge recombination kinetics: the changes in these parameters in the double mutant are also roughly additive (Williams et al., 1992b). In the photooxidized state, the main positive peak in the C=O region of the double mutant is observed at 1685 cm^{-1}. In LH(M160), the 9-keto C=O of P_M^+ is found at 1678 cm^{-1}. Assuming that the additivity is conserved for the frequency shifts observed in the ionized state of the double mutant and of the corresponding single mutants, the 9-keto C=O of P_L^+ in LH(L131) would be located at 1692 cm^{-1}, thus leading to a 35-cm^{-1} upshift of the 9-keto C=O of P_L upon photooxidation. On the other hand, positioning P_L^+ in LH(L131) at 1682 cm^{-1} instead of 1692 cm^{-1} would be more compatible with the general 20–25-cm^{-1} upshift observed upon photooxidation of P for both Wt and mutants (Table I). Thus, the definitive assignment of the 9-keto C=O mode of P_L^+ in LH(L131) is not resolved in this work.

In summary, large changes in the FTIR spectra are associated with the introduction of hydrogen bond donor amino acid residues at single sites near the 9-keto carbonyl groups of P. These changes support the conclusion that a hydrogen bond has been introduced to the dimer at the 9-keto carbonyl of P_L or P_M due to the single mutations Leu-L131 to His and Leu-M160 to His, respectively, or the corresponding double mutation. Furthermore, these new IR data allow the clear identification of the 9-keto C=O modes of P_L and P_M in Wt, while the problem of the 10a-ester C=O assignment in the $P^+Q_A^-/PQ_A$ spectrum of Wt requires further investigation.

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