Probing the Primary Donor Environment in the Histidine^{M200} → Leucine and Histidine^{L173} → Leucine Heterodimer Mutants of *Rhodobacter capsulatus* by Light-Induced Fourier Transform Infrared Difference Spectroscopy†

E. Nabedryk,*,‡ S. J. Robles,§ E. Goldman,§ D. C. Youvan,§ and J. Breton‡

Section de Bioénergétique, Département de Biologie Cellulaire et Moléculaire, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur-Yvette Cedex, France, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received May 4, 1992; Revised Manuscript Received August 20, 1992

ABSTRACT: Light-induced $P^+Q_B^-/PQ_B$ FTIR difference spectra of reaction centers (RCs) have been obtained from chromatophores lacking light-harvesting B800–850 antenna for *Rhodobacter capsulatus* wild type (WT) and for the two mutants His^{M200} → Leu and His^{L173} → Leu. The primary donor (P) in both mutants consists of a bacteriochlorophyll–bacteriopheophytin heterodimer. The most prominent difference between the WT and the mutant spectra is in the 1600–1200-cm^{-1} region. The WT spectrum displays large positive bands at ≈1290, 1500–1430, and 1580–1530 cm^{-1}. These three bands are either small or altogether absent in the heterodimer spectra. In addition, both heterodimer spectra compare well with the electrochemically generated BChla$^+$/BChla spectrum [Mäntele, W. G., Wollenweber, A. M., Nabedryk, E., & Breton, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 8468–8472]. These observations indicate that the positive charge is localized on the monomeric BChl in the heterodimers. The overall shape of the ester and keto C=O signals in the BChla$^+$/BChla spectrum is maintained in the in situ spectra although significant differences are observed in the frequency, width, and splitting of the bands. The shape of the signal at 1757/1744 cm^{-1} in His^{L173} → Leu is comparable to the 1751/1737-cm^{-1} signal of BChla$^+$/BChla in tetrahydrofuran, indicating a free 10a ester C=O of P_M in His^{L173} → Leu. The reduced amplitude of the negative 1740-cm^{-1} feature in both His^{M200} → Leu and WT spectra suggests a hydrogen-bonded 10a ester C=O for P_L. Furthermore, from the striking similarity between the 1751/1740-cm^{-1} signal of His^{M200} → Leu and WT, a predominant localization of the positive charge on the P_L side in WT is favored. The frequency range (1683–1696 cm^{-1}) observed for the 9-keto carbonyls of P demonstrates that these groups are free from interaction in situ. An increase in the 9-keto C=O frequency over WT is observed for both P and P^+ states in the heterodimers. This upshift and the overall shapes of the differential band are nonequivalent in the two heterodimers, demonstrating that the geometry of the BChl moiety is significantly different in each heterodimer. Thus, the WT spectrum cannot be reconstructed from the two heterodimers' spectra. The differences observed between the heterodimers' spectra and the WT spectrum indicate that the conformation and/or protein local environment of P_L and P_M in WT is different from that of P_L in His^{M200} → Leu and P_M in His^{L173} → Leu.

The reaction center (RC)[1] from purple photosynthetic bacteria is a membrane-bound protein–chromophore complex containing four bacteriochlorophylls (BChl), two bacteriopheophytins (BPhe), two quinones Q_A and Q_B, one nonheme iron, and one carotenoid, associated with the subunits L and M. The high-resolution X-ray structure of RC from *Rhodopseudomonas viridis* (Deisenhofer et al., 1985; Michel et al., 1986) and *Rhodobacter sphaeroides* (Yeates et al., 1988; Tiede et al., 1988; El-Kabbani et al., 1991) suggests a key role for some amino acid residues of the L and M subunit polypeptides in providing hydrogen bonding and ionic or hydrophobic interactions with the cofactors. In particular, the three-dimensional model of the RC reveals that the central Mg atom of each of the four BChl molecules appears to have the imidazole ring of a His as an axial ligand (Deisenhofer et al., 1985; Yeates et al., 1988; El-Kabbani et al., 1991). Two of the BChls constitute the primary electron donor (P) and interact primarily with the D helices of the L and M subunits, respectively. These two BChls of P (P_L and P_M) are tightly coupled by ring I overlap (i.e., the aromatic planes are nearly parallel and separated by about 3.3 Å). P_L and P_M are related by the 2-fold symmetry axis found in the L–M complex and the other associated cofactors. In *Rps. viridis* (Deisenhofer et al., 1985; Michel et al., 1986) and *Rb. sphaeroides* RC (El-Kabbani et al., 1991), the side chains of His^{M200} and His^{L173} have been proposed to be coordinated to the central Mg atom of P_M and P_L, respectively, although in one *Rb. sphaeroides* structure (Yeates et al., 1988), His^{L173} appears to be too far away to coordinate directly the central Mg of P_L. These His residues are conserved in the amino acid sequence of the *Rhodobacter capsulatus* RC (Komiya et al., 1988) for which there is not yet a crystal structure. However, site-directed mutations affecting the primary donor ligands were originally generated in *Rb. capsulatus* (Bylina & Youvan, 1988, 1990; Coleman & Youvan, 1990) and more recently in *Rb. sphaeroides* (Schenck et al., 1990). Substitution of His^{M200} by a Leu residue results in RCs for which the total pigment content is 5.7 ± 0.1 and the BChl/BPhe ratio is 1.0 ± 0.1

† Part of this work was supported by a HFSP grant (to J.B. and D.C.Y.), NIH (GM 42645) and DOE (DE-FG02-90ER20019) grants (to D.C.Y.), and an NIH Biotechnology Training Grant (to E.G.).

* To whom correspondence should be addressed.

‡ CEN Saclay.

§ MIT.

[1] Abbreviations: RC(s), reaction center(s); BChl, bacteriochlorophyll; P, primary electron donor; BPhe, bacteriopheophytin; Q_A (Q_B), primary (secondary) quinone electron acceptor; FTIR, Fourier transform infrared; WT, wild type; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

0006-2960/92/0431-10852$03.00/0

(Bylina & Youvan, 1988). This ratio is 1.01 ± 0.02 in His^{L173} → Leu (Bylina & Youvan, 1990). These results, taken together with the altered absorption spectra, indicate that the primary donor in His^{M200} → Leu and His^{L173} → Leu RCs now consists of a BChl/BPhe heterodimer. These heterodimer mutants are photochemically active and the unidirectionality of electron transfer to $BPhe_L$ is preserved (Kirmaier et al., 1988). Most of the spectroscopic studies (Bylina & Youvan, 1988; Kirmaier et al., 1989; Breton et al., 1989; Bylina et al., 1990; Hammes et al., 1990; DiMagno et al., 1990; Peloquin et al., 1990; Huber et al., 1990; McDowell et al., 1991) have focused on the kinetic and structural properties of the primary donor radical cation in the BChl/BPhe heterodimer, but the structural effects of the mutation at the level of both the pigment and the protein conformations and/or the amino acid–pigment interactions still remain to be clarified.

Light-induced Fourier transform infrared (FTIR) difference spectroscopy provides a useful means for studying changes of protein–cofactor interactions upon charge separation and stabilization during primary reactions in the bacterial photosynthetic RC [Mäntele et al. (1988a) and references therein]. Using this approach, the photooxidation of P as well as the photoreduction of the primary (Q_A) and secondary (Q_B) quinone acceptor (i.e., $PQ_A \rightarrow P^+Q_A^-$, $PQ_B \rightarrow P^+Q_B^-$, $Q_A \rightarrow Q_A^-$, and $Q_B \rightarrow Q_B^-$ transitions) have recently been characterized in *Rb. sphaeroides* and *Rps. viridis* RCs (Bagley et al., 1990; Nabedryk et al., 1990a; Thibodeau et al., 1990a,b; Buchanan et al., 1990; Breton et al., 1991a,b). The IR spectra should help clarify the role of several amino acid residues that, according to the X-ray structure, have important interactions with the cofactors. To determine how the BChl–protein interactions are selectively perturbed in the heterodimer mutants, we have investigated the effects of the two symmetry-related mutations, His^{M200} → Leu and His^{L173} → Leu, on the light-induced FTIR difference spectra of the $PQ_B \rightarrow P^+Q_B^-$ transition in *Rb. capsulatus* chromatophores. A preliminary report of this work has been presented in Breton et al. (1991c) and Nabedryk et al. (1992).

MATERIALS AND METHODS

WT and mutant RC (His^{M200} → Leu and His^{L173} → Leu) genes were expressed from plasmid pU2922 in the *Rb. capsulatus* strain U43, as previously reported (Bylina & Youvan, 1988, 1990). The genetic and biophysical properties of these genetically altered RCs have been previously reviewed (Coleman & Youvan, 1990). Chromatophores were purified by a two-step (0.6 and 1.2 M) sucrose gradient. Purified chromatophores were washed with distilled water before preparation of IR samples. Air-dried films of chromatophores were rehydrated for FTIR measurements.

IR measurements were performed on a Nicolet 60SX FTIR spectrometer equipped with a MCT-A detector and a KBr beam-splitter. Light-induced FTIR spectra were obtained under steady-state continuous illumination ($\lambda \geq 715$nm). Cycles of illumination were repeated several hundred times (Nabedryk et al., 1990a).

RESULTS

Light-induced FTIR difference spectra at 275 K between the charge-separated state $P^+Q_B^-$ and the neutral state PQ_B, designated as $P^+Q_B^-/PQ_B$ spectra, are displayed in Figure 1 for *Rb. capsulatus* chromatophores from WT (spectrum a), His^{M200} → Leu (spectrum b), and His^{L173} → Leu (spectrum c) mutants. In these spectra, negative bands arise from vibrations of the neutral species (i.e., P and Q_B), while positive

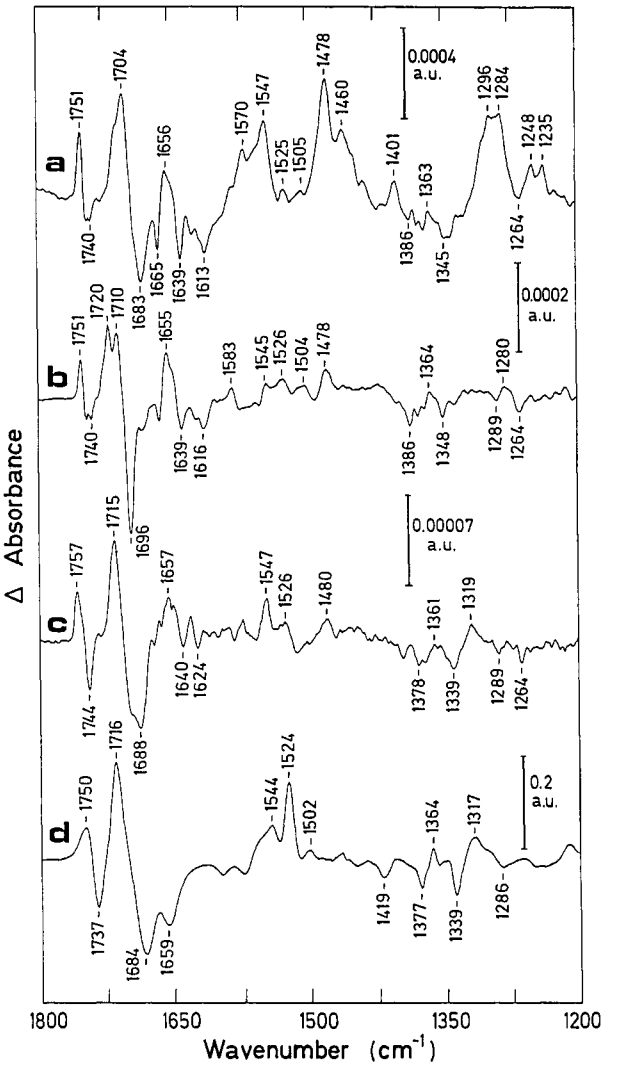

FIGURE 1: Light-induced FTIR difference spectra at 275 K between the charge-separated state $P^+Q_B^-$ and the uncharged state PQ_B for *Rb. capsulatus* chromatophores containing (a) wild-type RC, 17 920 interferograms coadded, (b) the heterodimer His^{M200} → Leu RC, 49 920 interferograms coadded, and (c) the heterodimer His^{L173} → Leu RC, 69 120 interferograms coadded. (d) Redox-induced FTIR difference spectrum at 295 K of the electrochemically generated BChla cation in deuterated tetrahydrofuran in the electrochemical cell described in Mäntele et al. (1988a,b). For all spectra the frequencies of the peaks are given at ±1 cm^{-1}. Spectral resolution, 4 cm^{-1}; a.u., absorbance units.

bands are associated with the radicals (i.e., P^+ and Q_B^-). For both the neutral and charge-separated states, contributions from protein backbone and side-chain groups interacting with P and/or Q_B could be expected. For comparison, the cation-minus-neutral difference spectrum of BChla (referred to as $BChla^+$/BChla spectrum) in deuterated tetrahydrofuran (Mäntele et al., 1988a) is shown in Figure 1d.

(1) WT Chromatophores. The FTIR light-minus-dark $P^+Q_B^-/PQ_B$ spectrum from WT chromatophores (Figure 1a) compares well with those previously reported for chromatophores and RCs of *Rb. capsulatus* and *Rb. sphaeroides* (Mäntele et al., 1985, 1988a; Nabedryk et al., 1987, 1990a; Hayashi et al., 1990).

In the C=O frequency region, the 1704/1683-cm^{-1} differential signal has been previously assigned to a shift of the 9-keto C=O group(s) of P upon photooxidation, while the positive signal at 1751 cm^{-1} has been attributed to the 10a ester C=O of P^+ (Mäntele et al., 1988a). Positive (≈1712 cm^{-1}) and weak negative (≈1693 cm^{-1}) shoulders are also

observed on the main 1704/1683-cm^{-1} differential signal. From the Q_B^-/Q_B difference spectrum obtained in situ after photoreduction of Q_B (Breton et al., 1991b), the semiquinone anion Q_B^- is expected to contribute to the positive signal at $\approx$1478 cm^{-1} and the neutral Q_B to the negative signal at 1264 cm^{-1}. The major part of the large positive signals at 1580–1530 and 1500–1430 cm^{-1}, as well as the positive band at 1296–1284 cm^{-1} (referred below as the $\approx$1290-cm^{-1} band) and a large band at $\approx$2600 cm^{-1} [data not shown; see Breton et al. (1992)] appear characteristic of the P^+ state of the BChl dimer. Below, such WT data will be compared with data obtained from mutants.

(2) $His^{M200} \rightarrow Leu$ Heterodimer Mutant. The light-induced FTIR $P^+Q_B^-/PQ_B$ spectrum of the $His^{M200} \rightarrow$ Leu heterodimer mutant (Figure 1b) shows significant differences compared to spectra of WT (Figure 1a) and of the $His^{L173} \rightarrow$ Leu heterodimer mutant (Figure 1c). The most striking of these differences is the increase in frequency and the sharpening of a negative band at 1696 cm^{-1} in $His^{M200} \rightarrow$ Leu, compared to the broad 1683-cm^{-1} negative signal in WT. The corresponding positive signal is upshifted and split in $His^{M200} \rightarrow$ Leu (1710–1720 cm^{-1}). In $His^{M200} \rightarrow$ Leu, the absorption increase at 1751 cm^{-1} and the structured feature at $\approx$1740 cm^{-1} are remarkably similar to the absorption changes observed in WT. In contrast to the three large positive signals seen in WT in the 1600–1200-cm^{-1} region, only small positive signals (notably at 1545, 1526, 1504, 1478, and 1364 cm^{-1}) are found in the heterodimer. In particular, the $\approx$1290-cm^{-1} signal and the broad $\approx$2600-cm^{-1} band [data not shown; see Breton et al. (1992)], both present in WT, are greatly reduced in $His^{M200} \rightarrow$ Leu. Small negative signals are observed at 1386, 1348, 1289, and 1264 cm^{-1}. The small positive band at 1478 cm^{-1} and the negative one at 1264 cm^{-1} are characteristic for the semiquinone anion formation of Q_B (Breton et al., 1991b).

(3) $His^{L173} \rightarrow Leu$ Heterodimer Mutant. The light-induced FTIR $P^+Q_B^-/PQ_B$ spectrum of the $His^{L173} \rightarrow$ Leu heterodimer (Figure 1c) also shows significant differences compared to the spectrum of WT. An upshift of the 9-keto and 10a ester C=O frequencies is observed in both P and P^+ states. In contrast to the $His^{M200} \rightarrow$ Leu mutant, where a sharpening of the negative band at 1696 cm^{-1} and a splitting of the positive band at 1710–1720 cm^{-1} were observed (Figure 1b), the $His^{L173} \rightarrow$ Leu spectrum contains a broad negative band at 1688 cm^{-1} with a shoulder at 1698 cm^{-1} and a sharp positive band at 1715 cm^{-1}. In the ester C=O absorption range, instead of the positive signal at 1751 cm^{-1} which is observed in both WT and $His^{M200} \rightarrow$ Leu, a differential signal at 1757/1744 cm^{-1} is found. Small positive signals are observed at 1547, 1526, 1480, 1361, and 1319 cm^{-1} as well as negative ones at 1378, 1339, 1289, and 1264 cm^{-1}. As in the $His^{M200} \rightarrow$ Leu mutant, the $\approx$1290- and $\approx$2600-cm^{-1} bands are of very low amplitude in $His^{L173} \rightarrow$ Leu (data not shown). Presumed quinone signals appear at 1480 and 1264 cm^{-1}.

DISCUSSION

It has been established from EPR and ENDOR/TRIPLE resonance measurements for bacterial RCs that the positive charge of P^+ is delocalized over both BChl molecules constituting the dimer (Norris et al., 1971; Feher et al., 1975; Lubitz et al., 1984). In *Rps. viridis* RC, calculations of electrostatic energies (Parson et al., 1990) suggest that the unpaired electron is not equally shared between the two BChls of the dimer. Spin density calculations performed on *Rb. sphaeroides* RC (Plato et al., 1990) also favor an asymmetrical charge distribution on P^+. Characterization of $His^{M200} \rightarrow$ Leu and $His^{L173} \rightarrow$ Leu mutants by EPR (Bylina et al., 1990) and ENDOR (Huber et al., 1990, 1992) spectroscopy has been recently performed, and it has been shown that the radical cation state of the donor is predominantly monomeric in nature. Since BPhe is more difficult to oxidize than BChl, it was concluded that the positive charge on the oxidized donor is localized on the BChl side of the heterodimer (P_L in $His^{M200} \rightarrow$ Leu, P_M in $His^{L173} \rightarrow$ Leu). In addition, recent ENDOR and TRIPLE resonance investigations of the primary donor cation radical in single crystals of *Rb. sphaeroides* RCs (native and heterodimer mutants) indicate that the spin density is asymmetrically distributed in the native dimer (Lendzian et al., 1990), favoring the P_L side by $\approx$2:1 (Huber et al., 1992). Using light-induced FTIR difference spectroscopy, we expect vibrational contributions from both halves of the dimer in WT, while contributions from the BChl molecule (P_L or P_M, respectively) should dominate the spectrum in the $His^{M200} \rightarrow$ Leu or $His^{L173} \rightarrow$ Leu heterodimer mutants.

(1) IR Band Assignment for P and P^+ in WT: Comparison with the X-ray Structural Model. On the basis of FTIR data for the electrochemically generated BChla radical cation, the largest signals observed in the C=O stretching frequency region (1760–1620 cm^{-1}) of the $P^+Q_B^-/PQ_B$ spectra of *Rb. sphaeroides* WT chromatophores or RCs have been previously related to the 10a ester and 9-keto C=O groups of P and P^+ (Mäntele et al., 1988a). As shown in Figure 1d, BChla cation formation in tetrahydrofuran leads to differential FTIR signals at 1750/1737 and 1716/1684 cm^{-1} which have been assigned to absorption changes of the non-hydrogen-bonded 10a ester and 9-keto C=O of monomeric BChla (Mäntele et al., 1988a). For pyroBChla, which lacks the 10a ester C=O, a single differential signal at 1710/1679 cm^{-1} was attributed to the 9-keto C=O (Leonhard et al., 1989). The $P^+Q_B^-/PQ_B$ spectrum of WT (Figure 1a) shows a negative peak at 1683 cm^{-1} (with a weak shoulder at $\approx$1693 cm^{-1}) and a positive peak at 1704 cm^{-1} with a clear shoulder at 1712 cm^{-1}. Both positive and negative signals are of comparable amplitude to those observed in the $BChla^+/BChla$ spectrum (Figure 1d). The 1712–1704/1683-cm^{-1} signals have been assigned to the keto C=O of P^+/P. It is worth noting that $P^+Q_B^-/PQ_B$ spectra of *Rb. sphaeroides* RC show a clear splitting at 1703 and 1715 cm^{-1} (Mäntele et al., 1985, 1988a; Nabedryk et al., 1990a; Bagley et al., 1990; Thibodeau et al., 1990a,b; Hayashi et al., 1990; Morita et al., 1991), instead of a main positive peak with a shoulder as observed for chromatophore membranes of *Rb. sphaeroides* and *Rb. capsulatus* (Figure 1a; Nabedryk et al., 1987; Morita et al., 1991). The small but highly reproducible difference that occurs between $P^+Q_B^-/PQ_B$ spectra obtained from isolated RCs and chromatophore membranes might be related to subtle geometrical changes occurring during RC extraction, such as a slight rotation of the ring I acetyl group or a modest change in the puckering of the BChl macrocycle (Thompson et al., 1991), which could affect the electron density on the 9-keto C=O of one of the two BChls in P^+.

It has been suggested that a negative band of variable amplitude at $\approx$1665 cm^{-1} in P^+Q^-/PQ spectra of *Rb. sphaeroides* RCs could arise from one keto C=O of the neutral special pair (Mäntele et al., 1988a). However, on the basis of polarized light-induced measurements on oriented films of *Rb. sphaeroides* RCs (Thibodeau et al., 1991), it has been established that the $\approx$1665-cm^{-1} signal corresponds to an IR transition moment oriented perpendicularly to the membrane. The X-ray model reveals that the two 9-keto C=O bonds of the special pair are almost perpendicular to the C-2 symmetry

Table I: Tentative Assignments of Peak Frequencies[a] in Light-Induced $P^+Q_B^-/PQ_B$ Difference Spectra from Wild-Type Chromatophores and $His^{M200} \rightarrow Leu$ and $His^{L173} \rightarrow Leu$ Heterodimers and in the Redox-Induced Difference Spectrum of Electrochemically Generated BChla Cation in Tetrahydrofuran

	WT		$His^{M200} \rightarrow Leu$		$His^{L173} \rightarrow Leu$			
	P	P^+	P	P^+	P	P^+	BChla	$BChla^+$
10a ester C=O	1740	1751	1740	1751	1744	1757	1737	1750
9-keto C=O	1683	1704	1696	1710 and/or 1720	1688	1715	1684	1716
	1693[b]	1712[c]			1698[c]			
peptide C=O		1656		1655		1657		
C – C (mainly BChl)		1547		1545		1547		1544
		1525		1526		1526		1524
				1504				1502
quinone	1264		1264	1478	1264	1480		

[a] Peak frequencies are given in reciprocal centimeters. [b] Very weak. [c] Shoulder.

axis (Allen et al., 1987). Therefore, the ≈1665-cm^{-1} band cannot be identified as a keto C=O and is more probably due to a peptide C=O in the environment of P.

In Figure 1a, the negative signal at 1683 cm^{-1} and possibly its weak shoulder at 1693 cm^{-1} are assigned to the overlap of the 9-keto C=O absorption of P_L and P_M. The vibrational frequency range observed for the keto C=O of P indicates that these groups are not hydrogen-bonded to the protein. The present IR data are fully consistent with the refined X-ray model of *Rb. sphaeroides* RC showing that no amino acid residue is in contact with the keto C=O of ring V of either P_L or P_M, making both BChls of the special pair equivalently non-hydrogen-bonded (Yeates et al., 1988; El-Kabbani et al., 1991). In the P^+ state, we have proposed that the keto C=O modes are upshifted to 1704 and 1712 cm^{-1} in chromatophores (Figure 1a) and to 1703 and 1715 cm^{-1} in RCs. The presence of two positive bands at 1703 and 1715 cm^{-1} in the $P^+Q_B^-/PQ_B$ FTIR spectra of *Rb. sphaeroides* RC has been consistently observed (Mäntele et al., 1985, 1988a; Bagley et al., 1990; Nabedryk et al., 1990a; Thibodeau et al., 1990a,b; Hayashi et al., 1990; Morita et al., 1991). This leads us to propose that the delocalization of the positive charge over both BChl macrocycles in P^+, on the time scale of vibrational spectroscopy (≈10^{-13} s), reveals different environments of the two keto carbonyls. Although it cannot be decided from the WT spectrum which of the two positive bands (1704 or 1712 cm^{-1} in chromatophores, 1703 or 1715 cm^{-1} in RCs) has to be associated with P_L^+ or P_M^+, it seems reasonable to infer that the BChl half of the dimer which carries the largest extent of the positive charge in WT would be responsible for the large FTIR differential signal at 1704/1683 cm^{-1} (Figure 1a). This 21-cm^{-1} shift of the keto C=O in situ is thus smaller than the 32-cm^{-1} shift observed for the 9-keto of $BChla^+$/BChla in tetrahydrofuran (Figure 1d). However, since the magnitude of the shift in vitro is strongly influenced by the solvent [56–70 cm^{-1} in methanol (Mäntele et al., 1988; Hayashi et al., 1990)], it is quite probable that, upon photooxidation of P, the shift of the 9-keto C=O vibration will be influenced by the microenvironment and local conformation of ring V. It thus seems premature to quantify the 21-cm^{-1} shift observed in situ in terms of the charge repartition over the two halves of the dimer in P^+.

It has to be noted that different band assignments for the keto C=O of P have been proposed for the interpretation of resonance Raman spectra of native RC using either Soret (Zhou et al., 1989) excitation (bands at 1660 and 1684 cm^{-1}) or near IR (Mattioli et al., 1991) excitation at 1064 nm (bands at 1679 and 1691 cm^{-1}). On the other hand, FT resonance Raman spectra of oxidized RCs show a single P^+ keto C=O band at 1717 cm^{-1} (Mattioli et al., 1991). This result was interpreted in terms of the localization of the positive charge on mainly one BChl in the dimer, presumably P_L. The absence or weakness of P_M^+ contributions could be due to the resonance conditions (Mattioli et al., 1991).

In the C=O stretching region of WT (Figure 1a), a positive band is observed at 1751 cm^{-1} with structured features at ≈1740 cm^{-1}. This 1751-cm^{-1} signal has been assigned to the 10a ester C=O of P^+ because a comparable positive band feature is present in the electrochemically generated $BChla^+$/BChla difference spectrum [Figure 1d and Mäntele et al. (1988a)] either in tetrahydrofuran (at 1750 cm^{-1} with a clear negative signal at 1737 cm^{-1}), or in methanol (at 1756 cm^{-1} without a negative signal) but is absent in the difference spectrum of the pyroBChla cation (Leonhard et al., 1989). For *Rb. sphaeroides* RCs, the absence of a clear negative signal at ≈1740 cm^{-1} led us to propose that at least one 10a ester C=O of P is hydrogen-bonded in the neutral state of the BChl dimer (Mäntele et al., 1988a). This interpretation is in agreement with the recent X-ray structural model of *Rb. sphaeroides* RC showing that the 10a ester C=O of P_L is within hydrogen-bonding distance (2.8 Å) to Ser L244 while no residue is present on the M side which can hydrogen-bond to the ester C=O of ring V of P_M (El-Kabbani et al., 1991).

In the $BChla^+$/BChla spectrum, the 2a acetyl C=O group of the neutral BChla is found at 1659 cm^{-1} in tetrahydrofuran [Figure 1d; see also Hayashi et al. (1990)] or at 1636 cm^{-1} in methanol (Mäntele et al., 1988a). However, in the $P^+Q_B^-/PQ_B$ spectrum of WT (Figure 1a), no clear assignment for acetyl groups is possible due to superimposed contributions, in the 1620–1665-cm^{-1} region, from peptide and quinone carbonyls, amino acid side chains as well as from the water OH bending vibration. For example, the positive band at 1656 cm^{-1} could arise from the acetyl C=O of P^+ but it also lies in the typical range for a peptide C=O of an α-helix (possibly in the D helix of the L or M subunits). This protein backbone change, which is not affected by the mutations (Figure 1b,c) would be of very low amplitude, involving the equivalent of one or two peptide bonds.

Band assignments are summarized in Table I. The 1600–1200-cm^{-1} region of the $P^+Q_B^-/PQ_B$ WT spectrum will be discussed below in comparison with heterodimers' spectra.

(2) 1600–1200-cm^{-1} Region of the Heterodimers' Spectra. The most prominent differences between the four spectra shown in Figure 1 are observed in the 1600–1200-cm^{-1} region. The large positive bands at ≈1290, 1500–1430, and 1580–1530 cm^{-1}, present in WT, are absent or very small in the spectra of both heterodimers and of $BChla^+$/BChla. In WT chromatophores and RCs, the major part of the three bands thus appears characteristic of a BChl dimer state of P^+. In addition, a broad band at 2600 cm^{-1} in WT, which shows very low amplitude in $His^{M200} \rightarrow Leu$ and $His^{L173} \rightarrow Leu$ spectra and is absent in the $BChla^+$/BChla spectrum (Breton et al.,

1992), has been recently correlated with an asymmetrical dimeric cation state of the photooxidized P in bacterial RCs.

Indeed, it appears that the spectra of the heterodimers resemble more closely the cation spectrum of monomeric BChla[2] than the WT spectrum. In the 1600–1200-cm^{-1} region, several corresponding bands with comparable frequencies and relative intensities are observed in the $His^{M200} \rightarrow Leu$, $His^{L173} \rightarrow Leu$, and $BChla^+/BChla$ spectra. All these observations are in good agreement with the conclusion derived from EPR and ENDOR experiments that shows the positive charge of the heterodimer to be largely localized on the BChl side (Bylina et al., 1990; Huber et al., 1990).

(3) C=O Stretching Region of the Heterodimers Spectra. The overall shape of the P^+/P signal, with positive and negative contributions, in the 1760–1680-cm^{-1} region (Figure 1b,c) is conserved in both mutants relative to WT and is comparable to the general shape of the $BChla^+/BChla$ cation spectrum in the corresponding region (Figure 1d). However, significant changes in frequency, width, and splitting of the bands are observed. The keto C=O of P shows a single negative band at 1696 cm^{-1} in $His^{M200} \rightarrow Leu$ while in $His^{L173} \rightarrow Leu$ a broad negative signal at 1688 cm^{-1} with a clear shoulder at 1698 cm^{-1} is found. In contrast, a single positive band appears at 1715 cm^{-1} in the $His^{L173} \rightarrow Leu$ mutant rather than the split signal (1710–1720 cm^{-1}) found in $His^{M200} \rightarrow Leu$. Compared to the $P^+Q_B^-/PQ_B$ spectrum of WT (Figure 1a), these changes reflect a perturbation of the 9-keto C=O, which experiences a large frequency upshift in both the neutral and cation radical states. These upshifts are taken to indicate a partial change of bond order which could result from either a redistribution of charge on the ring V of the BChl macrocycle of the heterodimer or a conformational change of this BChl.

It has been established from chlorophyll model studies that several factors can influence the vibrational modes of the 9-keto C=O: differences in the local environment (Krawczyk, 1989), changes in the dielectric constant (Koyama et al., 1986), and induction of ring strain in the BChl macrocycle which may affect the extent of conjugation of the 9-keto C=O (Andersson et al., 1989). In particular, perturbations at the level of the 10a ester C=O might alter the ring V conformation sufficiently to prevent the keto C=O from fully conjugating with the macrocycle and the result would be an increase of the keto frequency (Andersson et al., 1989; Leonhard et al., 1989; Nabedryk et al., 1990b; Heald & Cotton, 1990). After cation formation, an upshift of the 9-keto C=O of Chla, BChla, and BChlb appears to be a general property (Mäntele et al., 1988a; Nabedryk et al., 1990b; Heald & Cotton, 1990; Hayashi et al., 1990). In contrast, a downshift of the keto C=O has been reported upon generation of (bacterio)pheophytin and BChl anions (Mäntele et al., 1988a,b; Nabedryk et al., 1990c). These effects have been assigned to a partial change of bond order of the conjugated 9-keto C=O upon charge redistribution following addition or subtraction of one electron. In addition, resonance Raman spectra of porphyrin cation radicals suggest that BChl macrocycles may undergo significant conformational changes with oxidation [Heald & Cotton (1990) and references therein].

In the $His^{M200} \rightarrow Leu$ mutant, the single 1696-cm^{-1} peak is assigned to the 9-keto C=O of P_L (Table I). Its frequency implies that it is free from interaction with the protein: the upshift of $\approx$12 cm^{-1} compared to WT or BChla spectra is not the result of a difference in hydrogen-bonding. A single peak would be also expected for the 9-keto of P_L^+. It is thus intriguing to observe a split signal at 1710–1720 cm^{-1} in the $His^{M200} \rightarrow Leu$ mutant. Although it cannot be strictly ruled out that side-chain groups or anomalously shifted peptide carbonyls could in part contribute to the signals displayed in the 1720–1700-cm^{-1} region of the $P^+Q_B^-/PQ_B$ spectrum, this appears an unlikely possibility in view of the frequency range. On the other hand, the split signal could be due to two conformations for the 9-keto C=O of P_L in the P^+ state, possibly due to some inductive effect caused by a slightly different geometry of the 2a acetyl C=O group. In this respect, the lack of the His ligand could provide a less constrained environment of P in the heterodimer than in WT, leading to nonunique vibrational modes for the 9-keto C=O of the heterodimers.

The differential signals in the 1760–1680-cm^{-1} region of the $His^{L173} \rightarrow Leu$ spectrum, although upshifted, are the most comparable to the $BChla^+/BChla$ spectrum (Figure 1d). The keto C=O of P_M (1688–1698 cm^{-1}) in $His^{L173} \rightarrow Leu$ (Table I) appears free from interaction, and its frequency in P^+ (1715 cm^{-1}) closely corresponds to that observed for a BChla cation generated in tetrahydrofuran (1716 cm^{-1}). In the ester C=O region, a clear differential signal is observed at 1757/1744 cm^{-1}, slightly upshifted but otherwise comparable to the one observed at 1750/1737 cm^{-1} in the $BChla^+/BChla$ spectrum. It is assigned to an absorbance change of the 10a ester C=O of P_M (table I). The presence of this ester C=O band above 1735 cm^{-1} indicates that it is not hydrogen-bonded (Ballschmitter & Katz, 1969) in the neutral state of the heterodimer.

In contrast, the spectrum in the ester C=O region of $His^{M200} \rightarrow Leu$ appears to be very close to that of WT. In both spectra, increased absorption is observed at 1751 cm^{-1} with a structured signal at $\approx$1740 cm^{-1}. This absorption change at 1751 cm^{-1} for $His^{M200} \rightarrow Leu$ is assigned to the 10a ester C=O of P_L^+ (Table I). From the absence of a clear negative signal at $\approx$1740 cm^{-1} (as discussed above for WT), we speculate that the hydrogen-bonding interaction of the 10a ester C=O of P_L is retained in this mutant. Furthermore, in view of the remarkable similarity between WT and $His^{M200} \rightarrow Leu$ in the ester C=O region, we favor a predominant contribution in WT of the ester C=O from the P_L moiety to these signals. Within this scheme, it is also expected that the largest contribution to the 1712–1704/1683-cm^{-1} 9-keto signal in WT is due to P_L and thus we assigned the differential band at 1704/1683 cm^{-1} to P_L.

The present assignment to P_L of the largest FTIR signals in the ester and keto C=O stretching regions is in good agreement with the interpretation of new ENDOR data obtained recently on RC single crystals of *Rb. sphaeroides* (native and heterodimer mutants), showing that the unpaired electron is not equally distributed over the two halves of the special pair in WT, with about 68% of the spin density on P_L^+ (Huber et al., 1992). The ENDOR experiments also indicate that the methyl hyperfine coupling constants of the two mutants differ, suggesting that the environment (which is different on the L and M sides) has a major effect on the electronic structure of P^+. In agreement with these ENDOR data, the FTIR spectra of the two heterodimers are not only nonequivalent and different from WT, but they also differ from a pure $BChla^+/BChla$ spectrum, highlighting the influence of the BChl–protein interactions in the charge stabilization process.

The differences, at the molecular level, between WT and heterodimers are clearly illustrated in Figure 2, where the sum of the two heterodimer spectra is compared to the WT

[2] Since the IR difference spectrum of an electrochemically generated BPhe cation radical has not been reported, we cannot compare the heterodimers' spectra to a cation-minus-neutral FTIR spectrum of BPhe.

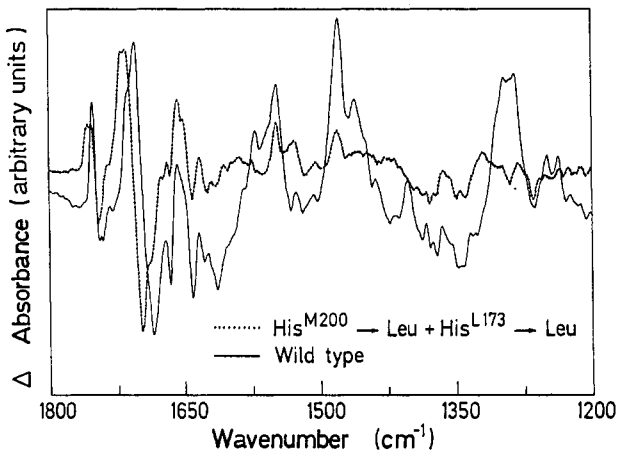

FIGURE 2: Comparison between the $P^+Q_B^-/PQ_B$ spectrum (—) of wild-type chromatophores (Figure 1a) and the sum (- - -) of the two $P^+Q_B^-/PQ_B$ heterodimers' spectra (Figure 1b,c) obtained by matching the peak-to-peak amplitude of the 9-keto C=O. A comparable sum (data not shown) can be obtained by matching the peak-to-peak amplitude of the 10a ester C=O of the two heterodimers' spectra.

spectrum. It is interesting to note that the overall shape of the keto signal is preserved and is comparable to WT and $BChla^+/BChla$. However, the negative and positive signals in the ester and keto C=O region of the sum are all upshifted. Clearly, the WT spectrum cannot be reconstructed from the spectra of the heterodimers. Two models can explain the differences between WT and the heterodimer mutants. The different extent of charge localization on each of P_L and P_M in the P^+ state of the WT and the heterodimers as well as difference between BChl–BChl and BChl–BPhe coupling and/or electrostatic effects on BPhe caused by the positive charge localized on the BChl could be responsible for the perturbation of the C=O vibrations. Significant spin redistribution induced in the porphyrin would thus lead to concomitant IR shifts. However, the observation of comparable upshifts for both the neutral and the cationic forms of P in the heterodimers compared to WT (Figure 2), makes this explanation rather unlikely. Alternatively, the conformation and/or protein local environment of P_L and P_M in WT would be different from that of P_L in $His^{M200} \rightarrow$ Leu and P_M in $His^{L173} \rightarrow$ Leu. In each mutant, molecular rearrangements in the BChl macrocycle and/or its substituents (such as changes in bond orientation or interaction or puckering of the macrocycle) may be imposed by the protein to adjust the pigment geometry to the new local amino acid environment in the heterodimer. These conformational changes will tend to minimize unfavorable contacts between the BChl macrocycle substituents and the nearby amino acid side chains.

ACKNOWLEDGMENT

We thank G. Feher for communicating unpublished data, E. J. Bylina, M. Huber, and J. Norris for helpful discussions, W. W. Parson for a critical reading of the manuscript, and S. Andrianambinintsoa and D. Dejonghe for preparing the purified chromatophores.

REFERENCES

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5730–5734.

Andersson, L. A., Loehr, T. M., Cotton, T. M., Simpson, D. J., & Smith, K. M. (1989) *Biochim. Biophys. Acta 974*, 163–179.

Bagley, K. A., Abresch, E., Okamura, M. Y., Feher, G., Bauscher, M., Mäntele, W., Nabedryk, E., & Breton, J. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 77–80, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Ballschmiter, K., & Katz, J. J. (1969) *J. Am. Chem. Soc. 91*, 2661–2677.

Breton, J., Bylina, E. J., & Youvan, D. C. (1989) *Biochemistry 28*, 6423–6430.

Breton, J., Thibodeau, D. L., Berthomieu, C., Mäntele, W., Verméglio, A., & Nabedryk, E. (1991a) *FEBS Lett. 278*, 257–260.

Breton, J., Berthomieu, C., Thibodeau, D. L., & Nabedryk, E. (1991b) *FEBS Lett. 288*, 109–113.

Breton, J., Bylina, E. J., Robles, S. J., Youvan, D. C., & Nabedryk, E. (1991c) *Biophys. J. 59*, 31a.

Breton, J., Nabedryk, E., & Parson, W. W. (1992) *Biochemistry 31*, 7503–7510.

Buchanan, S., Michel, H., & Gerwert, K. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel Beyerle, M.-E., Ed.) Springer Series in Biophysics, Vol. 6, pp 75–85, Springer, Berlin.

Bylina, E. J., & Youvan, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7226–7230.

Bylina, E. J., & Youvan, D. C. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 53–59, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Bylina, E. J., Kolaczkowski, S. V., Norris, J. R. & Youvan, D. C. (1990) *Biochemistry 29*, 6203–6210.

Coleman, W. J., & Youvan, D. C. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 333–367.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature 318*, 618–624.

DiMagno, T. J., Bylina, E. J., Angerhofer, A., Youvan, D. C., & Norris, J. R. (1990) *Biochemistry 29*, 899–907.

El-Kabbani, O., Chang, C.-H., Tiede, D., Norris, J., & Schiffer, M. (1991) *Biochemistry 30*, 5361–5369.

Feher, G., Hoff, A. J., Isaacson, R. A. & Ackerson, L. C. (1975) *Ann. N.Y. Acad. Sci. 244*, 239–259.

Hammes, S. L., Mazzola, L., Boxer, S. G., Gaul, D. F., & Schenck, C. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5682–5686.

Hayashi, H., Morita, E. H., & Tasumi, M. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 73–76, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Heald, R. L., & Cotton, T. M. (1990) *J. Phys. Chem. 94*, 3968–3975.

Huber, M., Lous, E. J., Isaacson, R. A., Feher, G., Gaul, D., & Schenck, C. C. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel Beyerle, M.-E., Ed.) Springer Series in Biophysics, Vol. 6, pp 219–228, Springer, Berlin.

Huber, M., Isaacson, R. A., Abresch, E. C., Feher, G., Gaul, D., & Schenck, D. C. (1992) *Biophys. J. 61*, A104.

Kirmaier, C., Holten, D., Bylina, E. J., & Youvan, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7562–7566.

Kirmaier, C., Bylina, E. J., Youvan, D. C., & Holten, D. (1989) *Chem. Phys. Lett. 159*, 251–257.

Komiya, H., Yeates, T. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012–9016.

Koyama, Y., Umemoto, Y. & Akamatsu, A., Uehara, K., & Tanaka, M. (1986) *J. Mol. Struct. 146*, 273–287.

Krawczyk, S. (1989) *Biochim. Biophys. Acta 976*, 140–149.

Lendzian, F., Endeward, B., Plato, M., Bumann, D., Lubitz, W., & Möbius, K. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel Beyerle, M.-E., Ed.) Springer Series in Biophysics, Vol. 6, pp 57–68, Springer, Berlin.

Leonhard, M., Wollenweber, A., Berger, G., Kléo, J., Nabedryk, E., Breton, J., & Mäntele, W. (1989) in *Techniques and New Developments in Photosynthesis Research* (Barber, J., & Malkin, R., Eds.) NATO ASI Series A, Vol. 168, pp. 115–118, Plenum Press, New York.

Lubitz, W., Lendzian, F., Scheer, H., Gottstein, J., Plato, M., & Möbius, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1401–1405.

McDowell, L. M., Gaul, D., Kirmaier, C., Holten, D., & Schenck, C. C. (1991) *Biochemistry 30*, 8315–8322.

Mäntele, W., Nabedryk, E., Tavitian, B. A., Kreutz, W., & Breton, J. (1985) *FEBS Lett. 187*, 227–232.

Mäntele, W. G., Wollenweber, A. M., Nabedryk, E., & Breton, J. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8468–8472.

Mäntele, W. G., Wollenweber, A. M., Rashwan, F., Heinze, J., Nabedryk, E., Berger, G., & Breton, J. (1988b) *Photochem. Photobiol. 47*, 451–456.

Mattioli, T. A., Hoffmann, A., Robert, B., Schrader, B., & Lutz, M. (1991) *Biochemistry 30*, 4648–4654.

Michel, H., Epp, O., & Deisenhofer, J. (1986) *EMBO J. 5*, 2445–2451.

Morita, E. H., Hayashi, H., & Tasumi, M. (1991) *Chem. Lett.*, 1853–1856.

Nabedryk, E., Tavitian, B. A., Mäntele, W., Kreutz, W., & Breton, J. (1987) in *Progress in Photosynthesis Research*, (Biggins, J., Ed.) Vol. 1, pp 177–180, Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

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

Nabedryk, E., Leonhard, M., Mäntele, W., & Breton, J. (1990b) *Biochemistry 29*, 3242–3247.

Nabedryk, E., Andrianambinintsoa, S., Berger, G., Leonhard, M., Mäntele, W., & Breton, J. (1990c) *Biochim. Biophys. Acta 1016*, 49–54.

Nabedryk, E., Robles, S. J., Goldman, E., Youvan, D. C., & Breton, J. (1992) *Biophys. J. 61*, A101.

Norris, J. R., Uphaus, R. A., Crespi, H. L., & Katz, J. J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 625–628.

Parson, W. W., Chu, Z.-T., & Warshel, A. (1990) *Biochim. Biophys. Acta 1017*, 251–272.

Peloquin, J. M., Bylina, E. J., Youvan, D. C., & Bocian, D. F. (1990) *Biochemistry 29*, 8417–8424.

Plato, M., Mobius, K., Lubitz, W., Allen, J. P., & Feher, G. (1990) in *Perspectives in Photosynthesis* (Jortner, J., & Pullman, B., Eds.) Vol. 22, pp 423–434, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schenck, C. C., Gaul, D., Steffen, M., Boxer, S. G., McDowell, L., Kirmaier, C., & Holten, D. (1990) in *Reaction Centers of Photosynthetic Bacteria* (Michel Beyerle, M.-E., Ed.) Springer Series in Biophysics, Vol. 6, pp 229–238, Springer, Berlin.

Thibodeau, D. L., Nabedryk, E., Hienerwadel, R., Lenz, F., Mäntele, W., & Breton, J. (1990a) *Biochim. Biophys. Acta 1020*, 253–259.

Thibodeau, D. L., Breton, J., Berthomieu, C., Bagley, K. A., Mäntele, W., & Nabedryk, E. (1990b) in *Reaction Centers of Photosynthetic Bacteria* (Michel Beyerle, M.-E., Ed.) Springer Series in Biophysics, Vol. 8, pp 87–98, Springer, Berlin.

Thibodeau, D. L., Nabedryk, E., & Breton, J. (1991) in *Spectroscopy of Biological Molecules* (Hester, R. E., Girling, R. B., Eds.) pp 69–70, The Royal Society of Chemistry, London.

Thompson, M. A., Zerner, M. C., & Fajer, J. (1991) *J. Phys. Chem. 95*, 5693–5700.

Tiede, D. M., Budil, D. E., Tang, J., El-Kabbani, O., Norris, J. R., Chang, C.-H., & Schiffer, M. (1988) in *The Photosynthetic Bacterial Reaction Center* (Breton, J., & Vermeglio, A., Eds.) pp 13–20, Plenum, New York.

Yeates, T. O., Komiya, H., Chirino, A., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7993–7997.

Zhou, Q., Robert, B., & Lutz, M. (1989) *Biochim. Biophys. Acta 977*, 10–18.

Registry No. BChla, 17499-98-8.