Mg Coordination by Amino Acid Side Chains Is Not Required for Assembly and Function of the Special Pair in Bacterial Photosynthetic Reaction Centers†

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ABSTRACT: A conserved histidine serves as the axial ligand to the Mg of bacteriochlorophylls in the photosynthetic reaction center (RC) and many other photosynthetic systems. The histidine axial ligands to each and both bacteriochlorophylls of the special-pair primary electron donor of the *Rhodobacter sphaeroides* RC have been replaced with glycine to create a cavity. In each case, RCs assemble and a normal special-pair comprised of Mg-containing bacteriochlorophylls is formed, as judged by many different spectroscopic and functional probes (e.g., absorption and Stark spectra, *P decay kinetics, $P^+Q_A^-$ recombination rate, and the redox potential of P). In contrast with heme proteins, where this strategy has been exploited to introduce exogenous organic ligands that can greatly affect the functional properties of the protein [DePillis, G. D., Decatur, S. M., Barrick, D., & Boxer, S. G. (1994) *J. Am. Chem. Soc. 116*, 6981−6982], addition of exogenous imidazole, pyridine, and ethanethiol has no measurable effect on the functional properties of the special pair in these cavity mutants. FT-Raman spectroscopy is used to provide more detailed information on local interactions around the special pair. Data in the core-size marker mode and carbonyl stretching region suggest that an adventitious ligand replaces histidine as the axial ligand to bacteriochlorophylls in the cavity mutants. We speculate that this ligand is water. Furthermore, the position of the core-size marker mode changes when the cavity mutant RCs are incubated with exogenous ligands such as imidazole, pyridine, or ethanethiol, suggesting that the axial ligand to the special pair BChls can be exchanged in the cavity mutants. Interestingly the temperature dependence of $P^+Q_A^-$ recombination kinetics is very similar in the cavity mutants and WT, suggesting that the axial ligands to the special pair are not significant contributors to the solvent reorganization energy for this reaction. These results lead to the surprising conclusion that the nature of the axial ligand to the special pair has little influence on the properties of the macrocycle, and that axial coordination from the protein by histidine is not required for bacteriochlorophyll binding or for efficient electron transfer in the RC.

All metal-containing macrocycles, such as hemes, chlorophylls, and bacteriochlorophylls, that are associated with globular or membrane proteins have at least one ligand from the protein to the central metal atom. In the case of heme proteins, the nature of this ligand has profound biological consequences and has been the object of extensive studies (Dawson, 1988; Liu et al., 1995). Although fewer structures are available for photosynthetic systems, most chlorophylls and bacteriochlorophylls are ligated to the protein by conserved histidine residues through the central Mg atom. Three interesting exceptions are found in the literature. In the antenna complex from *P. aestuarii*, one of seven bacteriochlorophylls (BChl)[1] is ligated to the protein by the backbone carbonyl oxygen of Leu 234, and another appears to have an ordered water molecule as the ligand (Tronrud et al., 1986). Second, in the recently solved structure of the LHII antenna complex from *Rps. acidophilia*, the carbonyl oxygen of a formylmethionine at the amino terminus of the α-subunit serves as the axial ligand to one of the three BChls (McDermott et al., 1995). Third, the structure of the light-harvesting chlorophyll a/b−protein complex obtained by electron crystallography of two-dimensional crystals suggests the following axial ligands to chlorophylls a and b: three side chain amides (Gln 131, Gln 197, Asn 183), three salt-bridged glutamates (Glu 139−Arg 142, Glu 65−Arg 185, Glu 180−Arg 70) and a peptide carbonyl (Gly 78), possibly bridged by a water molecule (Kuhlbrandt et al., 1994). The role of these axial ligands in modulating the properties of Mg-containing macrocycles is unknown.

Histidine appears to serve as the axial ligand to BChl in all known bacterial photosynthetic reaction centers (RCs). A schematic diagram of the special-pair primary electron donor, relevant neighboring residues, and ordered water molecules from the crystallographic coordinates of RCs from *Rb. sphaeroides* (Ermler et al., 1994) is shown in Figure 1. When the axial His ligand to either of the Mg atoms of the special pair, (M)202 or (L)173, is replaced with a bulky, noncoordinating ligand such as Leu or Phe, the special pair assembles; however, the Mg atom of the BChl whose axial His is replaced is lost. This forms a so-called heterodimer consisting of BChl and bacteriopheophytin (BPheo) with a greatly modified absorption spectrum and function (Bylina & Youvan, 1988; Schenck et al., 1990). When the axial

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[1] Abbreviations: RC, reaction center; His, histidine; BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; P, primary donor (bacteriochlorophyll dimer); B, monomer bacteriochlorophyll in the RC; H, bacteriopheophytin in the RC; Q_A, primary quinone; Im, imidazole; Py, pyridine; EtSH, ethanethiol; WT, wild-type; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; FT, Fourier transform.

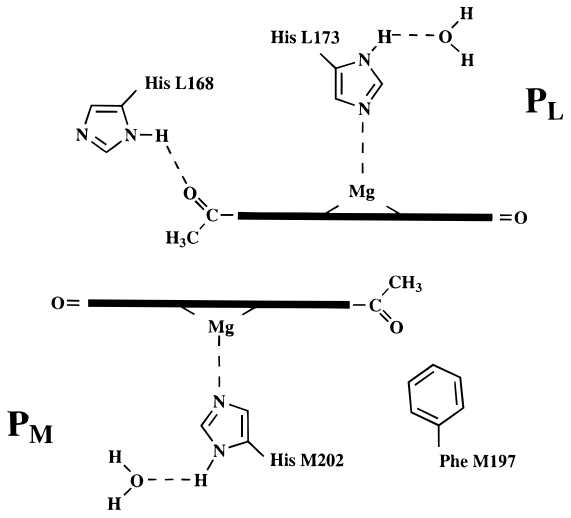

FIGURE 1: Diagram of the special-pair dimer of BChls with nearby residues, ordered water molecules, and numbering for the RC from *Rb. sphaeroides*.

histidine ligand on the M side of RCs from *Rb. capsulatus* is replaced by the coordinating amino acid Gln, the special pair also assembles but remains a homodimer of BChls (Bylina & Youvan, 1988); Gln substitution at His (L)173 gives rise to unstable RCs (Bylina, 1988). The axial His ligands to the monomeric BChls of the RC from *Rb. capsulatus* have been mutated to the coordinating amino acid Ser at (M)180 and both Ser and Thr at (L)153 without changing the pigment composition of the purified RCs (Bylina et al., 1990). Conversely, whenever Mg-free bacteriopheophytins are found in the RC, coordinating axial ligands from the protein are not in proximity, as in the functionally critical primary electron acceptor BPheo (H_L) and the BPheo in the B_M binding site of *Chloroflexus aurantiacus* RCs (Blankenship et al., 1983; Ovchinnikov et al., 1988; Pierson & Thornber, 1983). To date there has been no report of an enzyme that converts BChl into BPheo [the Mg atom is inserted early in the biosynthesis (Leeper, 1991)]; thus, it appears that Mg loss is catalyzed during the assembly of the RC polypeptides with the chromophores. Consistent with this notion, when a His residue is engineered into a position such that it would be capable of axial ligation, a Mg-containing BChl is found to replace BPheo in the H_L binding site (Kirmaier et al., 1991). Taken together, these results suggest the generalization that Mg-containing BChls are always ligated to photosynthetic proteins by coordinating amino acid residues. This idea has guided the investigation of chromophore binding sites in photosynthetic systems for which no structural information exists, such as the RC and light harvesting complexes from Photosystem II (Brunisholz & Zuber, 1992; Ruffle et al., 1992). In the present work we demonstrate that axial ligation of BChl in the special pair by amino acids from the protein is not required at all and that this ligand has surprisingly little effect in modulating the functional properties of Mg-containing macrocycles.

This work was originally motivated by a new class of cavity mutants of myoglobin (Mb). The heme iron in Mb is coordinated to His 93 in all Mbs. When this residue is replaced by glycine and the mutant protein H93G is expressed in bacteria grown in the presence of exogenous imidazole (Im), a stable protein is formed containing Im in the cavity created by the mutation (Barrick, 1994). Im serves as the axial ligand to heme in this protein denoted H93G-(Im). Significantly for what follows, when Im is absent in the growth medium, no stable heme-containing protein is recovered.[2] We have shown that it is possible to replace this Im ligand with a wide range of other small organic ligands by a simple diafiltration method (DePillis et al., 1994), thereby creating an enormous diversity of novel proteins. This general strategy has now been extended to several other heme proteins with similarly interesting results (McRee et al., 1994; Wilks et al., 1995). Thus, it was natural for us to apply this strategy to bacterial RCs. A preliminary report of these results was presented elsewhere (Goldsmith & Boxer, 1995).

EXPERIMENTAL PROCEDURES

The *puf*L and *puf*M genes were subcloned into pUC from M13 clones, SH1 and SB8. The mutations (M)H202G, (M)-H202L (the heterodimer mutant), and (L)H173G were created in the subclones with an oligo-directed, double-stranded mutagenesis kit from Stratagene (Chameleon). The genes were dideoxy sequenced to verify the existence of the mutations and shuttled into the pRKSCH *puf* operon which contains a His to Glu mutation at residue 32 of *puf*A. The pRKSCH operon was then conjugated to the antenna-containing deletion strain of *Rb. sphaeroides*, ΔLM1.1. The mutagenesis system is a modification of that described by Paddock et al. (1989). M13 clones, pRKSCH, ΔLM1.1, and protocols were generously provided by Dr. JoAnn Williams (Williams et al., 1992). The existence of the (M)H202G mutation was established further by purifying the *puf* operon from the *Rb. sphaeroides* (M)H202G strain (after conjugation), cloning the *puf*M fragment into pUC, and resequencing the construct.

The bacteria were grown nonphotosynthetically in YCC medium in either the presence or absence of 2 mM Im (it was found that Im concentrations in excess of 5 mM inhibited growth). RCs were isolated by standard methods (Paddock et al., 1988), with a final purification step by FPLC on DEAE Toyopearl 650-S. BChl/BPheo and total pigment/RC ratios were obtained as described in the literature (Kirmaier et al., 1991; van der Rest & Gingras, 1974). When studying the effects of exogenous ligands on purified RCs, protein was incubated with 10 mM exogenous ligand (pH 8.0) at 4.0 °C for at least 48 h.

Absorption and Stark effect spectra were obtained as described previously (Boxer, 1993). $P^+Q_A^-$ decay kinetics were obtained as described previously (Stocker et al., 1992). FT-Raman spectroscopy of reaction centers was carried out as described by (Mattioli et al., 1991) with the following exceptions: RCs at ∼250 μM were suspended in 10 mM Tris-HCl (pH 8.0), 0.1% LDAO, and 1 mM EDTA and poised in the reduced state with 5 mM ascorbic acid. The data were collected with a Bio-Rad FTS-40 Raman Spectrometer equipped with a CW diode-pumped Nd:Yag laser (1064 nm). The laser power at the sample was ∼500 mW, and samples were contained in 5 mm diameter quartz NMR tubes. Spectra of comparable signal/noise were collected with the laser power at 320 mW and found to be identical, within one wavenumber, to the spectra collected at 500 mW. The 1605 cm^{-1} core-size marker mode band does not change position at lower laser power. All spectra were recorded at

[2] The H93G apoprotein can also be expressed and reconstituted with heme. In this case, water is likely the axial ligand (Decatur and Boxer, unpublished results).

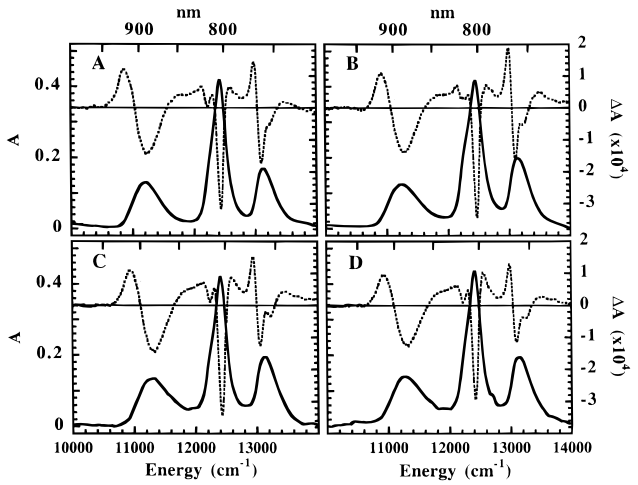

FIGURE 2: Absorption (solid line) and Stark (dotted line) spectra of (A) wild-type and cavity mutants: (B) (M)H202G, (C) (L)-H173G, and (D) (M/L)H202/173G in 1:1 glycerol/buffer at 77K. The absorption spectra were scaled to the same optical density at 804 nm. The Stark spectra were taken with an applied field of 1.8 $\times$ 10^5 V/cm and were scaled with the absorption, i.e., the stark spectra were not scaled to each other.

room temperature and were the average of 4096 interferograms at 4 cm^{-1} spectral resolution. The baseline was corrected by subtracting a polynomial from the spectra.

*P decays were obtained by fluorescence upconversion as described in detail previously (Stanley & Boxer, 1995). Data were collected at room temperature in a stirred 1 mm path length quartz cuvette with RCs at ~7 μM suspended in 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.1% Triton X-100 and poised in the Q_A^- state with 5 mM dithionite. RCs were excited with ~90 fs pulses at 804 nm (10 mW at 82 MHz) from a mode-locked Ti:sapphire laser. The 804 nm excitation of the RC leads to excitation of the accessory monomeric bacteriochlorophylls and subsequent ultrafast energy transfer in approximately 100 fs to the primary donor, P (Jia et al., 1995). The model function providing the best fit to the data was an instrument response function convoluted with a sum of three exponentials: one having a negative amplitude to account for energy transfer to the primary donor and two for the subsequent decay of *P. The *B→P energy transfer component of the decays is not accurately resolved owing to the time resolution of the instrument. Fluorescence decays collected in 10 nm steps between 910 and 940 nm were found to be identical.

Redox titrations were performed with an optically transparent thin layer electrode (OTTLE) (DeAngelis & Heineman, 1976) using a gold mesh working electrode modified with 4,4′-dithiodipyridine (Taniguchi et al., 1982) as described by (Moss et al., 1990). $K_3Fe(CN)_6$ at 150 μM was used as the mediator, and the ambient potential was measured with respect to a saturated calomel electrode. The fraction of reduced P was determined by monitoring the optical density of the band associated with the special pair (865 nm). For the titrations, RCs at 35 μM were suspended in 20 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, and 60 mM KCl.

RESULTS

Mutant reaction centers (M)H202G, (L)H173G, and (M/L)H202/173G were isolated from strains of *Rb. sphaeroides* and found to be stable. Neither the apparent stability nor functional properties (*vide infra*) of these RCs depend on whether the exogenous ligand Im is present in the growth medium and during the RC isolation process. The term "exogenous ligand" is used in the following to denote ligands deliberately added either during cell growth and protein isolation or to purified RCs. Unless otherwise stated, experiments were carried out on RCs that had no previous exposure to exogenous ligands. The absorption and Stark spectra of the three cavity mutants in the Q_Y region at 77 K are compared with WT in Figure 2. The band associated with the special pair (~890 nm at 77 K) in the cavity mutants is very similar to that of WT as are the monomeric B and H bands.[3] The absorption and $P^+Q_A^-$ difference spectra of the cavity mutants in the Q_x region were obtained at 77 K and found to be similar to those of the WT protein (data not shown). Both the absorption and Stark spectra of the cavity mutants were unchanged for RCs incubated in exogenous Im at concentrations 5000 times that of the RC. The absorption spectra were also unchanged for RCs incubated with a large excess of exogenous pyridine (Py) and ethanethiol (EtSH). Consistent with the absorption and Stark data, the pigment content and ratios for the cavity mutants are reasonably compatible with four BChls and two BPheos (see Discussion). The values measured for the mutant proteins (M)H202G, (L)H173G, (M/L)H202/173G, and (M)H202L (heterodimer) are 1.56 $\pm$ 0.05, 2.07 $\pm$ 0.07, 1.98 $\pm$ 0.07, and 0.95 $\pm$ 0.02, respectively, compared to the WT value of 1.76 $\pm$ 0.03. The total pigment/RC ratio was found to be between 5 and 5.5 for the mutant and WT RCs.

Table 1: Kinetics of $P^+Q_A^-$ Recombination and *P Spontaneous Fluorescence Decay Measured by Fluorescence Upconversion

RC	$P^+Q_A^-$ recombination τ (ms) 298 K	77 K	*P decay τ (ps) (amplitude)
WT	110 $\pm$ 2	19$\pm$ 2	2.5 $\pm$ 0.1 (80% $\pm$ 1) 9.0 $\pm$ 0.5 (20% $\pm$ 1)
(M)H202G	118 $\pm$ 2	21$\pm$ 2	3.0 $\pm$ 0.1 (79% $\pm$ 1) 16.1 $\pm$ 0.9 (21% $\pm$ 1)
(L)H173G	96 $\pm$ 2	16$\pm$ 2	3.00 $\pm$ 0.1 (81% $\pm$ 2) 13.7 $\pm$ 0.8 (19% $\pm$ 2)
(L/M)H202/173G	106 $\pm$ 2	20$\pm$ 2	3.3 $\pm$ 0.1 (81% $\pm$ 1) 17.7 $\pm$ 0.8 (19% $\pm$ 1)

The lifetimes of the $P^+Q_A^-$ state for the WT and cavity mutant proteins were obtained at 298 and 77 K by measuring the absorbance change at 865 and 890 nm, respectively, on the millisecond time scale after a subsaturating actinic flash at 532 nm (data not shown).[4] The results are found in Table 1. The $P^+Q_A^-$ recombination rate and the temperature dependence of this rate for the cavity mutants are different from WT values, but they differ by less than 15%. For samples of comparable optical densities at the excitation wavelength, the initial amplitudes of the bleach at 870 nm were comparable within 10%, suggesting that the quantum yield of $P^+Q_A^-$ formation is similar to WT for all three mutants. Incubation of purified RCs with Im, Py, and EtSH has no measurable effect on the recombination rate or its temperature dependence.

The fluorescence decay kinetics of *P for WT and the three cavity mutants at room temperature are shown in Figure

[3] In some cases, the amplitude of the band associated with the special pair was observed to decrease for the cavity mutants. The magnitude of the decrease appeared to correlate with the length of time (on the order of days) the RCs were stored at temperatures ≥4 °C. Addition of ascorbate caused only partial recovery of the band.

[4] Purified reaction centers were found to contain a considerable amount (>50%) of the secondary quinone, Q_B, and were Q_B depleted by a protocol found in Okamura et al. (1975) prior to data collection.

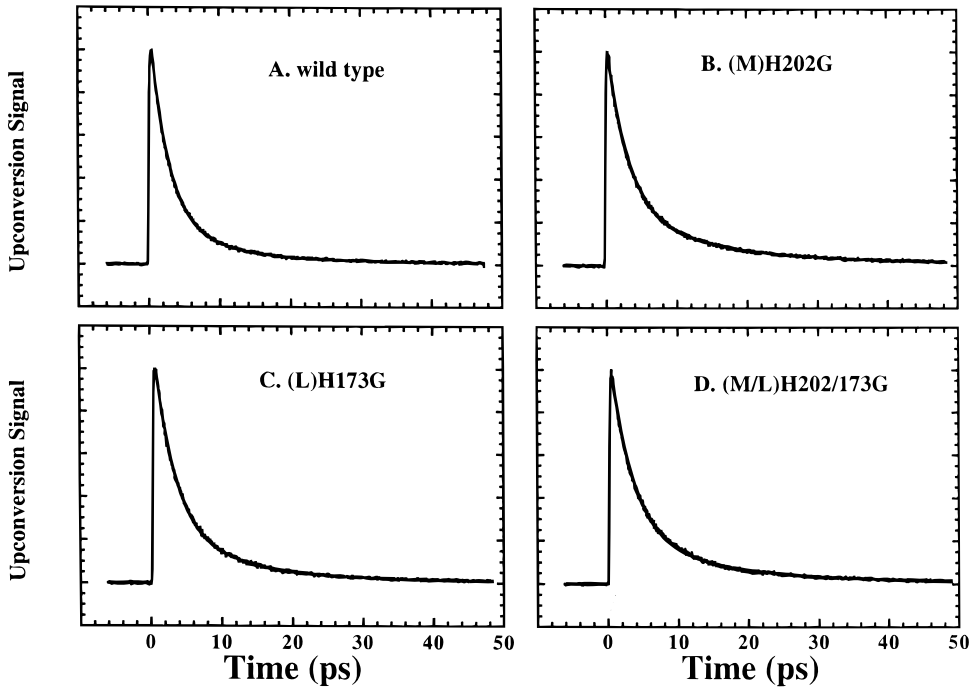

FIGURE 3: *P fluorescence decays of (A) wild-type and cavity mutants: (B) (M)H202G, (C) (L)H173G, and (D) (M/L)H202/173G measured by fluorescence upconversion at room temperature (λ_{exc} = 804 nm, λ_{det} = 920 nm). The number of counts at the maximum for each transient is approximately 10 000. Overlayed on the data are fits to a model function consisting of three exponentials, one with a negative ampitude, convoluted with the instrument response function as described in the text.

3, and results of the fit to the data are presented in Table 1. The lifetimes of the fast and slow decay components increase 20−35% and 50−100%, respectively, for the cavity mutants, while the relative amplitudes of these decay components are unchanged. Incubation of purified RCs with exogenous ligands had no measurable effect on the fluorescence decay kinetics of *P.

Spectroelectrochemical titrations were carried out to ascertain whether changes in the nature of the axial ligand to BChl perturbs the redox potential of the special pair. The data are shown in Figure 4. All titrations were found to fit well to the Nernst equation and were reversible. The redox potentials measured for the mutant proteins (M)H202G, (L)-H173G, and (M/L)H202/173G are 494, 511, and 495 mV, respectively, compared to the WT value of 505 mV (vs NHE). The error in the measurement is approximately ±5 mV. Incubation of purified RCs with exogenous ligands had no measurable effect on the redox potential of P.

FT-Raman data were collected between 500 and 1800 cm^{-1}. The spectra of WT and the cavity mutants between 1590 and 1750 cm^{-1} are shown in Figure 5, and the peak positions of the bands are found in Table 2. The spectra were obtained at room temperature with RCs poised in the reduced state with ascorbate as described previously (Mattioli et al., 1991). The general features of the WT spectrum are identical to those obtained previously by Mattioli and co-workers on WT *Rb. sphaeroides* RCs; however, the absolute positions of the bands are found to differ by a few cm^{-1}. Mattioli and co-workers, obtain bands at 1607, 1620, 1653, 1679, and 1691 cm^{-1}, whereas our spectra reveal bands at 1605, 1625, 1656, 1682, and 1691 cm^{-1}. Such discrepancies may arise from differences in RC sample conditions during the FT-Raman measurement, different RC preparation protocols, or differences in the way the spectra were baseline corrected.

The overall fingerprint of the FT-Raman spectra of WT is maintained in those of the cavity mutants, although subtle

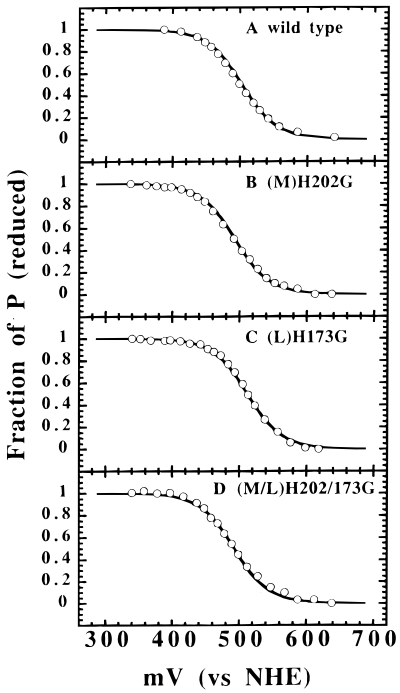

FIGURE 4: Spectroelectrochemical redox titrations of the special-pair in (A) wild-type and cavity mutants. (B) (M)H202G, (C) (L)-H173G, and (D) (M/L)H202/173G measured with an optically transparent thin layer electrode (OTTLE) as described in the text. The fraction of reduced P was determined by monitoring the optical density of the special pair (865 nm). The data are from oxidative titrations, and the solid lines are best fits of the data to the Nernst equation.

shifts are observed as shown in Figure 5 and Table 2. The WT spectrum has bands at ∼1625, ∼1656, ∼1691, and ∼1682 cm^{-1} assigned to the C_2 acetyl carbonyls (hydrogen-bonded and free) and the C_9 keto carbonyls (both free) of the special pair BChls of P_L and P_M, respectively (Mattioli et al., 1991). This region of the spectrum also contains a

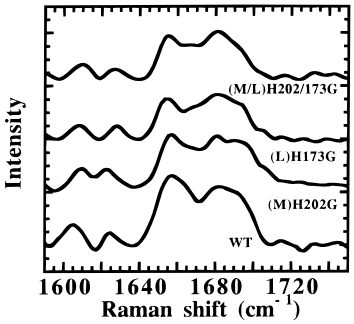

FIGURE 5: FT-Raman spectra of wild-type and cavity mutants: (M)-H202G, (L)H173G, and (M/L)H202/173G with the special pair poised in the reduced state with 5 mM ascorbate (room temperature, excitation at 1064 nm). The region of the special-pair bacteriochlorophyll core-size marker mode and carbonyl stretching region of the spectra is shown. Peak positions are tabulated in Table 2.

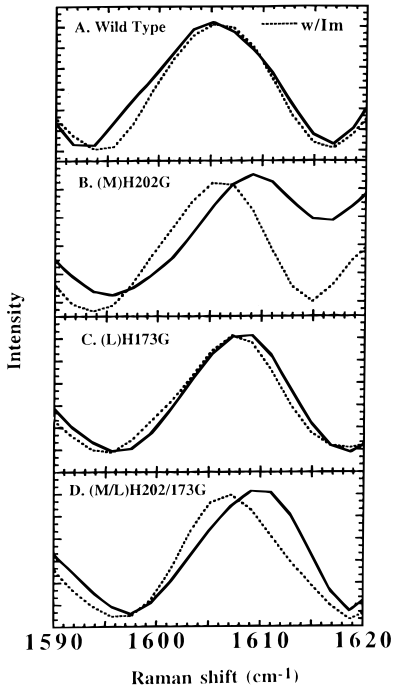

FIGURE 6: Expansion of the FT-Raman spectra in the region of the special-pair bacteriochlorophyll core-size marker mode for (A) wild-type and cavity mutants (B) (M)H202G, (C) (L)H173G, and (D) (M/L)H202/173G. Data were taken on RCs in the presence (dotted line) and absence (solid line) of 10 mM Im as described in text.

Table 2: Peak Positions of FT-Raman Bands in Special-Pair Core-Size Marker Mode and Carbonyl-Stretching Region of Spectra

RC	core-size marker mode (cm^{-1})	P_L C_2 acetyl (cm^{-1})	P_M C_2 acetyl (cm^{-1})	P_L C_9 keto (cm^{-1})	P_M C_9 keto (cm^{-1})
WT	1605	1625	1656	1691	1682
(M)H202G	1609	1622	1656	1690	1681
(L)H173G	1608	1628	1654	1691	1681
(L/M)H202/173G	1610	1627	1655	1691	1681

band at ~1605 cm^{-1} for WT assigned to a bacteriochlorophyll core-size marker mode with contributions primarily from a C_aC_m methine bridge stretch of the special-pair macrocycles (Mattioli et al., 1991). This core-size marker mode is found shifted to higher energy in the cavity mutants as shown in Figure 5, Table 2, and the solid line of Figure 6A−D. The values measured for the mutant proteins (M)-H202G, (L)H173G, and (M/L)H202/173G are 1609, 1608, and 1610 cm^{-1}, respectively. Upon incubation of purified RCs with exogenous Im, this core-size marker mode is found to shift to lower energy for the cavity mutants yet remains unchanged for WT as shown in the dotted line of Figure 6. The values measured for the cavity mutants (M)H202G, (L)-H173G, (M/L)H202/173G in the presence of exogenous Im are 1606, 1607, and 1607 cm^{-1}, respectively. As can be observed in Figure 6, the Im-induced shift in the core-size marker mode is larger for (M)H202G and the double mutant (M/L)H202/173G (3 cm^{-1}) than for (L)H173G (1 cm^{-1}). Identical exogenous ligand-induced shifts of the core-size marker mode were observed when mutant RCs were incubated in Py or EtSH (data not shown).

DISCUSSION

On the basis of the overall similarity of the pigment composition, absorption and Stark effect spectra, $P^+Q_A^-$ decay kinetics and quantum yield, *P decays, redox potential of P, and FT-Raman spectra, we conclude that, surprisingly, the special pairs in these cavity mutant RCs are relatively normal and functional. Furthermore, the addition of the exogenous ligands Im, Py, and EtSH to the cavity mutant RCs has no measurable effect on the functional properties of these proteins. In the following, we focus further on the subtle differences and implications of these results.

Wild-type RCs bind four BChls and two BPheos; thus, the expected ratio of BChl/BPheo is 2.0 and that of total pigments/RC is 6.0. The experimentally derived ratios both in WT and many mutants deviate from these ideal values, typically giving a ratio somewhat less than 2 and a pigment content somewhat less than 6. The small standard deviations reported in Results were obtained by repeating the extractions as many as six separate times on RCs from one preparation. The BChl/BPheo and total pigments/RC ratios measured here for the WT protein are 1.76 ± 0.03 and 5.56 ± 0.12, respectively, and are within the range reported previously by other investigators (Heller et al., 1995; Kirmaier et al., 1991). There is, at this time, no way to know whether these results for WT reflect the actual pigment composition of the protein, i.e., some fraction of the BChl is actually BPheo in the normal, functional RC, or whether the relative loss of BChl is associated with conversion of BChl to BPheo during the extraction from different sites in the protein (i.e., a process analogous to that which generates BPheo in the H-binding sites when the RC assembles). The X-ray crystal structures of *Rb. sphaeroides* and *Rps. viridis* RCs, even at the highest resolution currently available (2.65 and 2.3 Å, respectively) (Ermler et al., 1994; Deisenhofer et al., 1995), do not provide accurate enough information to foreclose the possibility that some of the BChl in isolated RCs is actually BPheo. For example, if 10% of each BChl in the RC were a BPheo, a pigment ratio of 1.5/1 is predicted. Although electron density attributable to the central Mg atoms is observed in the X-ray structures, it would be impossible to detect a 10% "impurity" at each site; in fact, at the current level of resolution, even a 20% occupancy of BPheo at each of the four BChl sites (and vice versa at the BPheo sites) would be compatible with the experimental data for *Rps. viridis* RCs (Hans Deisenhofer, personal communication). Likewise, the Mg/RC ratio of 3.9 ± 0.4 obtained by Mg atomic emission spectroscopy of WT RCs (Kirmaier et al., 1991) does not rule out a 10% loss of Mg at each BChl site.

As a further test of the reliabilty of the methodology, pigment extraction was carried out on the heterodimer

mutant, (M)H202L, giving the ratio 0.95 ± 0.02 (Results). This is consistent with what is expected if one of the BChls in the special pair is converted to BPheo and with values obtained by other investigators (Bylina & Youvan, 1988). The ratios measured for the cavity mutant proteins (L)H173G and (M)H202G/(L)H173G are 2.07 ± 0.07 and 1.98 ± 0.07 respectively, both higher than WT and giving the expected values for 4 BChls and 2 BPheos, while that measured for (M)H202G is 1.56 ± 0.05. Because (M)H202G is a subset of the double mutant, it would be surprising if Mg were lost in the single but not in the double mutant. All the cavity mutants have a much larger ratio than the (M)H202L heterodimer; thus, we conclude that none is a heterodimer, and the data for (L)H173G and the double mutant are entirely consistent with the normal WT complement of pigments. We note in passing that there has been much speculation during the past few years on the origin of heterogeneity in the kinetics of RCs measured with very good signal-to-noise ratios (Du et al., 1992; Peloquin et al., 1994; Woodbury et al., 1994; Hamm et al., 1993; Stanley & Boxer, 1995) (and likewise observed here for the cavity mutants; see Figure 3). It is possible that small variations in the Mg content of the monomeric BChl B_L or residual Mg in the chromophore in the H_L site could be responsible for this, rather than more exotic explanations based on theoretical conjecture (Marchi et al., 1993).

Both the electronic absorption and Stark spectra are also sensitive to pigment content and perhaps more importantly, to the interactions among the pigments. Examination of the spectra in Figure 2 suggests that the interactions responsible for the absorption spectrum of P in WT are also present in the three cavity mutants. The absorption spectrum alone, even at cryogenic temperatures, does not provide definitive information on pigment content, the classic case being borohydride-treated RCs (Struck et al., 1991), and to a lesser extent the "reverse heterodimer" mutant, (L)H173L (Schenck et al., 1990). However, the Stark effect spectrum is extremely sensitive to changes in the nature of the pigments comprising P. The Stark effect for the lowest energy electronic transition of P is dramatically larger in both the normal (M)H202L (Hammes et al., 1990) and reverse (L)-H173L (Schenck et al., 1990) heterodimers of *Rb. sphaeroides*. The effect is so large that even a 10% difference between the pigment composition of the special pair in the cavity mutants and WT could be readily observed, if it were present, in the Stark spectrum of the mutant proteins. However, the Stark spectra of the special pair in WT and the three cavity mutants are essentially identical; they simply track the small differences in position and lineshape of the absorption spectra.

The obvious question raised by these results is, what is the fifth ligand to Mg in the special pairs of the cavity mutants? All studies of chlorophylls or bacteriochlorophylls *in vitro* demonstrate that the Mg atom is at least five-coordinate, never four-coordinate (Closs et al., 1963; Freed & Sancier, 1954; Katz et al., 1963; Livingston et al., 1949; Storm & Corwin, 1964). It is conceivable that a different amino acid side chain or a carbonyl moiety from the main chain has replaced His as the axial ligand to BChl in the cavity mutants. Examination of the X-ray crystal structure of the RC shows that the nearest coordinating amino acid is His L168, which is 6.4 and 6.5 Å from Mg in P_M and P_L, respectively. Ligation by His L168 could stabilize only one Mg (not two in the double mutant) and would require gross structural changes for which we have no evidence. Another possibility is that the C_2 acetyl groups of BChls, P_L, and/or P_M (depending on which cavity mutant), have rotated 180°, positioning the carbonyl oxygen in proximity to the Mg of the other BChl, thereby providing the fifth ligand. Indeed, this conformation of the C_2 acetyl groups of P_L and P_M was initially proposed in the original crystal structure of the RC from *Rps. viridis* (Deisenhofer et al., 1984) prior to the results of Raman spectroscopy (Robert & Lutz, 1986) and further refinement of the X-ray data (Deisenhofer et al., 1995). Finally, an additional possibility is that an adventitious ligand has entered the cavity created by the removal of the His side chain, the most likely candidate being water. Ordered water molecules are found 2.81 and 2.47 Å from the Nδ1 atom of both His (M)202 and (L)173, respectively, in the RC from *Rb. sphaeroides* (cf. Figure 1) (Ermler et al., 1994). Interpretation of the FT-Raman spectra (below) supports this latter hypothesis.

FT-Raman spectra of RCs in the 1600−1750 cm^{-1} region have been shown previously to provide detailed information on the conformation of the special pair and its interactions with individual amino acids of the protein matrix (Mattioli et al., 1991, 1994).[5] The peaks at 1691 and 1682 cm^{-1} in the WT spectrum have been assigned to the two free C_9 keto carbonyl groups of P_L and P_M, while the bands at 1656 and 1625 cm^{-1} have been assigned to the free C_2 acetyl group of P_M and the hydrogen-bonded (with His L168) C_2 acetyl group of P_L (Mattioli et al., 1991, 1994). These assignments are consistent with the X-ray crystal structure (Ermler et al., 1994). Furthermore, the addition and/or removal of hydrogen-bond donating residues in proximity to the special-pair carbonyl groups by site-specific mutagenesis causes predictable shifts in the carbonyl stretching frequencies (Mattioli et al., 1994). As can be seen from Figure 5 and Table 2, the peaks corresponding to two free C_9 keto groups and a free and hydrogen-bonded C_2 acetyl group are also apparent in the spectra of the cavity mutants. This reveals that the carbonyl groups of the special pair BChls in the mutants are in a similar conformation to WT. If His L168 were the fifth ligand to BChl in any of the cavity mutants, the hydrogen bond that exists between it and the C_2 acetyl group of P_L would be lost and the 1625 cm^{-1} band in the FT-Raman spectrum would shift to higher energy by at least 25 cm^{-1} (Mattioli et al., 1994). This is not observed in the spectra of the cavity mutants. Moreover, if the C_2 acetyl group of P_M is the ligand to BChl P_L in (L)H173G or (M/L)H202/173G, the interaction of the carbonyl moiety with the electrophilic Mg of BChl would be expected to shift the 1656 cm^{-1} band to lower energy by at least 25 cm^{-1}, which is not observed. Therefore, the most likely possibility is that an adventitious ligand has entered the cavity created by the removal of the His side chain.

Absorption spectra of the RC, even at low temperature, do not provide definitive information on the nature of this adventitious ligand to the special pair BChls. The Q_Y transition of monomeric, five-coordinate BChl is relatively insensitive to the nature of the axial ligand (Callahan & Cotton, 1987; Cotton & Van Duyne, 1981). The higher energy Q_X transition of monomeric, five-coordinate BChl is

[5] Use of the 1064 nm emission from a Nd:YAG laser primarily interrogates the vibrational modes of the special-pair bacteriochlorophylls over those of the monomer BChls and BPheos, presumably due to preresonance with its red-shifted absorption band.

moderately sensitive (Callahan & Cotton, 1987; Cotton & Van Duyne, 1981); however, this region of the RC spectrum is congested by the transitions of both the special pair and the monomeric BChls, and the $P^+Q_A^-$ difference spectrum contains both a bleach from P and a band shift from B. Therefore, we have no direct information on the nature of the adventitious ligand in the absence of added exogenous ligands. Water is the most probable candidate, though we cannot rule out some other small ligand that is present in the cell, growth medium, or buffer.[6]

The band at ca. 1605 cm^{-1} in the FT-Raman spectrum of BChl has been shown to be a sensitive indicator of the coordination number of Mg because of the large amount of C_aC_m methine bridge stretching character in this vibration (Callahan & Cotton, 1987). In solution, the Mg atom of five-coordinate BChl is located above the plane of the macrocycle, and the band is observed at 1610 cm^{-1}. For six-coordinate BChl in solution, however, the Mg is forced into the plane of the macrocycle such that both the fifth and sixth ligands can interact with it. The porphyrin core expansion that results from the Mg atom displacement causes this so-called core-size marker mode to shift to 1595 cm^{-1} (Callahan & Cotton, 1987).[7] The peak position of this band is found to be insensitive to the nature of the axial ligand to monomeric BChl presumably because the core size is affected only by the number of ligands to the Mg atom (Callahan & Cotton, 1987).

The core-size marker mode band associated with the special-pair BChls in WT RCs is found at 1605 cm^{-1} and is shifted to higher energy in the cavity mutants (M)H202G, (L)H173G, and (M/L)H202/173G at 1609, 1608, and 1610 cm^{-1}, respectively (Figure 6). The value for WT is observed to be in between the five- and six-coordinate solution values of monomeric BChl, whereas it is close to the five-coordinate solution position of monomeric BChl in the cavity mutants. This observation is consistent with a model in which the Mg of the special-pair BChls are forced closer to the plane of the macrocycles by the ligating histidines in WT, increasing the porphyrin core-size of both BChls of the special pair.[8] We may then speculate that removal of the His side chains in the cavity mutants releases this strain and the Mg relaxes back closer to its five-coordinate solution position presumably because the coordinating ligand in the cavity mutants (e.g., H_2O) is smaller than His. We have attempted to displace whatever adventitious ligand is bound to the Mg atom of the special-pair BChls by incubating RCs with the coordinating ligands Im, Py, and EtSH. As can be seen from Figure 6, the addition of Im shifts the peak position of the core-size marker mode back to lower energy by 3 cm^{-1} in the cavity mutants (M)H202G and (M/L)H202/173G, by 1 cm^{-1} for (L)H173G, and it has no effect on this mode for WT. Identical exogenous ligand-induced shifts are also observed for Py and EtSH (data not shown). These data suggest that the exogenous ligands are entering the cavity, displacing the bound adventitious ligand (likely H_2O), and forcing Mg closer to the plane of the BChl as in WT, presumably due to the steric constraints of the cavity.

Despite this evidence that ligands can be exchanged in and out of the cavity, we have observed no measurable effects of incubating RCs with exogenous ligands on the absorption and Stark spectra, $P^+Q_A^-$ recombination rate and temperature dependence thereof, *P decay kinetics, and redox potential of P for the cavity mutants. It may be concluded that the nature of the axial ligand has little influence on the properties of the special pair in RCs. Consistent with these observations, the $P^+Q_A^-$ recombination rate of the (M)-H200Q, (L)H173Q, (M/L)H200/173Q mutants of *Rb. capsulatus* differs by no more than 7% of the WT value at room temperature (Bylina & Youvan, 1988). In addition, the EPR line width of the oxidized special-pair and zero-field splitting parameters of the special pair triplet state at 80 K and 6 K, respectively, for (M)H200Q and (L)H173Q RCs contained in chromatophore membranes are essentially identical to WT (Bylina et al., 1990). It has been shown that little unpaired spin density resides on the central Mg atom in P^+ (Lendzian et al., 1993). Furthermore, the profound influence of the axial ligand on the reactivity and electronic properties of Fe-containing heme macrocycles involves interactions of the Fe d orbitals with orbitals of the ligand while Mg is d^0. The small changes in phenotype observed for our cavity mutants may simply reflect subtle variations in the conformation of the special-pair caused by the loss of the connection to the protein scaffold. This may be surmised by the small shifts in the FT-Raman bands assigned to the C_2 acetyl and C_9 keto groups of the special-pair in the mutants compared to WT (Figure 5 and Table 2).

We examined the temperature dependence of the $P^+Q_A^-$ decay with the hypothesis that if additional water were present in the immediate vicinity of P, this might alter the solvent reorganization energy, which would be reflected in a change in the temperature dependence of the $P^+Q_A^-$ decay. Indeed, electrostatic calculations carried out by Parson and co-workers have predicted that movements of the axial His ligands to P are responsible for as much as one third of the reorganization energy for the oxidation of P to P^+ (1 out of 3 kcal/mol) (Parson et al., 1990). However, the temperature dependence of the $P^+Q_A^-$ decay varies little for the cavity mutants with respect to WT, and it is unchanged by the incubation of RCs with exogenous ligands.

Finally, we note that many investigators have proposed models of the location of chlorophyll or bacteriochlorophyll binding sites in chlorophyll-binding proteins based on conserved His residues (Brunisholz & Zuber, 1992; Ruffle et al., 1992). The recent demonstration by X-ray crystallography that one of the BChls in LHII of *Rhodopseudomonas acidophilia* (McDermott et al., 1995) is bound to the N-terminal formylmethionine of the α-subunit runs counter to earlier predictions that His 12 of the β-subunit was a ligand

[6] Heller and co-workers have recently characterized the double mutants (L)F97V/F121H and (L)F97C/F121H from *Rb. sphaeroides* and find that both bind BChl in place of $BPheo_L$ despite the fact that the introduced His is in an unlikely position to serve as the axial ligand to BChl; the side chain being ~3.7 Å away from ring V of $BPheo_L$ (Heller et al., 1995). The residue that is located directly above the axial position is the small amino acid Ala while Leu M214 is at this same position on the opposite face of the macrocycle. As there are no other coordinating amino acids in proximity to $BPheo_L$, Heller and co-workers have also postulated the existence of water as the axial ligand, presumably found in the mutant as opposed to WT because of a favorable interaction with the newly introduced His.

[7] These data were obtained by Soret excitation resonance Raman. We have collected FT-Raman spectra of both five- and six-coordinate monomeric BChl in solution and find the position of the core-size marker mode to be similar at 1611 and 1597 cm^{-1}, respectively (data not shown). The spectra of five- and six-coordinate BChl were obtained with 10 mM BChl in neat ethyl ether and neat, dry tetrahydrofuran, respectively, as described in Callahan and Cotton (1987) and Mattioli et al. (1991).

[8] Here the assumption is made that the core-size marker mode band observed for the special pair in RCs is, at first order, the sum of the core-size marker mode bands of the two BChl monomers that make up the dimer.

for BChl (Brunisholz & Zuber, 1992). Furthermore, sequence alignments of photosystem II RCs with RCs from bacterial systems reveal no His residues in proximity to the monomeric Chl binding sites (Michel & Deisenhofer, 1988), while pigment extraction of PSII RCs supports the existence of a full complement of Chls (Nanba & Satoh, 1987). The cavity mutant RCs presented here demonstrate unambiguously that His side chain coordination to BChl is not essential for assembly and function of isolated RCs.

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