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Directed mutations affecting spectroscopic and electron transfer properties of the primary donor in the photosynthetic reaction center

(protein engineering/pigment composition/heterodimer/*Rhodobacter capsulatus*)

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ABSTRACT **Oligonucleotide-mediated mutagenesis has been used to change the histidine residues that act as axial ligands to the central Mg^{2+} ions of the "special pair" bacteriochlorophylls in the reaction center of *Rhodobacter capsulatus*. Histidine-173 of the L subunit has been replaced with glutamine, while histidine-200 of the M subunit has been replaced with glutamine, leucine, or phenylalanine. When leucine or phenylalanine is introduced at M200, one of the special pair bacteriochlorophylls is converted to bacteriopheophytin, which generates a heterodimer at the special pair binding site. The pigment composition of the reaction center is unaltered when either histidine is replaced with glutamine. All of these mutant reaction centers are photochemically active, although the electron transfer properties of heterodimer-containing reaction centers are altered. These mutations begin to define the structural parameters that determine whether bacteriochlorophyll or bacteriopheophytin will be incorporated into the tetrapyrrole binding sites of the photosynthetic reaction center. Our results demonstrate that the properties of the photosynthetic reaction center can be changed by directed mutagenesis, which makes this complex an excellent model for testing theories of electron transfer in biological systems.**

The photosynthetic reaction center (RC) offers a model system for investigating a diverse set of structure–function problems. This complex mediates extremely efficient electron transfer reactions that result in high quantum yields for conversion of light to chemical energy (for review, see refs. 1 and 2). More is known about the composition and structure of RCs from the *Rhodospirillaceae* than from any other photosynthetic organism. X-ray structures have been determined for both *Rhodopseudomonas viridis* (3, 4) and *Rhodobacter sphaeroides* (5, 6). Amino acid residues binding prosthetic groups are almost completely conserved between these two species and *Rhodobacter capsulatus*. Such extensive sequence similarity (see review in ref. 7) facilitates mutagenesis experiments in *R. capsulatus*, since genetic tools are most advanced for this species (8). RC and light-harvesting genes have been deleted from the *R. capsulatus* chromosome (9). Such deletions may be complemented by plasmids (10) bearing either wild-type or mutagenized copies of the RC genes. Recently, RCs were generated and characterized in which Ile^{229} of the L subunit (Ile^{L229} is part of the secondary quinone binding site) was changed to each of 17 other amino acids (11).

The primary electron donor in bacterial RCs consists of a "special pair" or dimer of bacteriochlorophyll (Bchl) molecules. After light absorption, an electron is transferred with 100% quantum efficiency from the excited primary donor to an intermediate acceptor bacteriopheophytin (Bphe) molecule in ≈3 psec (1). EPR (12) and electron nuclear double resonance (ENDOR) (13) experiments indicate that the unpaired electron of the oxidized primary donor is delocalized over two Bchls. The x-ray structure reveals a close interaction, with ring I overlap of these two special pair Bchl molecules (3, 6). Histidines that correspond to L173 and M200 in *R. capsulatus* act as axial ligands to the central Mg^{2+} ions of the Bchl dimer, which is located between the L and M subunits near the periplasmic side of the membrane (14). Resonance Raman experiments (15) indicate that these Mg^{2+} ions are pentacoordinate, which is consistent with crystallographic data. In this paper, we report the properties of *R. capsulatus* RCs in which the axial histidine ligands of the special pair Bchls have been replaced with other amino acid residues by using site-directed mutagenesis. Characterization of these RCs will provide useful information for testing the validity of electron transfer theories (16) in biological systems.

MATERIALS AND METHODS

DNA Methods. All procedures were essentially as described by Maniatis *et al.* (17). M13 phage were maintained in *Escherichia coli* strain JM103. Plasmid pU29 derivatives were maintained in *E. coli* strain HB101. Plasmid pU2922 derivatives were conjugated from *E. coli* S17-1 donors (18).

Mutagenesis. Mutations were generated with the two-primer method of Zoller and Smith (19) with the modifications of Bylina and Youvan (10). YIB11, an M13mp18 derivative containing the 1375-base-pair *Eco*RI–*Kpn* I fragment of pU29 (10), was used for constructing $His^{L173} \rightarrow Gln$. YIB9, an M13mp18 derivative containing the 929-base-pair *Kpn* I–*Bam*HI fragment of pU29 (10), was used for construction of $His^{M200} \rightarrow Gln$. The M13 template carrying the $His^{M200} \rightarrow Gln$ mutation, which contains a *Stu* I site at the M200 codon, was used to improve the mutagenesis efficiency (11) for other M200 mutations. DNA inserts were shuttled into pU29 derivatives as described (11). The shuttling of $His^{L173} \rightarrow Gln$ into a pU29 derivative (which contains a *Bam*HI site at the L228 codon) was verified by the loss of the *Bam*HI site. The shuttling of $His^{M200} \rightarrow Gln$ into pU29 was verified by the presence of an additional *Stu* I site. The shuttling of $His^{M200} \rightarrow Leu$ or Phe into pU29($His^{M200} \rightarrow Gln$) was verified by the loss of the *Stu* I site. The double glutamine mutation ($His^{L173,M200} \rightarrow Gln$) was constructed by shuttling $His^{M200} \rightarrow Gln$ into pU29 ($His^{L173} \rightarrow Gln$) and was verified by the presence of an additional *Stu* I site. Mutations were shuttled from these pU29 derivatives to pU2922 derivatives. Plasmid pU2922, which replicates in both *E. coli* and *R. capsulatus*, is the result of a fusion between a pBR322

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Abbreviations: RC, reaction center; Bchl, bacteriochlorophyll; Bphe, bacteriopheophytin; LDAO, lauryldimethylamine oxide.

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derivative carrying the *puf* operon (pU22; see ref. 10) and a pRK292 derivative (20) in which the *Eco*RI site was removed. (The construction of this plasmid will be described elsewhere.) RC mutations were introduced into pU2922 by replacing the *Eco*RI–*Sac* I fragment of a pU2922 derivative with an *Eco*RI–*Sac* I fragment of the pU29 derivative containing the mutagenized *puf* operon. The correct construction was verified by restriction analysis. Antisense oligonucleotides were synthesized by using an Applied Biosystems 381A DNA synthesizer.

Phenotypic Assays. RC mutations carried on pU2922 derivatives maintained in *E. coli* strain S17-1 (18) were conjugated into *R. capsulatus*. *R. capsulatus* deletion strain U43 (which lacks the RC, light harvesting I, and light harvesting II complexes) served as the background (9) for the determination of mutant phenotypes. *R. capsulatus* cultures were grown semiaerobically in supplemented RCV medium (21). Photosynthetic growth assays were conducted by spotting mutants on PYE-rich medium (21) supplemented with 2 mM $MgCl_2$ and 2 mM $CaCl_2$ and testing for growth within an anaerobic jar immersed in a water tank at 32°C. The tank was illuminated with two 500-W quartz-halogen lamps, which provided a light intensity of approximately 20 mW/cm^2.

RC Preparation and Analysis. RCs were purified with a previously described DEAE chromatography method (22) with the following modifications. Chromatophores with RCs containing L-subunit mutations were diluted to an A_{875} of 7–8 and were solubilized in 0.3% lauryldimethylamine oxide (LDAO). The 0.6% LDAO wash step was eliminated and was replaced with a 0.05% LDAO wash. Columns were eluted in the cold room.

Optical spectra were recorded with a Perkin–Elmer Lambda diode array model 3840 spectrometer linked to a Perkin–Elmer model 7300 workstation. Flash-induced optical absorbance changes were measured as described previously (23). RCs in 10 mM potassium phosphate, pH 7.4/10 mM KCl/0.05% LDAO were illuminated with a brief flash of light (15 μs at half height), and the recovery of the bleaching of the primary donor was observed at 850 nm. The addition of 1 mM prometon (2-methoxy-4,6-bis[isopropylamino]-*s*-triazine) prevented electron transfer to the secondary quinone. The recovery could be fit with a single exponential for each sample. RC pigment content was determined by analyzing the near infrared pigment bands after the extraction of RCs with acetone/methanol, 7:2 (vol/vol). The procedure was similar to those used previously (24, 25). Since absolute extinction coefficients have not been determined for *R. capsulatus* RCs, the *R. sphaeroides* value of 288 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient at 802 nm (24) was used for the determination of total pigment content. The extinction coefficients necessary for determination of the pigment ratios have been reported previously for Bchl and Bphe in organic solvents (25).

RESULTS

RC Assembly. The axial histidine ligands of the special pair Bchls were changed by site-directed mutagenesis (Table 1). His^{M200} was changed to glutamine, leucine, or phenylalanine, whereas His^{L173} was changed to glutamine. A double mutation was constructed in which both histidines were replaced with glutamine ($His^{L173,M200} \rightarrow$ Gln). Absorption spectra of chromatophore membranes from each of these mutants show that in each case the RC is stably assembled (data not shown). In the light harvesting II-deficient background strain U43, RC absorption at 750 nm and 800 nm is clearly seen in addition to light harvesting I absorption at 875 nm.

RCs were purified from chromatophore membranes of each mutant. RCs containing mutations at His^{L173} deteriorate during purification. A reduction in the RC 850-nm absorption band is observed in preparations from the $His^{L173} \rightarrow$ Gln and $His^{L173,M200} \rightarrow$ Gln mutants when their chromatophores are solubilized in 1.5% LDAO. RCs similar to the wild type can be obtained from these two mutants by reducing the LDAO concentration used for solubilization, but the 850-nm absorption band is unstable and disappears after brief storage of the samples at 4°C.

Ground-State Absorption Spectra. The room temperature absorption spectra of RCs isolated from each of the mutants are shown in Fig. 1. The Soret bands of these RCs (data not shown) are very similar to the wild type. The lowest-energy singlet electronic transitions of the RC chromophores (Q_y region) near 750, 800, and 850 nm are assigned predominantly to the Bphe Q_y transitions, the monomeric Bchl Q_y transitions, and the special pair Bchl Q_y transitions, respectively. Spectra from RCs containing glutamine substitutions resemble the absorption spectrum from wild-type RCs. The long wavelength absorption band in $His^{M200} \rightarrow$ Gln appears to be slightly blue-shifted. At 77 K, the shoulder at 590 nm on the wild-type Bchl Q_x band is much more pronounced in the Bchl Q_x band of $His^{M200} \rightarrow$ Gln (Fig. 2).

The absorption spectra of the $His^{M200} \rightarrow$ Leu and $His^{M200} \rightarrow$ Phe RCs show significant differences from the wild type (Fig. 1). The special pair absorption band at 850 nm, most or all of which has been lost in the purified RCs from these two mutants, is replaced by a broad, diffuse band. The monomeric Bchl Q_y band is blue-shifted $\approx$5 nm in these RCs. Although the ratio of the Bphe Q_y band to the monomeric

Table 1. Directed mutations of the axial histidine ligands of the special pair Bchls of the RC

Mutation	Nucleotide change	Bchl/Bphe ratio	Pigment content	Photosynthetic growth	Rate of charge recombination between primary donor and primary quinone
None		1.9 ± 0.1	5.8 ± 0.2	+	$(150\ ms)^{-1}$
$His^{M200} \rightarrow$ Leu	CAC → CTC	1.0 ± 0.1	5.7 ± 0.1	−	$(97\ ms)^{-1}$
$His^{M200} \rightarrow$ Phe	CAC → TTC	1.1 ± 0.1	5.8 ± 0.2	−	$(93\ ms)^{-1}$
$His^{L173} \rightarrow$ Gln	CAC → CAG	1.9 ± 0.1	5.5 ± 0.1	+	$(150\ ms)^{-1}$
$His^{M200} \rightarrow$ Gln	CAC → CAA	1.9 ± 0.1	5.8 ± 0.2	+	$(160\ ms)^{-1}$
$His^{L173,M200} \rightarrow$ Gln		2.0 ± 0.1	ND	+	$(140\ ms)^{-1}$

Nomenclature such as $His^{M200} \rightarrow$ Leu includes the wild-type residue (histidine), the subunit and position of this residue (amino acid number 200 of the M subunit), and the newly incorporated residue (leucine). The nucleotide sequences given are of the mutagenized codon. Pigment extraction data reported is the average of a minimum of three trials. Concentration of $His^{L173,M200} \rightarrow$ Gln RCs was too dilute for reliable determination of total pigment. Mutants that grew within 2 days under the conditions described in *Materials and Methods* were considered photosynthetically competent. The charge recombination rates correspond to the recovery of RC absorbance at 850 nm in the presence of 1 mM prometon (a triazine family herbicide) after illumination with a brief flash of light. The herbicide blocks electron transfer from the primary quinone anion to the secondary quinone. Data for wild-type and $His^{M200} \rightarrow$ Leu RCs are presented in Fig. 4. ND, not determined.

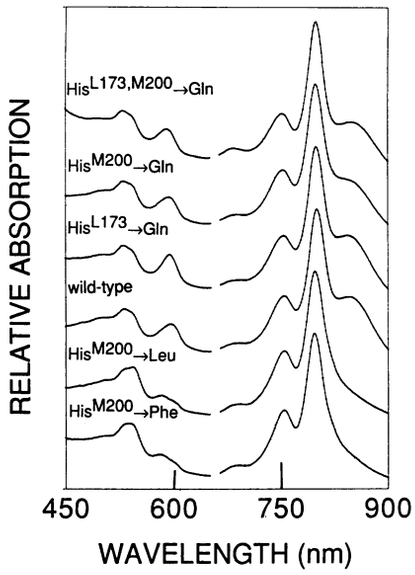

FIG. 1. Room temperature ground-state absorption spectra of genetically altered RCs. The spectrometer changes filters at 650 nm. Although these absorption bands are reasonably attributed to specific transitions, the close proximity of the six pigments probably results in the mixing of their optical transitions (26).

Bchl Q_y band is similar to the wild type in these two RCs, the Q_x transitions are very different. The Bchl Q_x bands are reduced in magnitude, and the Bphe Q_x bands are increased in magnitude relative to the corresponding bands in wild-type RCs. At 77 K, the Bchl Q_x region consists of two well-defined absorption bands near 580 and 600 nm, while the Bphe Q_x band at 545 nm is approximately twice as large as the Bphe Q_x band at 531 nm (Fig. 2). This is reminiscent of the Q_x region of RCs from *Chloroflexus aurantiacus* (28), in which one of the RC Bchls is replaced with Bphe. This suggests that

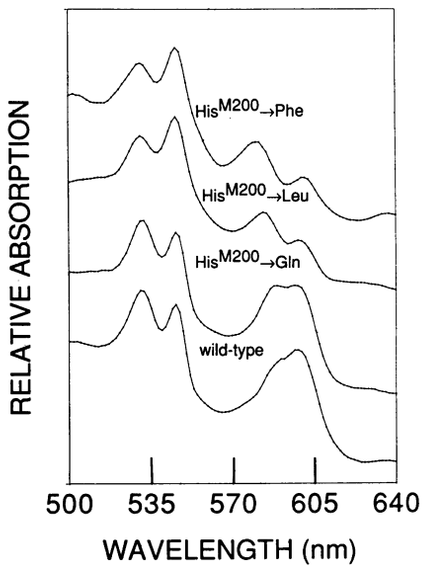

FIG. 2. Low-temperature (77 K) absorption spectra in the Q_x region of purified RCs with amino acid substitutions at M200. The samples contain 50% (vol/vol) glycerol. The absorbance bands near 540 nm can be attributed to the Bphe Q_x transitions, and those near 600 nm can be attributed to the Bchl Q_x transitions. The spectral changes and band splitting of the Bchl Q_x transitions may reflect changes in the coordination of the central Mg^{2+} ion of RC Bchl in some of these RCs, since previous studies of monomeric Bchl-*a in vivo* have shown that the position of the Bchl Q_x transition depends on whether the central Mg^{2+} ions are pentacoordinate or hexacoordinate (27).

Bphe replaces one of the Bchls in the His^{M200} → Leu and His^{M200} → Phe RCs. As previously reported,† the primary donor in His^{M200} → Leu and His^{M200} → Phe RCs may now consist of a Bchl–Bphe heterodimer.

RC Composition. Pigment extractions were performed on RCs from these five mutations and the wild type to determine their pigment content. The Bchl/Bphe ratios and the total pigment content for these RCs are listed in Table 1. The total pigment content of the wild-type and mutant RCs is in good agreement with the expected value of 6. However, the values are consistently about 5% low. This reduction may be due to differences between the value of the absolute extinction coefficient of wild-type *R. sphaeroides* used in these determinations and the actual extinction coefficients from wild-type or mutant *R. capsulatus* RCs.

The Bchl/Bphe pigment ratios of wild-type and glutamine-substituted RCs are in good agreement with the expected Bchl/Bphe ratio of 2. Since the total pigment analysis gives a value of approximately 6, we conclude that they contain four Bchls and two Bphes. A Bchl/Bphe ratio of 1 is obtained from RCs of His^{M200} → Leu and His^{M200} → Phe. Taken together with measurements of their total pigment content, these results clearly indicate that these two RCs each contain three Bchls and three Bphes. Since the mutations are directed at the axial ligand of the special pair Bchl predominantly associated with the M subunit ($Bchl_M$) and the changes in the absorption spectra affect bands associated with the special pair, the data indicate that His^{M200} → Leu and His^{M200} → Phe RCs each contain a heterodimer in which $Bchl_M$ of the special pair is replaced with a Bphe. Experiments are underway to determine if these heterodimers exist in the membrane *in vivo*.

Electron Transfer. The His^{L173} → Gln, His^{M200} → Gln, and His^{L173,M200} → Gln mutants are all photosynthetically competent, which indicates that they contain functional RCs. Oxidized-minus-reduced difference spectra from these glutamine-substituted RCs are very similar to the wild type (Fig. 3). Their rates of charge recombination between the special pair cation and the primary quinone anion are also similar to the rate determined for wild-type RCs (Table 1).

RCs whose primary donor consists of a heterodimer of Bchl and Bphe are photochemically active. Although the mutations His^{M200} → Leu and His^{M200} → Phe result in the loss of photosynthetic growth (Table 1), these RCs exhibit reversible bleaching of their long wavelength absorption bands by actinic light. The rate of charge recombination between the oxidized heterodimer and the primary quinone anion has increased in His^{M200} → Leu and His^{M200} → Phe RCs relative to the wild type (Table 1 and Fig. 4). Horse heart cytochrome *c* reduces the oxidized heterodimer species of His^{M200} → Leu RCs *in vitro* with kinetics similar to those observed with wild-type RCs (Fig. 4). The oxidized-minus-reduced difference spectra from these two RCs (Fig. 3) are similar to the wild-type spectrum between 700 and 820 nm. However, the bleaching of the Bchl bands at 600 and 850 nm is lost in the heterodimer spectra. A broad tail between 820 and 900 nm is now present in their difference spectra. In addition, there appears to be a small bleaching component centered at 548 nm.

DISCUSSION

Properties of the Heterodimer. We have shown that RCs whose primary donor consists of a Bchl–Bphe heterodimer,

†Bylina, E. J. & Youvan, D. C., North Atlantic Treaty Organization Advanced Research Workshop on the Structure of the Photosynthetic Bacterial Reaction Center: X-Ray Crystallography and Optical Spectroscopy with Polarized Light, September 20–25, 1987, Cadarache, France.

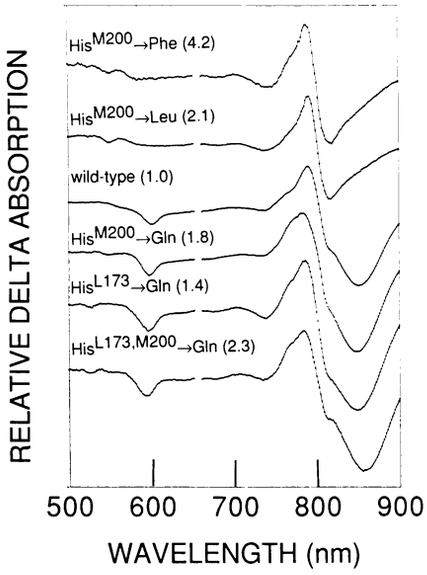

FIG. 3. Oxidized-minus-reduced (light-minus-dark) absorption difference spectra of purified RCs at room temperature. Light-induced oxidized spectra were measured under constant illumination. The numbers in parentheses indicate the expansion factors used for each of the difference spectra relative to the wild type after normalization of the 800-nm Bchl Q_y transition in the dark spectra. These spectral differences are due to both the loss of absorption bands of the primary donor after its oxidation and electrochromic effects on the other pigments (29). Assignment of the absorption changes in the difference spectrum leads to an understanding of molecular events that occur during charge separation.

which results from the replacement of $Bchl_M$ of the special pair with Bphe, are photochemically active and possess an accelerated back reaction from the primary quinone anion to the oxidized primary donor. The replacement of the special pair by a heterodimer has a significant effect on the RC ground-state absorption spectrum. Colonies containing the $His^{M200} \rightarrow$ Leu mutation have enhanced light harvesting I fluorescence (data not shown). This increased fluorescence may be due to a reduced quantum yield in heterodimer-containing RCs, a large number of closed RCs in the membrane, or an uncoupling of the light-harvesting complexes from the RC. *R. capsulatus* strains bearing these heterodimer-containing RCs are unable to grow photosynthetically. This inability to grow photosynthetically in spite of *in vitro* RC activity has been observed in other RC mutations (unpublished results) and may be due to pleiotropic membrane effects, such as the improper insertion of the RC complexes into the membrane or a disruption of RC interactions with the other protein complexes involved in the photosynthetic apparatus.

A comparison of the oxidized-minus-reduced difference spectra of $His^{M200} \rightarrow$ Leu and $His^{M200} \rightarrow$ Phe RCs with wild-type RCs reveals interesting characteristics of the heterodimer-containing RCs. Both RCs have a derivative-like feature centered around 800 nm, which in wild-type RCs has been ascribed to an electrochromic blue shift on the monomeric Bchls upon the formation of the oxidized primary donor (30, 31). Difference spectra of these RCs appear to contain a bleaching band similar to the wild type near 815 nm, which has been ascribed to the high-energy exciton component of the primary donor. This is somewhat surprising, considering that the 850-nm band, ascribed to the low-energy exciton component of the primary donor, is drastically altered and that the Q_x excitonic components of the primary donor appear to be absent in the 600-nm region. Because the Q_y band of the heterodimer is considerably broadened and more diffuse than the corresponding band in wild-type RCs, the Q_x transition of the remaining Bchl in the heterodimer may also be extensively broadened, making it difficult to detect. This broadening may explain both the loss of a

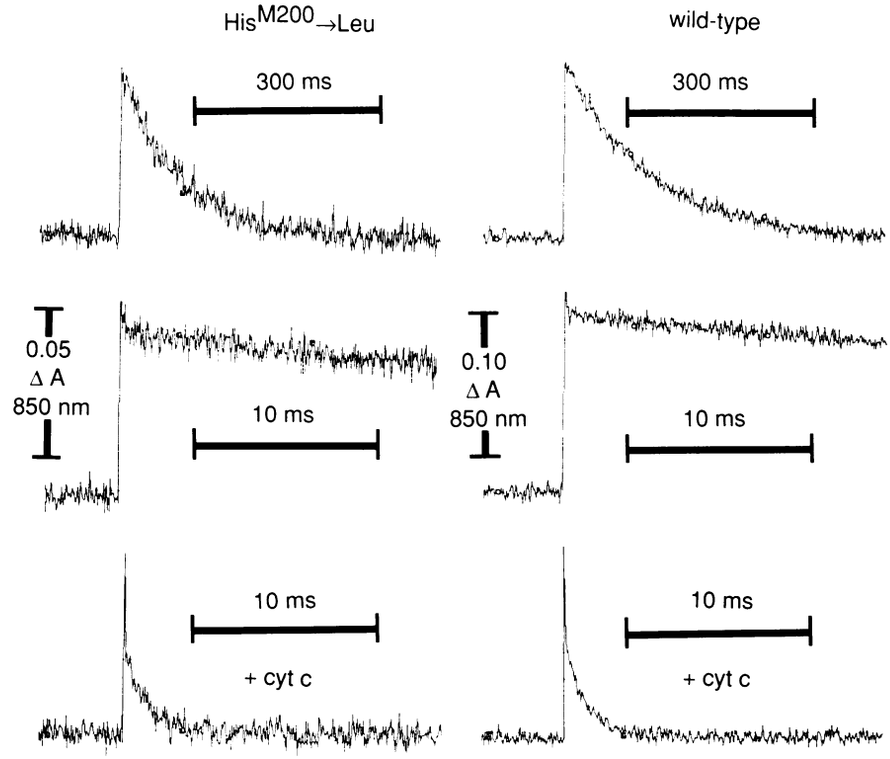

FIG. 4. Reduction of the oxidized primary donor with exogenous cytochrome *in vitro*. RCs (A_{800} = 0.3) in 10 mM potassium phosphate, pH 7.4/10 mM KCl/1 mM prometon/0.05% LDAO were illuminated with a brief flash of light in the presence and absence of 20 μM horse heart cytochrome *c*. The reduction of the oxidized primary donor was observed at 850 nm. This reduction in the presence of cytochrome consists of a slow ($t_{1/2} \approx 1$ ms) and fast component ($t_{1/2} < 50$ μs) in both wild-type and $His^{M200} \rightarrow$ Leu RCs.

bleaching band at 600 nm and the large absorption decrease in the Bchl Q_x region of these RCs, which is greater than would be expected for the loss of a single Bchl from the RC. Similar broadening is not observed in the Bphe Q_x region, which contains a bleaching band at 548 nm. Linear dichroism experiments will better characterize these optical transitions involving the Bchl–Bphe heterodimer and indicate if the orientation of the pigments has been changed in these RCs.

The properties of a primary donor consisting of a Bchl–Bphe heterodimer may be considerably altered relative to a Bchl–Bchl homodimer. In view of the different redox properties of Bchl and Bphe (32), we might expect asymmetry in the distribution of electron density in the heterodimer. Since Bchl is easier to oxidize than Bphe, the positive charge of an oxidized heterodimer may be principally localized on the Bchl half of the heterodimer. This type of charge asymmetry would lead to an increase in the monomeric character of the primary donor, which would result in a more positive midpoint potential and a broadened EPR linewidth of the oxidized heterodimer.

Specificity of Tetrapyrrole Binding. The Bchl axial ligand mutations presented here have begun to define the structural parameters that determine whether Bchl or Bphe will be bound to a particular site. Bchl and Bphe are incorporated into the RC in a structurally specific manner, even though these two species differ only by the central Mg^{2+} ion that is found in Bchl and is replaced with two protons in Bphe. In the crystal structure, the histidine residues that act as the axial ligands to the Mg^{2+} ions of the Bchls are absent in the corresponding positions in the Bphe binding sites (14). The mutations at M200 indicate that replacing the axial histidine ligand to one of the special pair Bchls with a hydrophobic residue, which has no lone pairs of electrons available for Mg^{2+} coordination (i.e., leucine or phenylalanine), is enough to change the tetrapyrrole species found at this site. A leucine residue (M212) points toward the center of the Bphe predominantly associated with the L subunit. Introducing histidine in place of this leucine residue may result in the replacement of the Bphe with Bchl.

Photosystem II. The core of the photosystem II reaction center is very similar in structure to the bacterial RCs of the *Rhodospirillaceae*. Sequence comparisons have suggested that the D1 and D2 proteins of photosystem II are analogous to the L and M subunits of photosynthetic bacteria (33). Functionally important amino acids residues are conserved throughout these subunits (34). A photochemically active photosystem II RC complex has been isolated, which contains only D1, D2, and cytochrome *b*-559 (35). However, structural differences do exist between these two complexes. Whereas the axial histidine ligands of the Bchl dimer from bacterial RCs are conserved in D1 (His^{198}) and D2 (His^{198}), the axial histidine ligands of the monomeric Bchls (L153 and M180) from bacterial RCs are not conserved. Our results show that histidine is not required for binding the primary donor Bchls in RCs. Mutagenesis of the axial histidine ligands of the accessory Bchls in bacterial RCs indicates that these histidines can also be replaced with other amino acid residues (unpublished results). We propose that the two chlorophyll *a* molecules of the photosystem II RC that are not bound to His^{198} of D1 and His^{198} of D2 are associated with non-histidine residues in the regions of D1 and D2 that connect the postulated C and D helices (36) in each of the subunits.

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