Site-Directed Mutations Near the L-Subunit D-Helix of the Purple Bacterial Reaction Center: A Partial Model for the Primary Donor of Photosystem II†

W. J. Coleman,*,‡,§ T. A. Mattioli,|| D. C. Youvan,⊥ and A. W. Rutherford‡

Section de Bioénergétique and Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, CEA CNRS URA 2096, Centre d'Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France, and KAIROS Scientific Inc., Building 62, 3350 Scott Boulevard, Santa Clara, California 95054

Received September 29, 1996⊗

ABSTRACT: We have engineered a photosynthetically competent mutant of the purple non-sulfur bacterium *Rhodobacter capsulatus* which seeks to mimic the behavior of the primary electron donor (P) of the plant photosystem II (PS II) reaction center (RC). To construct this mutant (denoted D1-ILMH), four residues in the bacterial L subunit were mutagenized, such that an 11-residue segment was made identical to the analogous segment from the D1 subunit of PS II. The electronic properties of the bacteriochlorophyll (Bchl) dimer which constitutes the primary donor are substantially altered by these modifications, to the degree that the dimer becomes functionally much more "monomeric". The changes include (1) an increase in the values of the zero-field splitting (ZFS) parameters, as measured by electron paramagnetic resonance (EPR), for the spin-polarized triplet state, ^{3}P, from $|D| = 185 \times 10^{-4}$ cm^{-1} and $|E| = 31 \times 10^{-4}$ cm^{-1} in wild-type (WT) chromatophore membranes to $|D| = 200 \times 10^{-4}$ cm^{-1} and $|E| = 44 \times 10^{-4}$ cm^{-1} in the mutant and (2) an increase in the EPR line width of the oxidized state, P^+, from 0.97 mT in WT to 1.09 mT in D1-ILMH RCs. However, unlike the PS II primary donor (P680), the orientation of ^{3}P in the D1-ILMH mutant is the same as in WT bacteria and does not display the unusual orientation found for PS II. And whereas the redox couple P/P^+ has a very high midpoint potential in PS II, P/P^+ in the D1-ILMH mutant has a *lower* midpoint (90 mV more negative) than in WT *Rb. capsulatus*. In addition, Raman measurements indicate that the hydrogen bond between His^{L168} and the C_2 acetyl carbonyl oxygen of the Bchl on the active electron transfer pathway (P_A) is absent in the mutant, due to the fact that His^{L168} in the WT sequence has been replaced by a leucine in D1-ILMH. However, the Raman data also reveal the presence of a new hydrogen bond in the D1-ILMH RCs, between the C_9 keto carbonyl oxygen of P_A and an unknown hydrogen-bond donor. Thus, although the protein environment around one of the Bchls of the special pair is significantly changed in D1-ILMH, the chimeric RC does not, as a result of these changes, have a primary donor that is oriented like the one in PS II.

Photosynthetic energy conversion in cyanobacteria and plants begins with a photoinduced charge separation inside the membrane-bound pigment−protein complex known as photosystem II (PS II)[1] [for a review, see Diner et al. (1991)]. As in the bacterial reaction center (RC), a series of electron transfer reactions within the core of PS II stores the absorbed light energy by creating an oxidized chlorophyll species ($P680^+$) and a reduced quinone. The component P680 (Döring et al., 1967) initiates the charge separation step and thus is designated as the primary electron donor. The transient species $P680^+$ is re-reduced to P680 by removing an electron from water, a cyclic reaction which ultimately evolves molecular oxygen (Debus, 1992).

P680 is one of the fundamental catalysts of light to chemical energy conversion in plants, but little is known about the chemical nature of the P680 chlorophyll(s), except for the fact that among all of the naturally occurring biological redox compounds, the couple $P680/P680^+$ is a very powerful oxidant. Having an estimated midpoint redox potential of at least +1.0 V (Jursinic & Govindjee, 1977; Klimov et al., 1979), it is capable of oxidizing water to molecular oxygen in a four-electron reaction (+0.8 V/electron). The reason for the exceptionally high redox potential of the PS II primary donor is still not known, but it has been attributed to an effect of the protein environment around the Chl(s) (van Gorkom & Schelvis, 1993). However, even in organic solvents Chl *a* is not as oxidizing as it is in PS II [see Watanabe and Kobayashi (1991)]. The putative analog to P680 in the purple bacterial RC is a bacteriochlorophyll (Bchl) dimer having an E_m between +0.44 and +0.55 V (Prince & Dutton, 1978; Moss et al., 1991; Williams et al., 1992; Nagarajan et al., 1993; Jia et al., 1994), which means it is not an unusually strong oxidant.

One possible reason for the electrochemical difference between P680 and its bacterial RC counterpart (aside from

† This work was supported by NIH RO1 GM42645 (D.C.Y.), U.S. Department of Energy, Division of Energy Biosciences DE-FG03-96ER20211 (D.C.Y.), a National Science Foundation Postdoctoral Fellowship (W.J.C.), and a Human Frontier Science Program Long-Term Research Fellowship (W.J.C.).

* Author to whom correspondence should be addressed. E-mail: wcoleman@kairos-scientific.com.

‡ Section de Bioénergétique.

§ Present address: KAIROS Scientific Inc.

|| Section de Biophysique des Protéines et des Membranes.

⊥ KAIROS Scientific Inc.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: P680, primary donor of photosystem II; PS II, photosystem II; RC, reaction center; Bchl, bacteriochlorophyll; Chl, chlorophyll; Bphe, bacteriopheophytin; EPR, electron paramagnetic resonance; ZFS, zero-field splitting; $P_{A/B}$, special-pair bacteriochlorophyll on the A/B branch; LH I/II, light-harvesting complex I/II; Q_A, primary quinone; LDAO, lauryldimethylamine *N*-oxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

S0006-2960(96)02458-0 CCC: $14.00

chemical differences inherent to the chlorin rings) is that the PS II primary donor appears to involve Chls that are much less strongly interacting than those in the bacterial RC. The excitonic coupling between the pigments in the PS II RC has been studied by means of optical and magnetic resonance (EPR) spectroscopy, monitoring various states of the primary donor (e.g., P, P*, P^+, and ^3P) [see, e.g., Lösche et al. (1988), Tentenkin et al. (1989), Braun et al. (1990), He et al. (1990), van Kan et al. (1990), van Mieghem et al. (1991), van der Vos et al. (1992), Carbonera et al. (1994), Kwa et al. (1994), Schelvis et al. (1994), Durrant et al. (1995), and Hillman et al. (1995)].

Another feature of the primary donor which is unique to PS II is the orientation of the P680 triplet state. Within the structure of the purple bacterial RC, the two Bchls of the dimer can be denoted as P_A (associated with the L subunit) and P_B (associated with the M subunit). Measurements of the triplet in oriented samples of purple bacterial membranes (Hales & Das Gupta, 1979; Tiede et al., 1988) showed that the Bchl(s) on which the triplet resides is/are oriented perpendicular to the plane of the membrane, and this orientation was confirmed by the results of X-ray crystallography (Michel et al., 1986a,b). Low temperature (4K) EPR studies of the triplet in single crystals of *Rhodopseudomonas viridis* RCs indicated that the triplet resides primarily on the P_A side of the dimer (Norris et al., 1987). In PS II preparations under comparable conditions, however, the triplet is completely localized on a Chl that has been shown to be oriented nearly parallel (at an angle of 30°) relative to the plane of the membrane (Rutherford, 1985; van Mieghem et al., 1991). It is difficult to reconcile this result with the apparent homology between PS II and the bacterial RC, since RCs that have similar primary, secondary, and tertiary structure would be expected to have the same or similar orientations for the pigments. One solution to this paradox could be that the PS II triplet state does *not* reside on the Chls that are equivalent to the bacterial special pair but that it lies instead on one of the monomeric (accessory) Chls. The latter are oriented at approximately 30° relative to the special-pair Bchls in the bacterial RC. If the monomeric Chls maintain the same orientation in PS II, then this hypothesis could perhaps explain the "anomalous" orientation of the PS II triplet (Rutherford, 1986, 1988; van Mieghem et al., 1991). Another possibility is that the three-dimensional structure of the PS II protein in the vicinity of the special pair differs enough from the bacterial structure that it turns one or both of the Chls by 60° relative to the bacterial RC (Rutherford, 1986; van Mieghem et al., 1991; Noguchi et al., 1993).

At the present time, our understanding of the PS II primary donor is limited by the lack of information about the three-dimensional structure of the protein. However, the structure of the simpler anoxygenic bacterial RC (with which PS II may have shared a common ancestor; Rutherford & Nitschke, 1996) is believed to be generally analogous to that of PS II (Trebst, 1986; Coleman & Govindjee, 1987; Michel & Deisenhofer, 1988) and is known to atomic resolution from X-ray crystallography (Michel et al., 1986a,b; Deisenhofer et al., 1995). This simpler system is also quite amenable to site-directed mutagenesis and protein purification (for the *Rhodobacter capsulatus* system, see Coleman and Youvan, 1990). Taking advantage of the high degree of local sequence identity between the two RC proteins, we have modified the *Rb. capsulatus* RC so that several contiguous

	168					173				
1) D1-ILMH (PS II)	N	I	**L**	M	H	P	F	H	M	L	G
2) *Rb. capsulatus* WT	N	F	H	Y	N	P	F	H	M	L	G
3) *Rb. capsulatus* $His^{L173} \rightarrow$Leu	N	F	H	Y	N	P	F	**L**	M	L	G
4) *Rsp. rubrum* WT	N	F	H	Y	N	P	A	H	M	L	G
5) *Rb. sphaeroides* WT	N	F	H	Y	N	P	A	H	M	I	A
6) *Rx. gelatinosus* WT	H	F	H	Y	N	P	A	H	M	L	A
7) *Erythrobacter* WT	H	F	H	Y	N	P	A	H	M	L	A
8) *Rps. viridis* WT	N	W	H	Y	N	P	G	H	M	S	S
9) *C. aurantiacus* WT	N	F	**F**	Y	N	P	F	H	A	I	G
10) *Rb. sphaeroides* $His^{L168} \rightarrow$Phe	N	F	**F**	Y	N	P	A	H	M	I	A

FIGURE 1: Sequence comparison of the reaction center L-subunit region surrounding P_A in the *Rb. capsulatus* D1-ILMH mutant, several wild-type bacterial species, and two other bacterial mutants. P_A is one of a pair of bacteriochlorophylls that constitute the primary electron donor. His^{L168} has been shown to be capable of forming an H-bond to the C_2 acetyl carbonyl of P_A. His^{L173} provides the axial ligand to the P_A Bchl. Sequence differences at these two positions are highlighted in boldface type. Sequences are from the following sources: (1) This work and Metz et al. (1990), (2) Youvan et al. (1984), (3) Bylina and Youvan (1990), (4) Bélanger et al. (1988), (5) Williams et al. (1984), (6) Nagashima et al. (1994), (7) Liebetanz et al. (1991), (8) Michel et al. (1986b), (9) Ovchinnikov et al. (1988), and (10) Murchison et al. (1993). The FHYN sequence in WT *Rb. capsulatus* was changed to the corresponding ILMH sequence found in the D1 subunit of WT PS II by site-directed mutagenesis.

residues in the vicinity of one of the Bchls of the primary donor (P_A) have been replaced by the analogous residues taken from the consensus sequence of PS II. The X-ray crystal structure of the purple bacterium *Rps. viridis* indicates that the L-subunit polypeptide (equivalent to the D1 subunit in PS II) makes extensive contacts with the chromophores of the "active" electron transfer pathway of the RC (Michel et al., 1986a,b; Deisenhofer et al., 1995). In particular, a small region of the L-subunit D-helix covers the protein-exposed face of P_A. A single histidine residue (His^{L173}, or residue number 173 of the L subunit) forms a coordinate bond with the central magnesium atom of the Bchl (P_A). The other nitrogen atom within the imidazole ring of this histidine appears to form a hydrogen bond to a bound water molecule. The imidazole side chain of another nearby histidine (His^{L168}) is hydrogen-bonded to the C_2 acetyl carbonyl group of P_A and to a second nearby water molecule (Deisenhofer et al., 1995).

Although the (B)chl-binding residue appears to be conserved between bacteria and plants, there are nevertheless interesting differences between the two systems in the protein segment that surrounds P_A and the water molecule bound to His^{L173}. In order to explore the possible consequences of this difference in the sequence, we have mutagenized (as a group) four amino acid residues in this portion of the bacterial L subunit, in order to generate the conserved 11-residue sequence found in the plant D1 subunit (Figure 1). The goal of the protein engineering experiment described here is to determine how the environment around the Chls contributes to the unusual chemical properties of the primary donor of PS II. We have used the *Rb. capsulatus* RC as a scaffold on which to build a partial PS II-like structure, in order to see whether the spectroscopic properties and other functional aspects of the RC are correspondingly transformed. By using this type of "evolutionary engineering" it may be possible

to assign some of the novel functional characteristics of PS II to very specific regions of the protein.

MATERIALS AND METHODS

Mutant Construction. In vitro oligonucleotide-mediated mutagenesis was performed according the method of Kunkel et al. (1987) using the Bio-Rad Muta-Gene kit. A 61-mer antisense oligonucleotide containing six mismatches was used to replace four residues simultaneously. The mutagenic oligonucleotide had the following sequence (six mismatches which generate the I-L-M-H sequence are underlined): 5′ CAG GCT GAT GCC CAG CAT GTG GAA CGG GTG CAT GAG GAT GTT ACC GTA GGT GTA GCC GGT G 3′. Inserting the 4-bp CATG sequence in the Leu^{L166} and Met^{L167} codons also introduces a new *Nla*III restriction site which was used to screen the products of the mutagenesis reactions. The mutated *Hind*III−*Kpn*I (L subunit) fragment from M13 was then cloned into plasmid pU2925 (Robles et al., 1990), and this plasmid was used to complement the *Rb. capsulatus* U43 *puf* deletion background (Youvan et al., 1985). (The D1-ILMH construction in pU2925/U43 is LH I^- LH II^-.) After repurification of the mutant strain, the plasmid pU2925 was extracted and the L- and M-subunit genes were subcloned into the appropriate M13 vectors for dideoxy sequencing (Sanger et al., 1977). The entire L- and M-subunit genes were analyzed to ensure that the sequence was correct throughout both polypeptides.

Photosynthetic Growth Test. Spot assays for photosynthetic growth were performed according to the methods described by Robles et al. (1990) and Bylina et al. (1989). The light intensity was approximately 20 mW cm^{-2}. Cells were spotted onto MPYE (malate/peptone/yeast extract) plates (Weaver et al., 1975) and grown in anaerobic bags at 32 °C.

Bacterial Culture and Chromatophore and Reaction Center Preparation. Cells of both WT and the D1-ILMH mutant were grown aerobically in the dark (Prince & Youvan, 1987) in a mixture of $^1/_3$ MPYE and $^2/_3$ RCV (Weaver et al., 1975) media. Chromatophore membranes and RCs were isolated according to previously published methods (Robles et al., 1990; Prince & Youvan, 1987), with the exception that oriented chromatophores were resuspended in MOPS-based buffer (pH 7.0). All other preparations were resuspended in HEPES-based buffer (pH 8.0).

Optical Spectroscopy and P/P^+ Redox Titration. Optical absorption spectra of RCs were obtained on a Perkin-Elmer 3840 diode array spectrophotometer. Kinetics of the $P^+Q_A^-$ → PQ_A recombination reaction were measured at room temperature as described previously (Prince & Youvan, 1987), except that 0.1 mM prometryne [2,4-bis(isopropylamino)-6-(methylthio)-1,3,5-triazine] was used in place of ametryne. Chemical titration of D1-ILMH and WT chromatophores to determine the midpoint redox potential of the primary donor was performed in an anaerobic cuvette using a method similar to that described in Dutton (1971). The fraction of oxidized centers was measured by optically monitoring the sample in a time-resolved laser flash absorption apparatus using a germanium diode to detect the absorption difference between 1250 and 1260 nm (Mathis & Setif, 1981). The measuring electrode was calibrated using a saturated quinhydrone solution at pH 7. Titrations were done in both the reductive and oxidative directions to make certain that the reaction was fully reversible.

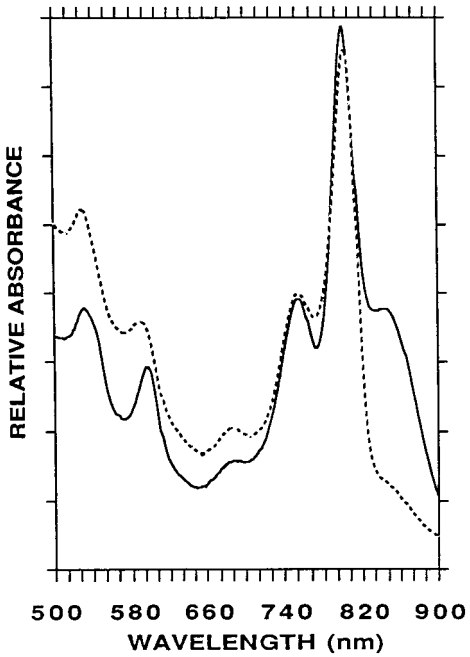

FIGURE 2: Room-temperature ground-state electronic spectra of wild-type (—) and D1-ILMH (- - -) reaction centers from *Rb. capsulatus*. Spectra were recorded with 1 mM sodium ascorbate added to the buffer medium (20 mM HEPES, pH 8.0, and 0.05% LDAO).

EPR Spectroscopy. Chromatophores for P^+ measurements were put into 3-mm (i.d.) quartz tubes and frozen in the presence of sodium ascorbate. EPR spectra were recorded on a Bruker ER200D X-band spectrometer equipped with an Oxford Instruments liquid helium cryostat. Partially oriented chromatophores for EPR were dried onto mylar sheets as described by van Mieghem et al. (1991). The mylar sheets were submerged in buffer containing 400 mM MOPS (pH 7.0), 100 mM sodium dithionite, 1 mM EDTA, and 70% glycerol. Samples were illuminated in the EPR cavity by the method described by van Mieghem et al. (1991).

Raman Spectroscopy. Spectra were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module (Mattioli et al., 1991). The excitation source was a diode-pumped Nd−YAG laser delivering 180 mW of continuous 1064-nm radiation. Reaction centers were poised in their P neutral (fully reduced) state using ascorbate. RC samples (ca. 100 OD at 800 nm) were contained in a sapphire cell holder and their spectra were recorded at room temperature. Spectral resolution is 4 cm^{-1}.

RESULTS

Photosynthetic Growth. Rb. capsulatus cells containing the D1-ILMH mutations were found to be photosynthetically competent when grown on plates at high light intensity. The mutant grows at a rate roughly comparable to that of WT. Reaction centers can be isolated with a good yield from the mutant using LDAO as the solubilizing detergent, and they are stable in solution at room temperature.

Optical Measurements. Comparison of the ground-state electronic spectra of the D1-ILMH and WT RCs at room temperature in the presence of 1 mM ascorbate (Figure 2) shows that the intensity of the Q_Y transition of P is greatly reduced in the D1-ILMH mutant. The wavelength maximum of the Q_Y transition is also blue-shifted by about 4 nm to approximately 846 nm. (It should be noted that, in WT *Rb. capsulatus* RCs isolated in LDAO, the Q_Y transition is at

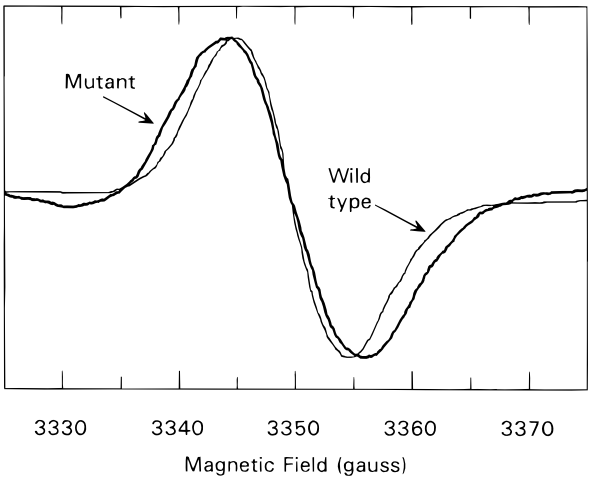

FIGURE 3: EPR spectra (15 K) of P^+ in chromatophores of wild-type (thin trace) and the D1-ILMH mutant (thick trace). Spectra were recorded under continuous illumination at 15 K in buffer containing 20 mM HEPES (pH 8.0), 30% ethylene glycol, 1 mM sodium ascorbate, and 1 mM EDTA. Spectrometer settings were as follows: microwave power, 35 dB (0.063 mW); modulation amplitude, 2 G; microwave frequency, 9.42 GHz; time constant, 80 ms.

850 nm.) The band corresponding to the unresolved Q_Y transitions of the monomeric Bchls is slightly red-shifted to about 802 nm. The band corresponding to the Q_Y transitions of the bacteriopheophytins is unaffected by the mutations. The Q_X transition of the Bchls is blue-shifted by about 6 nm to 594 nm. In the near-IR region of the spectrum, the P^+ cation band is slightly shifted from 1250 nm in WT to 1254 nm in chromatophores from the mutant (data not shown). The rate of $P^+Q_A^- \rightarrow PQ_A$ charge recombination in RCs at room temperature [$(110\ ms)^{-1}$; data not shown] is slightly slower than the rate for WT RCs [$(80\ ms)^{-1}$); Prince & Youvan, 1987].

EPR Measurements. Measurement of the EPR line width of the photooxidized P^+ at 15 K (Figure 3) indicates that it is slightly broadened from 0.97 mT in WT chromatophores to 1.09 mT in the D1-ILMH mutant. The photoinduced spin-polarized triplet state of P was also monitored in both mutant and WT chromatophores at 4 K (Figure 4). The spectra show that the zero-field splitting (ZFS) parameters calculated from the peak splittings are significantly increased by incorporating PS II sequence into the WT reaction center. The values obtained are $|D| = 200 \times 10^{-4}\ cm^{-1}$ and $|E| = 44 \times 10^{-4}\ cm^{-1}$ for D1-ILMH and $|D| = 185 \times 10^{-4}\ cm^{-1}$ and $|E| = 31 \times 10^{-4}\ cm^{-1}$ for WT. By comparison, the values measured for monomeric Bchl in organic solvents are $(224-238) \times 10^{-4}\ cm^{-1}$ for $|D|$ and $(53-69) \times 10^{-4}\ cm^{-1}$ for $|E|$ (Thurnauer, 1979), and the values for *Rb. capsulatus* $His^{M200} \rightarrow$ Leu (heterodimer) RCs are $|D| = 210 \times 10^{-4}\ cm^{-1}$ and $|E| = 60 \times 10^{-4}\ cm^{-1}$ (Bylina et al., 1990). The orientation of the triplet axes in the mutant with respect to the membrane plane is indistinguishable from the orientation in WT *Rb. capsulatus*; that is, the Bchl ring plane is oriented at an angle of 90° with respect to the plane of the membrane (Figure 4).

Midpoint Potential of P/P^+. A redox titration of P/P^+ in chromatophores is shown in Figure 5. Fitting these data with an n=1 Nernst curve gives an E_m of +397 ± 10 mV (vs NHE) for the D1-ILMH mutant. This value is approximately 90 mV *lower* than in WT chromatophores (+483 mV). Both the mutant and the WT chromatophores show $n = 1$ behavior.

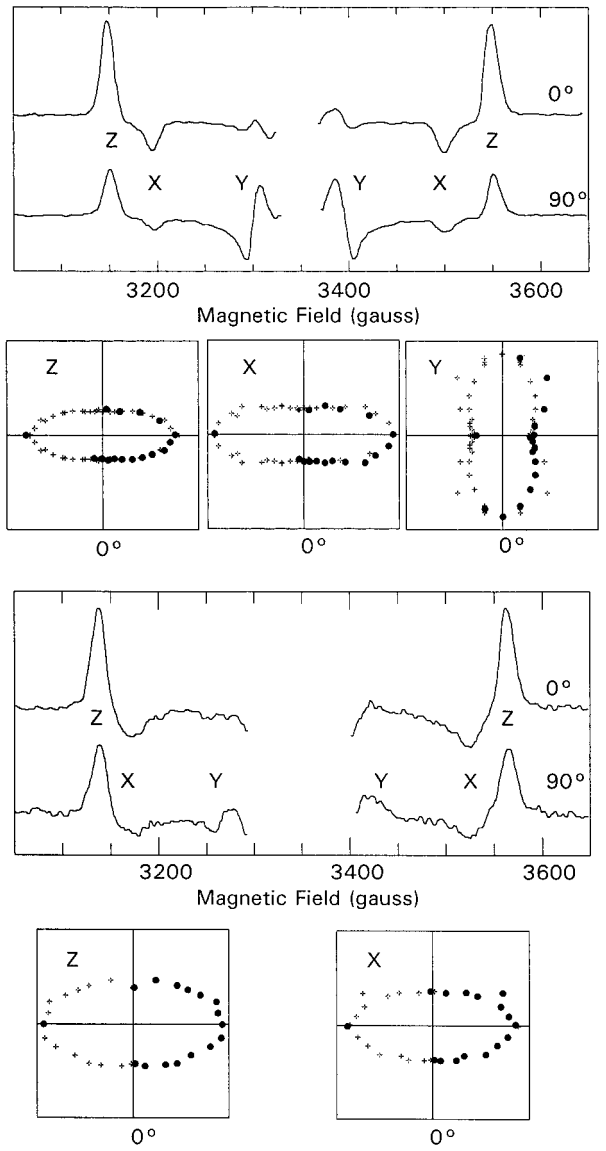

FIGURE 4: EPR spectra and orientation dependence of the *x*-, *y*-, and *z*-triplet peaks of the spin-polarized triplet state of the primary donor in chromatophores partially dried on mylar sheets. (Upper-panels) Wild-type *Rb. capsulatus*. (Lower panels) *Rb. capsulatus* D1-ILMH mutant. These are light-minus-dark spectra. The top spectrum for each sample shows the signal amplitude when the sheets are oriented parallel (0°) to the magnetic field. The bottom spectrum shows the amplitude for the perpendicular (90°) orientation. Polar plots of the orientation dependence of the triplet peaks are shown below the spectra. The filled circles are the data points; the other symbols are the same data points replotted in their symmetry-related positions as an aid to visual assessment of the data. The orientation of the *y*-peaks for the D1-ILMH mutant could not be determined. The ^3P *z*-axis is approximately perpendicular to the Bchl ring plane, whereas the *x*- and *y*-axes are in the plane of the macrocycle. The weaker triplet signals obtained for the mutant reflect a lower RC concentration in the samples used.

FT Raman Spectroscopy of the Bchl/Protein Interactions. Figure 6 shows the FT Raman spectra for reduced RCs (in their P neutral state) from wild-type and the D1-ILMH mutant excited at 1064 nm. The WT P spectrum of *Rb. capsulatus* is essentially the same as that of *Rhodobacter sphaeroides* (Mattioli et al., 1991, 1992). The 1607-cm^{-1} band arises from a C_aC_m stretching mode. Its observed frequency and narrow bandwidth (14 cm^{-1} FWHM) indicate that each of the two Bchl *a* molecules constituting the primary donor possesses one axial ligand. The 1622-cm^{-1} band arises from the C_2 acetyl carbonyl of P_A, which is

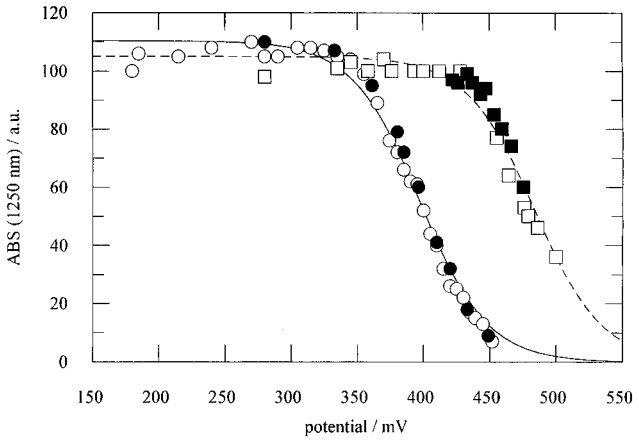

FIGURE 5: Redox titration of the D1-ILMH mutant (circles) and WT *Rb. capsulatus* (squares) in chromatophore membranes. Both oxidative titrations (open symbols) and reductive titrations (filled symbols) were performed, and these were fitted to an $n = 1$ Nernst curve.

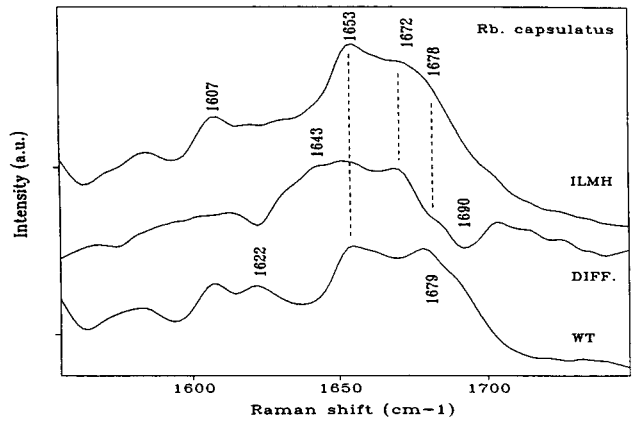

FIGURE 6: Near-infrared Fourier transform (pre)resonance Raman (FTRR) spectra of *Rb. capsulatus* WT RCs (bottom), D1-ILMH mutant RCs (top), and the difference (center).

strongly H-bonded to His^{L168}; this residue is conserved in *Rps. viridis*, *Rb. sphaeroides*, *Rb. capsulatus*, and *Rhodospirillum rubrum*. The 1653-cm^{-1} band is assigned to the non-H-bonded C_2 acetyl carbonyl of P_B. The 1679- and 1690-cm^{-1} bands are assigned to the non-H-bonded C_9 keto carbonyl groups of P_B and of P_A, respectively (Mattioli et al., 1991, 1994). The D1-ILMH mutations replace His^{L168} with a leucine residue and thus eliminate the H-bond to the C_2 acetyl carbonyl of P_A. Accordingly, an upshift in frequency of the 1622-cm^{-1} band ought to be observed. When the WT and D1-ILMH P spectra in Figure 6 are compared, the 1622-cm^{-1} band is conspicuously absent in the D1-ILMH spectrum, confirming this prediction. The upshift of this band is most clearly evident in a difference spectrum of P (D1-ILMH minus WT), where the negative band at 1622 cm^{-1} and a broad positive band at ca. 1643 cm^{-1} can be seen. Also evident in the D1-ILMH spectrum is the loss of the 1690-cm^{-1} band, indicating a change in vibrational frequency of the C_9 keto carbonyl group of P_A. The difference spectrum clearly indicates that the 1690-cm^{-1} band has downshifted to 1670 cm^{-1}.

DISCUSSION

Structural Aspects. On the basis of the apparent homology between the PS II and *Rb. capsulatus* RC polypeptides, we have made a bacterial mutant in the L subunit which is expected to be structurally similar to the D1 subunit of PS II in the region surrounding the Bchl on the L-subunit side of the special pair (P_A). This region includes five residues that make up most of the turn between the amphipathic cd-helix and the transmembrane D-helix, as well as the first six residues of the D-helix. Although the 11 residues in this segment are likely to fold in a manner that is crudely similar to the same region in PS II, the small-scale interactions between the protein backbone or the side chains and the chlorophyll(s) may well be different. Substituting the I-L-M-H sequence for F-H-Y-N changes the side-chain molar volume (Zamyatnin, 1972) in this segment of the protein only slightly, however, decreasing it by 4.9 $Å^3$. Nevertheless, the effect that this small increase in available volume might have on the water molecule located in this region (which is present in the *Rps. viridis* structural model and is presumed to be present in *Rb. capsulatus*) is not known. In addition, two aromatic residues (Phe and Tyr) are replaced by two aliphatic residues (Ile and Met). This would have the effect of decreasing the π-electron density near P_A.

248

1) *Rps. viridis* WT	T G A F G T I A S G P F W T
2) *Rb. capsulatus* WT	W S A C C M L V S G T I Y F
3) *Rsp. rubrum* WT	W S I I C M I L S G P I Y T
4) *Rb. sphaeroides* WT	F S A L C M I I T G T I W F
5) *Rx. gelatinosus* WT	W S A V C I V I S G P F W T
6) *Erythrobacter* WT	W S A I C I I I S G P V W T
7) *C. aurantiacus* WT	S A D L C I L L S G W P V Q
8) PS II (*psb*A3)	I **G** I W F **T** - **A** M **G** V S T M

286

FIGURE 7: Sequence comparison of the region surrounding Thr^{L248} in *Rps. viridis*, several bacterial species (including *Rb. capsulatus* WT and the D1-ILMH mutant), and PS II (the *psb*A3 gene from *Synechocystis* PCC 6803). Thr^{L248} provides an H-bond to the C_9 keto carbonyl of P_A in *Rps. viridis*. The alignment of the PS II sequence emphasizes the apparent similarity between $Thr^{D1\text{-}286}$ and the Thr^{L248} of *Rps. viridis*, although other alignments are possible. Residues in PS II that are identical to those in *Rps. viridis* are highlighted in boldface type. Sequence data are from the same sources as in Figure 1.

Sequence Comparisons in the Region Surrounding P_A. Figure 1 shows an alignment of the D-helix region, which contains the binding site for P_A (including His^{L173}, whose imidazole side chain coordinates the Mg atom of the Bchl, and His^{L168}, whose side chain forms a hydrogen bond to the C_2 acetyl carbonyl of the Bchl ring). The sequence of the D1-ILMH mutant is compared to the L-subunit sequences of various bacteria having purple-type RCs, including *Erythrobacter*, the green filamentous bacterium *Chloroflexus aurantiacus*, and two site-directed mutants. His^{L173} is conserved in all of the WT sequences. His^{L168} is conserved in all of the WT sequences except PS II and *C. aurantiacus*. Replacement of His^{L168} in the *Rb. sphaeroides* $His^{L168} \rightarrow$ Phe mutant (Murchison et al., 1993) would therefore be expected to delete a hydrogen bond in a way that is similar to *C. aurantiacus* and the D1-ILMH mutant (Ivancich et al., 1995, 1996). Likewise, one can also compare (Figure 7) sequences in a region of the L subunit containing, in *Rps. viridis*, a residue that forms a hydrogen bond to the C_9 keto carbonyl of P_A (Thr^{L248}) (Deisenhofer et al., 1995). The *Rps. viridis* RC is the only one that has a Thr at this position; all

of the other RCs have residues that are incapable of donating side-chain hydrogen bonds.

Our observation that the D1-ILMH mutant grows photosynthetically means that the chimeric RC is both stable and photochemically active. It also indicates that inserting the four-residue I-L-M-H sequence into the heart of the RC does not disrupt the protein and that the resulting folding pattern must therefore be compatible with the overall WT structure. The stability of the mutant protein is nevertheless surprising, since these residues are expected to form a substantial part of the binding site for P_A. However, this sequence also appears to comprise a somewhat variable region among the many bacterial L-subunit polypeptides that have been sequenced to date and could perhaps modulate the properties of the Bchl dimer.

Optical Aspects. The effects of the four mutations on the spectroscopic properties of the RC appear to be localized to the primary donor. The shift of the Q_Y transition to shorter wavelength and the apparent decrease in intensity suggest a partial reduction in the excitonic coupling between the two Bchls that comprise the dimer. But unlike the L-subunit heterodimer (His^{L173} → Leu) mutation, where the intensity of this band is shifted out into the near IR and broadened due to the replacement of P_A by a Bphe (Bylina & Youvan, 1990), the D1-ILMH mutant shows no increase in the amplitude of the Bphe absorption bands, and Raman measurements clearly indicate that there is a 5-coordinate Mg atom in P_A (see below). However, there is no indication from the optical absorption spectrum that any of the primary donor Bchls have been converted from Bchl into Bphe as in the heterodimer mutants [His^{L173} → Leu and His^{M200} → Leu (Bylina & Youvan, 1988, 1990)]. This is confirmed by FT resonance Raman measurements (see below).

Numerous spectroscopic studies indicate that excitonic couplings between any of the PS II pigments, including P680, are weak (on the order of 100−200 cm^{-1}) compared to the coupling between the two Bchl molecules constituting the special pair in the bacterial RC; the strength of the excitonic interaction involving P680 in PS II is similar to that experienced by the pigments that are considered monomers in the bacterial RC [see, e.g., Lösche et al. (1988), Tentenkin et al. (1989), Braun et al. (1990), He et al. (1990), van Kan et al. (1990), van Mieghem et al. (1991), van der Vos et al. (1992), Carbonera et al. (1994), Kwa et al. (1994), Schelvis et al. (1994), Durrant et al. (1995), and Hillman et al. (1995)].

Given the current level of understanding of the pigment interactions in the bacterial RC, we can only offer possible suggestions as to why the D1-ILMH RCs show a slightly less red-shifted and less intense Q_Y transition of P. The apparent decrease in the relative intensity of the 850-nm band could be the result of a small blue shift due to weakened coupling between the two halves of the dimer or to a very slight change in the orientation of the two halves of the dimer relative to each other, which could increase the intensity of the upper exciton [$P_{(+)}$] band at ∼810 nm at the expense of the lower exciton [$P_{(-)}$] band at ∼850 nm [for discussion, see Hartwich et al. (1995)]. For comparison, in the His^{L168} → Phe point mutant in *Rb. sphaeroides* (which, like the D1-ILMH mutant, contains no hydrogen bond to the C_2 acetyl carbonyl oxygen of P_A), the Q_Y transition of P is blue-shifted by 15 nm (Murchison et al., 1993), suggesting a less strongly interacting dimer. However, in the *Rb. sphaeroides* mutant the intensity of the Q_Y transition is unaffected by the mutation. It has been suggested that His^{L168} could play a structural role in maintaining the geometry between P_A and P_B (Mattioli et al., 1995).

Raman Spectroscopy. Comparing RCs from the D1-ILMH mutant with those of WT *Rb. capsulatus*, one can make three conclusions. First, it is clear from the 1607-cm^{-1} C_aC_m stretching mode that the Mg atoms of both Bchls of the special pair remain 5-coordinate in the mutant. This rules out any possibility that the EPR results could be explained by conversion from Bchl into Bphe at the L-side BChl of the special pair (P_A), as happens in the His^{L173} → Leu "heterodimer" mutant of *Rb. capsulatus* (Bylina & Youvan, 1990). This conclusion is also supported by the lack of any noticeable change in the intensity of the Q_Y transitions of the Bphes relative to the Bchls in the near-IR electronic spectrum of the RC.

Second, the loss of the 1622-cm^{-1} band and its apparent upshift to ca. 1643 cm^{-1} is consistent with a loss of the H-bond between His^{L168} and the C_2 acetyl carbonyl of P_A. In this respect, the mutant is very similar to *C. aurantiacus*, which contains a Phe substitution in its L subunit at a sequence position equivalent to His^{L168} (Ovchinnikov et al., 1988). The *C. aurantiacus* RC cannot therefore have the same H-bond to the C_2 acetyl carbonyl of P_A (Ivancich et al., 1996). There are also similarities in the ZFS parameters in the EPR triplet spectra of the mutant and *C. aurantiacus* (see below).

Finally, there is an apparent downshift in the 1690-cm^{-1} band (assigned to the free C_9 keto carbonyl of P_A in WT) to 1670 cm^{-1} in the D1-ILMH mutant. This 20-cm^{-1} downshift of the vibrational frequency of the keto stretching mode suggests that the C_9 keto carbonyl of P_A is now H-bonded. In the *Rps. viridis* RC, this H-bond is donated by the side chain of Thr^{L248}. No such side chain is available, however, at this position in the L subunit of *Rb. capsulatus*, where this residue is a Met. It is possible that the H-bond donor in the mutant is a backbone amide, or water, or that the nearby side chain of Cys^{L247} is close enough to form a weak H-bond (Ermler et al., 1994), especially since removal of the H-bond with the C_2 acetyl carbonyl may have resulted in a structural rearrangement. In the WT *Rb. sphaeroides* structure, Cys^{L247} interacts with the C_{10} carbomethoxy carbonyl of P_A (Ermler et al., 1994) and might be close enough to provide a weak H-bond if the orientation of P_A is altered slightly or if the protein environment around P_A is changed. [A recent study of protein structural databases indicates that Cys side chains can indeed form H-bonds with carbonyl oxygen atoms (Gregoret et al., 1991).] It is worth noting that, in PS II, there is no Cys anywhere near the position that is equivalent to *Rb. capsulatus* L247, and although there is a possibility that $Thr^{D1\text{-}286}$ might be equivalent to Thr^{L248} in *Rps. viridis*, the sequence similarity is not overwhelming (Figure 7). In *C. aurantiacus*, the residue equivalent to *Rps. viridis* Thr^{L248} is an Ile, and the residue equivalent to *Rb. capsulatus* Cys^{L247} is also a Cys. The next residue (Met^{L248}) forms an energetically unfavorable contact with the C_9 keto carbonyl of P_A in the *Rb. sphaeroides* structure (Ermler et al., 1994). In addition, all of these sequences except *C. aurantiacus* have Ser at L244, although in the WT X-ray crystallographic structure of *Rb. sphaeroides* this Ser is closer to the carbomethoxy group of P_A (Ermler et al., 1994). The PS II D1 subunit contains no obvious H-bond candidates at either position, although it does have several hydroxyl-containing side chains at other positions, most notably Thr^{286}, Ser^{291}, and Thr^{292} (*Synechocystis* PCC

6803 PS II numbering). It is important to note that introduction of an unexpected H-bond does not occur in the previously reported $His^{L168} \rightarrow Phe$ mutant in *Rb. sphaeroides* (Murchison et al., 1993; Mattioli et al., 1994). However, Noguchi et al. (1993) have concluded, on the basis of their FT-IR measurements of PS II, that the keto carbonyl of P680 is H-bonded to D1 residues Ser^{291} or Thr^{292}. This interpretation assumes that P680 is similar to the purple bacterial special pair.

Redox Measurements of P/P$^+$. The lowering of the apparent redox midpoint potential of the primary donor in the D1-ILMH mutant by about 90 mV compared to WT is nearly identical to the effect observed in the $His^{L168} \rightarrow Phe$ mutant of *Rb. sphaeroides* (Murchison et al., 1993). In the *Rb. sphaeroides* mutant, this reduction in E_m was attributed to the loss of the H-bond between His^{L168} and the C_2 acetyl carbonyl group of P_A. The correlation between histidine-donated H-bonds to the conjugated carbonyl groups of P and its rise in oxidation potential has been recently reported (Lin et al., 1994; Mattioli et al., 1995). It is also significant that the *C. aurantiacus* RC naturally contains the same $His \rightarrow Phe$ "mutation", and the E_m of its primary donor couple has been measured at +386 mV (Ovchinnikov et al., 1988), which is about 70−100 mV more negative than typical *Rhodobacter* primary donor couples. In *Rb. sphaeroides*, a $Met^{L248} \rightarrow Thr$ mutation does not affect the midpoint potential of P/P^+ (Wachtveitl et al., 1993), but this mutant also contains the WT His^{L168}. The lowering of the midpoint potential of P/P^+ in the D1-ILMH RCs could at least partly explain the slight decrease in the rate of $P^+Q_A^-$ charge recombination relative to WT.

Electron Paramagnetic Resonance Spectroscopy. One indication of the degree to which the two Bchls of the special pair interact is given by EPR measurements of the triplet state of the primary donor. It has been known for some time that the triplet state of WT P680 (^{3}P680) has zero-field splitting parameters ($|D| = 286 \times 10^{-4}$ cm^{-1} and $|E| = 44 \times 10^{-4}$ cm^{-1}; Rutherford et al., 1981) that are virtually identical to those of monomeric Chl *a* in solution. These values for PS II contrast with those for the bacterial RC (Budil & Thurnauer, 1991), where, for example, the $|D|$ parameter measured for the Bchl *a* dimer in *Rb. capsulatus* (Prince & Youvan, 1987) is ∼15% smaller than for Bchl *a* monomers in solution. The narrowing is due in part to sharing of the triplet spin density between the two constituent halves of the dimer, as well as to a contribution from charge transfer (Norris et al., 1987; Budil & Thurnauer, 1991). The orientation of the triplet axes with respect to the plane of the membrane is also consistent, in the bacterial case, with the idea that the triplet resides on one or both of the Bchls of the special pair, whose ring planes are oriented nearly perpendicular to the plane of the membrane (Norris et al., 1987). PS II is unusual, however, in that the triplet-bearing species is oriented nearly parallel to the plane of the membrane (∼30° away from it) (van Mieghem et al., 1991). This could conceivably give it the same orientation as one of the monomeric (accessory) Bchls in the bacterial RC, and indeed it was suggested that such a Chl (if it exists as an analog to one of the monomeric Bchls) could be the macrocycle on which the ^{3}P680 resides (Rutherford, 1986; 1988; van Mieghem et al., 1991). At the time the original suggestion was made, however, the measured orientation of the ^{3}P680 *x*- and *y*-axes did not seem to correspond well to the molecular axes of the monomeric Bchls (van Mieghem et al., 1991). In the case of Bchl, the magnetic *x*- and *y*-axes correspond fairly closely to the Q_X and Q_Y optical transition axes of the molecule (Budil & Thurnauer, 1991). In the case of Chl *a*, however, recent work has shown that there is a shift of ∼45° of the optical axes away from the triplet axes (van der Vos, 1992). Thus the suggestion that P680 may reside on a Chl which is a structural counterpart of the monomeric Bchl is perhaps more likely than was first thought.

A second indication of spin delocalization and interaction between Bchls comes from EPR measurements of the radical cation (P^+) state of the primary donor. In WT RCs of *Rps. viridis*, *Rb. capsulatus*, and *Rb. sphaeroides*, an observed narrowing of the EPR spectrum of P^+ relative to monomeric Bchl *a* in solution has been explained by proposing that it arises from hyperfine interactions between the spin of the unpaired electron and the nuclear spins of both Bchls of the dimer. This interaction narrows the EPR line width by a factor of $2^{-1/2}$ (Norris et al., 1971; McElroy et al., 1972). In *Rps. viridis*, however, the unpaired spin appears to be slightly more localized on one half of the dimer than it is in the *Rb. capsulatus* or *Rb. sphaeroides* RCs (Prince et al., 1976). This difference, as well as the difference in the triplet zero-field splitting parameters, has been attributed to the species-dependent differences in the hydrogen-bonding interactions between the Bchls and the protein (Norris et al., 1987; Tiede, et al., 1988; El-Kabbani et al., 1991). It is interesting, therefore, that the D1-ILMH mutant, which appears to have a hydrogen bond to the C_9 keto group of P_A, has a broader P^+ EPR line width than the two WT *Rhodobacter* RCs, implying that for this mutant the spin density of the unpaired electron in the P^+ state is more localized on one of the two Bchl molecules.

Rautter et al. (1995) have recently reported the EPR line widths of P^+ from several mutant strains of *Rb. sphaeroides* in which H-bonds to the C_2 and C_9 conjugated carbonyl groups of the Bchl molecules have been genetically added or removed, resulting in various H-bonding patterns for P. For the mutant in which the strongly conserved H-bond to the C_2 acetyl carbonyl of P_A was removed by replacing His^{L168} by a Phe ($His^{L168} \rightarrow Phe$), the EPR line width is quite similar to WT (0.97 mT for the mutant vs 0.96 mT for WT). Similarly, for a mutant where the H-bond was introduced onto the C_9 keto carbonyl of P_A ($Leu^{L131} \rightarrow His$), the P^+ EPR line width (0.98 mT) is also comparable to WT. For the double mutant ($His^{L168} \rightarrow Phe + Leu^{L131} \rightarrow His$), whose primary donor possesses only one H-bond on the C_9 keto carbonyl of P_A (an H-bond pattern similar to the D1-ILMH mutant), the EPR line width was found to significantly increase to 1.12 mT; this broadening implies that the unpaired spin density is less symmetrically shared between the constituent Bchl molecules of P, relative to WT, and approaches a "monomeric" situation. A similar broadening of the P^+ EPR line width was observed in this work for the D1-ILMH mutant (see Table 1). On the basis of electron nuclear double resonance (ENDOR) experimental data and a simple orbital energy scheme model, Rautter et al. (1995) concluded that for the $His^{L168} \rightarrow Phe + Leu^{L131} \rightarrow His$ mutant, 78% of the unpaired spin density in P^+ is asymmetrically located on the P_B Bchl; for the WT case, 68% of the unpaired spin density is located on P_A. The similarities in both the P^+ EPR line widths and the H-bonding patterns of the *Rb. sphaeroides* double mutant and the *Rb. capsulatus* mutant reported here suggest that a similar change in

Table 1: Summary of Optical, EPR, and Redox Data[a]

	wild type	D1-ILMH
photosynthetic growth	yes	yes
λ_{max} for Q_Y transition of P	850 nm	846 nm
λ_{max} for P^+ cation band	1250 nm	1254 nm
$P^+Q_A^- \rightarrow PQ_A$ rate	$(80\ ms)^{-1}$	$(110\ ms)^{-1}$
ZFS for ^3P	$\|D\| = 185 \times 10^{-4}\ cm^{-1}$	$\|D\| = 200 \times 10^{-4}\ cm^{-1}$
	$\|E\| = 31 \times 10^{-4}\ cm^{-1}$	$\|E\| = 44 \times 10^{-4}\ cm^{-1}$
orientation of P	90° to membrane plane	90° to membrane plane
ΔH_{p-p} for P^+	0.97 mT	1.09 mT
E_m for P/P^+	483 ± 10 mV	397 ± 10 mV
H-bonded substituent (P_A)	C_2 acetyl carbonyl	C_9 keto carbonyl

[a] For measurements of λ_{max} for the Q_Y transition of P, the $P^+Q_A^- \rightarrow PQ_A$ recombination kinetics, and the Raman spectra, RCs isolated with LDAO were used. All other measurements listed were performed on chromatophore preparations.

unpaired electron spin density localization has also occurred in the D1-ILMH mutant.

As outlined above, two other parameters which indicate how the unpaired spin is shared between the two halves of the dimer are the zero-field splitting parameters $|D|$ and $|E|$ for the triplet state. The former reflects the relative size of the molecule or supermolecule over which the electron spin density is distributed, while the latter reflects the symmetry of the molecular orbitals (Budil & Thurnauer, 1991). The increase in the $|D|$ and $|E|$ values for the D1-ILMH mutant strongly indicates that the triplet is localized on one half of the dimer. In WT *Rps. viridis*, in which the C_9 keto carbonyl of P_A is hydrogen-bonded, the triplet is also localized more on P_A than on P_B , although charge-transfer contributions are thought to contribute to substantial narrowing of the ZFS parameters (Norris et al., 1987). Once again, the degree of delocalization is believed to depend on the protein environment around the dimer (El-Kabbani et al., 1991). In the D1-ILMH mutant, the observed values for the ZFS parameters fall almost halfway between those of WT *Rb. capsulatus* and the *Rb. capsulatus* $His^{M200} \rightarrow$ Leu heterodimer mutant (Bylina et al., 1990). In the latter case, P_B is replaced by a Bphe, so that the triplet is likely to reside exclusively on P_A. An even closer analogy could be drawn between the D1-ILMH RC and the *C. aurantiacus* RC. In the latter, the residue equivalent to His^{L168} is a Phe, and the EPR spectrum of the triplet state of the primary donor is virtually identical to the spectrum of the D1-ILMH mutant ($|D| = 201 \times 10^{-4}$ cm^{-1} and $|E| = 45 \times 10^{-4}\ cm^{-1}$; Blankenship et al., 1984). This correspondence suggests that the $His^{L168} \rightarrow$ Leu mutation may be sufficient to decouple (at least partially) the two halves of the Bchl dimer and cause an asymmetric distribution of the triplet electron spin. The question remains as to which residues are the most important in determining the the localization of the triplet and the radical cation in the D1-ILMH mutant. Although it appears that removing His^{L168} is sufficient, this may only be because, in this mutant, it allows a slight rearrangement of the system, such that the C_9 keto carbonyl of P_A can gain a new hydrogen-bond donor.

The increase in the degree of localization of the triplet state in the D1-ILMH mutant is not nearly as dramatic as in the PS II RC, where the values of the ZFS parameters for $^3P680$ are very close to those for a monomeric Chl *a* in organic solvents (Thurnauer et al., 1975). Nevertheless, this increase demonstrates that relatively few mutations can have a large impact on the extent to which the two (B)chls of the special pair interact, even though the overall structure of the (B)chl binding site may not be substantially changed. The latter suggestion is supported by measurements of the orientation of the triplet axes in the D1-ILMH mutant. The orientation of the Bchl ring plane(s) relative to the membrane plane can be determined by measuring the three axes of the triplet state in a sample containing oriented membranes. In the D1-ILMH mutant, the orientation of the triplet is indistinguishable from WT (i.e., 90° to the plane of the membrane) and is not like that of PS II.

The D1-ILMH mutations lie at the starting point of a region of the L subunit that comprises a major part of the protein environment of P_A. We might therefore expect that the environment of P_A resembles that seen by its structural counterpart in PS II. If so, the fact that the mutations did not perturb the orientation of the triplet may be taken as an indication that other features of the PS II protein are responsible for this unusual orientation. In addition, it might be argued that the counterpart of P_A in PS II is likely to have its ring plane oriented perpendicular to the membrane. It has been proposed previously that, given the sequence and structural similarities between PS II and purple bacteria, and given that the P_A/P_B Chls are most likely to be ligated by histidines in transmembrane helices, steric constraints make it unlikely that the counterparts to the bacterial P_A and P_B could be tilted at 30° to the plane of the membrane (van Mieghem et al., 1991). This situation is consistent with the model preferred by van Mieghem et al. (1991) in which P680 is made up of the monomeric Chl weakly interacting with the counterparts of P_A and/or P_B (where the counterparts of P_A and P_B are more weakly coupled than in the bacterial reaction center, as discussed above).

General Conclusions. The D1-ILMH mutant described in this work replaces, in the vicinity of P, a four amino acid segment that varies somewhat among bacterial species (see Figure 1) with another segment which is strongly conserved in cyanobacteria, algae, and plants (Svensson et al., 1991). One question addressed by this work was whether such a change would dramatically alter the orientation of one of the Bchl molecules of P in the bacterial RC. Such a reorientation was not observed. What was observed, however, was a repositioning of at least one Bchl molecule of P with respect to the residues in its binding pocket. This repositioning is significant enough to allow a new, unexpected H-bond to form on the C_9 keto carbonyl of P_A. If one considers the structure of the *Rb. sphaeroides* RC (Ermler et al., 1994) and a structural model of the *Rb. capsulatus* RC (Foloppe et al., 1995), there is no obvious candidate for the donor, and the exact nature of the repositioning is not clear. In PS II, the excitonic coupling is relatively weak between the Chl molecules that are analogous to the bacterial primary donor. The presence of the I-L-M-H sequence in PS II could contribute to a "repositioning" of the pigments comparable to what is seen

in this bacterial mutant. Although this sequence change does not greatly reduce the excitonic coupling of the two Bchl molecules of P in *Rb. capsulatus*, a similar structural "repositioning" could decouple the Chl molecules of P680 to a greater degree because the Q_Y absorption band of Chl is weaker in oscillator strength than that of Bchl. In addition, Chl molecules do not possess a conjugated C_2 acetyl carbonyl, which extends the π-system in Bchl *a*. Therefore, it is entirely plausible that the structural shift exerted by the I-L-M-H sequence could contribute to the decoupling of the Chl molecules in PS II. Finally, one must also consider the fact that the mutant described here addresses only the L or D1 half of the question. Further work will be needed to study the contribution of the M or D2 side of the RC structure.

One important physical parameter of the primary donor which was altered in the *Rb. capsulatus* D1-ILMH mutant was the redox midpoint potential of the P/P^+ couple. The lowering of this midpoint potential by almost 90 mV appears to be due mainly to the rupturing of the H-bond to the acetyl carbonyl of P_A that is donated by His^{L168} (Murchison et al., 1993; Lin et al., 1994; Mattioli et al., 1994, 1995). In engineering a bacterial mutant to mimic the redox properties of the highly oxidizing species P680, it might seem that removing H-bonds from the Chl conjugated carbonyl groups is counterproductive. The fact that the PS II RC does not possess a histidine residue in the position equivalent to L168 such that its side chain can form an H-bond with the acetyl carbonyl of P_A [a situation which is conserved in all other purple-type RCs except *C. aurantiacus* (Mattioli et al., 1992)] could be rationalized by considering that Chl *a* does not possess an acetyl carbonyl group, unlike Bchl *a*; thus, this highly conserved interaction in bacterial RCs is not possible in Chl *a*-containing PS II RCs. Since the Chl *a* molecule(s) constituting P680 possess(es) only one conjugated carbonyl group (the C_9 keto carbonyl), whereas both the Bchl molecules of the bacterial primary donor possess two conjugated carbonyl groups, it would seem that the option of increasing the redox potential of the P680 species by adding H-bonds is severely limited compared to the bacterial primary donor. One may look to other possible factors which might explain the high oxidation potential of P680.

In designing a primary donor with an unusually high oxidation potential, one might intuitively consider several factors that could elevate the midpoint potential of a Chl molecule. One obvious factor would be to use a Chl molecule which has an intrinsically higher redox potential; in this respect, Chl-type molecules are more oxidizing than Bchl-type molecules. The next consideration would be to minimize as much as possible any strong excitonic interaction which would reduce the redox potential; in this case, one would want a "monomeric" species. An axial ligand other than a histidine imidazole could also influence the redox potential of a Chl molecule. Mutagenesis of $His^{D1\text{-}198}$ in *Synechocystis* PCC 6803, for example, lowers the apparent redox potential of $P680/P680^+$ by up to 90 mV, although the P680 triplet-state ZFS parameters, as measured by low-temperature EPR, are unaffected in several of the mutants (Coleman et al., 1995). According to the high-resolution structure of *Rps. viridis*, the analogous His residue in the bacterial RC (His^{L173}) coordinates the Mg atom of P_A with one imidazole nitrogen and a bound water molecule with the other (Deisenhofer et al., 1995). The water molecule, in turn, forms an H-bond to the C_9 keto carbonyl of the M-side monomeric Bchl. If a bound water molecule exists in PS II, this raises an intriguing question as to which of these possible interactions might be involved in lowering the redox potential of $P680/P680^+$ in the $His^{D1\text{-}198}$ mutants. Indeed, the mutant $His^{L173} \rightarrow$ Gly in *Rb. sphaeroides*, which may contain water as an axial ligand to P_A, has a midpoint potential that is indistinguishable from that of WT (Goldsmith et al., 1996). Finally, the inclusion of positive charges around the electron-donating Chl molecule(s) (in the form of charged/polar amino acids or metals) could also elevate the oxidation potential of P.

ACKNOWLEDGMENT

We thank Matt Scholl and Sandra Adrianambinintsoa for technical assistance. We thank Paul Mathis for helpful advice concerning the optical measurements and Alain Boussac and Fraser Macmillan for help with computing. D.C.Y. and W.J.C. initiated this work in the Departments of Applied Biological Sciences and Chemistry at the Massachusetts Institute of Technology.

REFERENCES

Bélanger, G., Berard, J., Corriveau, P., & Gingras, G. (1988) *J. Biol. Chem. 263*, 7632−7638.

Blankenship, R. E., Mancino, L. J., Feick, R., Fuller, R. C., Miachnicki, J., Frank, H. A., Kirmaier, C., & Holten, D. (1984) in *Advances in Photosynthesis Research* (Sybesma, C., Ed.) Vol. 1, pp 203−206, Martinus Nijhoff/Dr. W. Junk, The Hague, The Netherlands.

Braun, P., Greenberg, B. M., & Scherz, A. (1990) *Biochemistry 29*, 10376−10387.

Budil, D. E., & Thurnauer, M. C. (1991) *Biochim. Biophys. Acta 1057*, 1−41.

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. 1, pp 53−59, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Bylina, E. J., Jovine, R. V. M., & Youvan, D. C. (1989) *Bio/Technology 7*, 69−74.

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

Carbonera, D., Giacometti, G., & Agostini, G. (1994) *FEBS Lett. 343*, 200−204.

Coleman, W. J., & Govindjee (1987) *Photosynth. Res. 13*, 199−223.

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

Coleman, W. J., Nixon, P. J., Vermaas, W. F. J., & Diner, B. A. (1995) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. 1, pp 779−782, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.

Deisenhofer, J., Epp, O., Sinning, I., & Michel, H. (1995) *J. Mol. Biol. 246*, 429−457.

Diner, B. A., Nixon, P. J., & Farchaus, J. W. (1991) *Curr. Opin. Struct. Biol. 1*, 546−554.

Döring, G., Stiehl, H. H., & Witt, H. T. (1967) *Z. Naturforsch. 22B*, 639−644.

Durrant, J. R., Klug, D. R., Kwa, S. L. S., van Grondelle, R., Porter, G., & Dekker, J. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4798−4802.

Dutton, P. L. (1971) *Biochim. Biophys. Acta 226*, 63−80.

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

Ermler, U., Michel, H., & Schiffer, M. (1994) *J. Bioenerg. Biomembr. 26*, 5−15.

Foloppe, N., Ferrand, M., Breton, J., & Smith, J. C. (1995) *Proteins: Struct., Funct., Genet. 22*, 226−244.

Goldsmith, J. O., King, B., & Boxer, S. G. (1996) *Biochemistry 35*, 2421−2428.

Gregoret, L. M., Rader, S. D., Fletterick, R. J., & Cohen, F. E. (1991) *Proteins: Struct., Funct., Genet. 9*, 99−107.
Hales, B. J., & Das Gupta, A. (1979) *Biochim. Biophys. Acta 548*, 276−286.
Hartwich, G., Scheer, H., Aust, V., & Angerhofer, A. (1995) *J. Biol. Chem. 263*, 7632−7638.
He, W.-Z., Telfer, A., Drake, A., Hoadley, J., & Barber, J. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 431−434, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Hillman, B., Brettel, K., van Mieghem, F., Kamlowski, A., Rutherford, A. W., & Schlodder, E. (1995) *Biochemistry 34*, 4814−4827.
Ivancich, A., Feick, R., Ertlmaier, A., & Mattioli, T. A. (1995) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. 1, pp 671−674, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Ivancich, A., Feick, R., Ertlmaier, A., & Mattioli, T. A. (1996) *Biochemistry 35*, 6126−6135.
Jia, Y., DiMagno, T. J., Chan, C.-K., Wang, Z., Du, M., Hanson, D. K., Schiffer, M., Norris, J. R., & Fleming, G. R. (1993) *J. Phys. Chem. 97*, 13180−13191.
Jursinic, P., & Govindjee (1977) *Photochem. Photobiol. 26*, 617−628.
Klimov, V. V., Allakhverdiev, S. I., Demeter, S., & Krasnovsky, A. A. (1979) *Dokl. Acad. Nauk SSSR 249*, 227−230.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Kwa, S. L. S., Eijckelhoff, C., van Grondelle, R., & Dekker, J. P. (1994) *J. Phys. Chem. 98*, 7702−7711.
Liebetanz, R., Hornberger, U., & Drews, G. (1991) *Mol. Microbiol. 5*, 1459−1468.
Lin, X., Murchison, H. A., Nagarajan, V., Parson, W. W., Williams, J. C., & Allen, J. P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10265−10269.
Lösche, M., Feher, G., & Okamura, M. Y. (1988) in *The Photosynthetic Bacterial Reaction Center: Structure and Dynamics* (Breton, J., & Verméglio, A., Eds.) pp 151−164, Plenum, New York.
Mathis, P., & Setif, P. (1981) *Isr. J. Chem. 21*, 316−320.
Mattioli, T. A., Hoffmann, A., Robert, B., Schrader, B., & Lutz, M. (1991) *Biochemistry 30*, 4648−4658.
Mattioli, T. A., Robert, B., & Lutz, M. (1992) in *The Photosynthetic Bacterial Reaction Center: Structure, Spectroscopy and Dynamics* (Breton, J., & Verméglio, A., Eds.) pp 127−132, Plenum, New York.
Mattioli, T. A., Williams, J. C., Allen, J. P., & Robert, B. (1994) *Biochemistry 33*, 1636−1643.
Mattioli, T. A., Lin, X., Allen, J. P., & Williams, J. C. (1995) *Biochemistry 34*, 6142−6152.
McElroy, J. D., Feher, G., & Mauzerall, D. C. (1972) *Biochim. Biophys. Acta 267*, 363−374.
Metz, J., Nixon, P., & Diner, B. (1990) *Nucleic Acids Res. 18*, 6715.
Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1−7.
Michel, H., Epp, O., & Deisenhofer, J. (1986a) *EMBO J. 5*, 2445−2451.
Michel, H., Weyer, K. A., Gruenberg, H., Dunger, I., Oesterhelt, D., & Lottspeich, F. (1986b) *EMBO J. 5*, 1149−1158.
Moss, D. A., Leonhard, M., Bauscher, M., & Mäntele, W. (1991) *FEBS Lett. 283*, 33−36.
Murchison, H. A., Alden, R. G., Allen, J. P., Peloquin, J. M., Taguchi, A. K. W., Woodbury, N. W., & Williams, J. C. (1993) *Biochemistry 32*, 3498−3505.
Nagarajan, V., Parson, W. W., Davis, D., & Schenck, C. C. (1993) *Biochemistry 32*, 12324−12336.
Nagashima, K. V. P., Matsuura, K., Ohyama, S., & Shimada, K. (1994) *J. Biol. Chem. 269*, 2477−2484.
Noguchi, T., Inoue, Y., & Satoh, K. (1993) *Biochemistry 32*, 7186−7195.
Norris, J. R., Uphaus, R. A., Crespi, H. L., & Katz, J. J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 625−628.
Norris, J. R., Lin, C. P., & Budil, D. E. (1987) *J. Chem. Soc., Faraday Trans. 1 83*, 13−27.
Ovchinnikov, Y. A., Abdulaev, N. G., Zolotarev, A. S., Shmukler, B. E., Zagarov, A. A., Kutuzov, M. A., Telezhenskaya, I. N., & Levina, N. B. (1988) *FEBS Lett. 231*, 237−242.
Prince, R. C., & Dutton, P. L. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 439−453, Plenum, New York.
Prince, R. C., & Youvan, D. C. (1987) *Biochim. Biophys. Acta 890*, 286−291.
Prince, R. C., Leigh, J. S., & Dutton, P. L. (1976) *Biochim. Biophys. Acta 440*, 622−636.
Rautter, J., Lendzian, F., Schulz, C., Fetsch, A., Kuhn, M., Lin, X., Williams, J. C., Allen, J. P., & Lubitz, W. (1995) *Biochemistry 34*, 8130−8143.
Robles, S. J., Breton, J., & Youvan, D. C. (1990) *Science 248*, 1402−1405.
Rutherford, A. W. (1985) *Biochim. Biophys. Acta 807*, 189−201.
Rutherford, A. W. (1986) *Biochem. Soc. Trans. 14*, 15−17.
Rutherford, A. W. (1988) in *Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models* (Stevens, S. E., & Bryant, D. A., Eds.) pp 163−177, The American Society of Plant Physiologists, Rockville, MD.
Rutherford, A. W., & Nitschke, W. (1996) in *Origin and Evolution of Biological Energy Conservation* (Baltscheffsky, H., Ed.) pp 143−175, VCH, New York.
Rutherford, A. W., Paterson, D. R., & Mullet, J. E. (1981) *Biochim. Biophys. Acta 635*, 205−214.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Schelvis, J. P. M., van Noort, P. I., Artsmaa, T. J., & van Gorkom, H. J. (1994) *Biochim. Biophys. Acta 1184*, 242−250.
Stocker, J. W., Taguchi, A. K. W., Murchison, H. A., Woodbury, N. W., & Boxer, S. G. (1992) *Biochemistry 31*, 10356−10362.
Svensson, B., Vass, I., & Styring, S. (1991) *Z. Naturforsch. 46c*, 765−776.
Taguchi, A. K. W., Stocker, J. W., Alden, R. G., Causgrove, T. P., Peloquin, J. M., Boxer, S. G., & Woodbury, N. W. (1992) *Biochemistry 31*, 10345−10355.
Tentenkin, V. L., Gulyaev, B. A., Seibert, M., & Rubin, A. B. (1989) *FEBS Lett. 250*, 459−463.
Thurnauer, M. C. (1979) *Rev. Chem. Intermed. 3*, 197−230.
Thurnauer, M. C., Katz, J. J., & Norris, J. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3270−3274.
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: Structure and Dynamics* (Breton, J., & Verméglio, A., Eds.) pp 13−20, Plenum, New York.
Trebst, A. (1986) *Z. Naturforsch. 41*, 240−245.
van der Vos, R., van Leeuwen, P. J., Braun, P., & Hoff, A. J. (1992) *Biochim. Biophys. Acta 1140*, 184−198.
van Gorkom, H. J., & Schelvis, J. P. M. (1993) *Photosynth. Res. 38*, 297−301.
van Kan, P. J. M., Otte, S. C. M., Kleinherenbrink, F. A. M., Nieveen, M. C., Aartsma, T. J., & van Gorkom, H. J. (1990) *Biochim. Biophys. Acta 1020*, 146−152.
van Mieghem, F. J. E., Satoh, K., & Rutherford, A. W. (1991) *Biochim. Biophys. Acta 1058*, 379−385.
Wachtveitl, J., Farchaus, J. W., Das, R., Lutz, M., Robert, B., & Mattioli, T. A. (1993) *Biochemistry 32*, 12875−12886.
Watanabe, T., & Kobayashi, M. (1991) in *Chlorophylls* (Scheer, H., Ed.) pp 287−315, CRC Press, Boca Raton, FL.
Weaver, P. F., Wall, J. D., & Gest, H. (1975) *Arch. Microbiol. 105*, 207−216.
Williams, J. C., Steiner, L. A., Feher, G., & Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7303−7307.
Williams, J. C., Alden, R. G., Murchison, H. A., Peloquin, J. M., Woodbury, N. W., & Allen, J. P. (1992) *Biochemistry 31*, 11029−11037.
Youvan, D. C., Bylina, E. J., Alberti, M., Begusch, H., & Hearst, J. E. (1984) *Cell 37*, 949−957.
Youvan, D. C., Ismael, S., & Bylina, E. J. (1985) *Gene 38*, 19−30.
Zamyatnin, A. A. (1972) *Prog. Biophys. Mol. Biol. 105*, 107−123.

BI962458C