An examination of how structural changes can affect the rate of electron transfer in a mutated bacterial photoreaction centre

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A series of reaction centres bearing mutations at the (Phe) M197 position were constructed in the photosynthetic bacterium *Rhodobacter sphaeroides*. This residue is adjacent to the pair of bacteriochlorophyll molecules (P_L and P_M) that is the primary donor of electrons (P) in photosynthetic light-energy transduction. All of the mutations affected the optical and electrochemical properties of the P bacteriochlorophylls. A mutant reaction centre with the change Phe M197 to Arg (FM197R) was crystallized, and a structural model constructed at 2.3 Å (1 Å = 0.1 nm) resolution. The mutation resulted in a change in the structure of the protein at the interface region between the P bacteriochlorophylls and the monomeric bacteriochlorophyll that is the first electron acceptor (B_L). The new Arg residue at the M197 position undergoes a significant reorientation, creating a cavity at the interface region between P and B_L . The acetyl carbonyl substituent group of the P_M bacteriochlorophyll undergoes an out-of-plane rotation, which decreases the edge-to-edge

distance between the macrocycles of P_M and B_L . In addition, two new buried water molecules partially filled the cavity that is created by the reorientation of the Arg residue. These waters are in a suitable position to connect the macrocycles of P and B_L via three hydrogen bonds. Transient absorption measurements show that, despite an inferred decrease in the driving force for primary electron transfer in the FM197R reaction centre, there is little effect on the overall rate of the primary reaction in the bulk of the reaction-centre population. Examination of the X-ray crystal structure reveals a number of small changes in the structure of the reaction centre in the interface region between the P and B_L bacteriochlorophylls that could account for this faster-than-predicted rate of primary electron transfer.

Key words: membrane protein, mutagenesis, photosynthesis, reaction centre, X-ray crystallography.

INTRODUCTION

The photoreaction centres of bacteria, algae and plants are Nature's solar batteries. These integral membrane proteins are responsible for the conversion of light energy into a biologically useful form. High-resolution structural information was first presented for the reaction centre from the purple bacterium *Rhodospirillum rubrum* [1], and subsequently for the complex from *Rhodobacter sphaeroides* [2,3]. In recent years, *Rb. sphaeroides* has emerged as the bacterium of choice for studying the mechanism of light-energy transduction in the reaction centre, this research mainly involving spectroscopic studies, often combined with site-directed mutagenesis [4–6]. These reaction centres are also used as model systems in which to study the general principles of electron-transfer reactions in proteins [7–10].

In all reaction centres, the absorption of light triggers the transfer of an electron between cofactors located on opposite sides of a protein/lipid membrane, generating a transmembrane electrical potential. In the *Rb. sphaeroides* reaction centre, the primary donor of electrons (P) is the first singlet excited state (denoted P^*) of a pair of bacteriochlorophyll (BChl) molecules (denoted P_L and P_M) that are held within the protein

scaffold close to the periplasmic side of the membrane. The first step in electron transfer is the movement of an electron from P^* to a molecule of bacteriopheophytin (BPhe), denoted H_L , that is located approximately half way across the membrane dielectric, forming the radical pair $P^+H_L^-$ in 3–4 ps at room temperature (see reviews in [6,11]). There is now good evidence that this 'primary electron transfer' involves the anion of the accessory BChl (B_L) located between P and H_L , with the radical pair $P^+B_L^-$ being formed as a short-lived intermediate. Secondary electron transfer then involves the movement of an electron from H_L^- to the adjacent ubiquinone Q_A , forming the $P^+Q_A^-$ radical pair in approx. 200 ps [6,11].

During the course of extensive mutagenesis-based structure/function studies, relatively little high-resolution crystallographic information on the structure of mutant *Rb. sphaeroides* reaction centres has been forthcoming (reviewed in [12]). As a result, most of the interpretation of the origins of the altered spectroscopic and electron-transfer properties of mutant reaction centres has been based on assumptions concerning the changes in structure that accompany a particular mutation.

In this report, we describe changes in the spectroscopic and electrochemical properties of the reaction centre that accompany

Abbreviations used: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; LDAO, lauryldimethylamine oxide; FT-Raman, Fourier-transform Raman spectroscopy; E_m , mid-point potential; P, primary donor of electrons; SADS, species-associated difference spectra.

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mutation of residue Phe M197, which is located in the M subunit close to the acetyl carbonyl substituent group of the P_M BChl. It has been demonstrated that replacement of Phe M197 by His or Tyr affects the electrochemical properties of the P BChls [13–15]. Five mutations were introduced, to His, Tyr, Asp, Arg and Lys. In the case of the His, Tyr and Asp mutants, the results of a spectroscopic analysis of the properties of the reaction centre are consistent with the view that the new M197 residue donates a hydrogen bond to the acetyl carbonyl group of the P_M BChl. In the Arg and Lys mutants, interpretation of the spectroscopic data is less straightforward. We have used X-ray crystallography to examine in detail the effects of the Phe M197-to-Arg mutation (FM197R), and present a structural model at a resolution of 2.3 Å for a reaction centre with this mutation. We discuss how the changes in the structure of the protein that are observed can account for the spectroscopic and electrochemical properties of the FM197R reaction centre, and how they may influence the rate of primary electron transfer in the complex.

MATERIALS AND METHODS

Mutagenesis

The mutations Trp M115 to Phe (WM115F) and Phe M197 to Arg (FM197R), Lys (FM197K), His (FM197H), Tyr (FM197Y) and Asp (FM197D) were introduced to the *pufM* gene using mismatch oligonucleotides. The template for mutagenesis was plasmid pALTSB, which consisted of a 998-bp *SalI*–*BamHI* restriction fragment encompassing the 3' end of the *pufL* gene plus the whole of the *pufM* gene cloned into the plasmid pALTER-1 (Promega) [16]. The double mutation FM197R/WM115F was constructed by introducing the WM115F change into the derivative of pALTSB containing the FM197R mutation. The changes in the sequence of the *pufM* gene were confined to the target M197 and M115 codons, and were confirmed by DNA sequencing. Mutated *SalI*–*BamHI* restriction fragments carrying either a single mutation at M197 or the double mutation FM197R/WM115F were then transferred to plasmid pRKEH10D for expression in the double-deletion/insertion mutant strain DD13 [17]. The resulting strains, named according to the mutation carried (FM197R, FM197R/WM115F and so on), lacked the LH1 and LH2 antenna complexes and contained the mutant reaction centre as the sole pigment–protein complex.

Preparation of experimental material

Details of the growth of the mutant bacterial strains under dark/semi-aerobic conditions are given elsewhere [18]. Intracytoplasmic membranes were isolated by breakage of harvested cells in a French pressure cell, as described previously [18]. Membranes for spectroscopic studies were isolated by sucrose step ultracentrifugation [18], and membranes for reaction-centre purification were pelleted by ultracentrifugation [16]. Reaction centres were purified as described in detail in [16]; the procedure employed solubilization of the reaction centre in lauryldimethylamine oxide (LDAO) followed by anion-exchange chromatography on DE52 (Whatman) and Sepharose Q (Pharmacia) columns and gel filtration on a Sephadex 200 preparative-grade column (Pharmacia). The method used for growing trigonal crystals of the *Rb. sphaeroides* reaction centre was based on that described previously [16,19]. Crystals with space group $P3_121$ were grown by sitting-drop vapour diffusion from droplets containing 9 mg/ml reaction centre, 0.09 % (v/v) LDAO, 3.5 % (w/v) 1,2,3-heptanetriol and 0.75 M potassium phosphate (pH 7.5). The drops were equilibrated against a reservoir of 1.5 M potassium phosphate. Trigonal crystals ap-

peared within 1–4 weeks and grew as prisms of variable size, ranging from 0.3 to 1.0 mm in the longest dimension. The crystals had unit cell dimensions of $a = b = 141.2$ Å, $c = 187.3$ Å, $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$. Crystals of the FM197R reaction centre, produced in the same way, had unit cell dimensions of $a = b = 142.4$ Å, $c = 188.1$ Å, $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$.

X-ray crystallography

X-ray diffraction data were collected at room temperature on a 30-cm MAR image-plate on station 9.6 (FM197R) and 9.5 (FM197R/WM115F) of the Daresbury synchrotron facility (Daresbury, Cheshire, U.K.). The data were processed using the Denzo and Scalepack packages [20]. Collection and refinement statistics are given in Table 1. Rigid body refinement was performed using XPLOR 3.1 [21], with the co-ordinates of the wild-type reaction centre [16] as a starting model. In the case of the FM197R/WM115F reaction centre, this was followed by restrained maximum-likelihood refinement in REFMAC [22], with waters fitted by ARPP [23]. In the Figures, structures are illustrated using the programs Molscript [24], Raster3D [25], O [26] and MINIMAGE [27]. The Fischer numbering system for BChl is used throughout this report.

Spectroscopy

With the exception of Fourier-transform Raman spectroscopy (FT-Raman) measurements, all spectroscopic analysis was carried out on membrane-bound reaction centres. Steady-state absorption spectra of reaction-centre-only membranes were obtained using a Beckman DU640 spectrophotometer; sodium ascorbate was added to ensure full reduction of the P BChls. FT-Raman spectra of purified reaction centres were acquired at room temperature with a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous diode-pumped Nd:YAG laser. Sodium ascorbate was present to prevent photo-oxidation of the P BChls. Other experimental procedures were essentially as described previously [18].

Time-resolved difference spectra were recorded at 77 K on a 30 Hz amplified dye laser system as described in [28]. The excitation pulse was of approx. 200 fs duration and was centred at 880 nm. Spectra were recorded between several tens of fs before the excitation pulse and 450 ps after this pulse, at time intervals that increased between 40 fs and 50 ps. Global analysis of the data set recorded over different wavelength regions was performed as described in detail elsewhere [28]. A correction for the wavelength dependence of time 0 due to group velocity dispersion was performed as described in [28].

The mid-point potential (E_m) of the P/P^+ redox couple was estimated by measuring oxidation of P at 542 nm on a ms timescale following a single flash of red light of approx. 10 μ s duration from a xenon flash lamp. The ambient redox potential was adjusted by addition of potassium ferricyanide or sodium dithionite, and was monitored using a combination platinum electrode (Kent). For all of the spectroscopic measurements, details of the sample conditions are given in the Figure legends.

Accession numbers

Atomic co-ordinates for the FM197R/WM115F and FM197R/YM177F reaction centres have been deposited in the Protein Data Bank, with the accession codes 1E6D and 1MPS, respectively.

Table 1 Crystallographic statistics for data collection and refinement

Ubi, ubiquinone; Spo, spheroidenone. For R_{merge} and completeness values, figures within parentheses refer to the statistics for the outer resolution shell, which was 2.38–2.3 Å for FM197R/WM115F and 2.64–2.55 Å for FM197R. $R_{\text{merge}} = \sum_i \sum_h |I(h) - \langle I(h) \rangle| / \sum_i \sum_h I(h)$, where $I(h)$ is the intensity of reflection h , $\sum_i$ is the sum over all reflections and $\sum_h$ is the sum over all i measurements of reflection h . R-factor is defined by $\sum_i |F_o| - |F_c| / \sum_i |F_o|$. Free-R was calculated with 5% reflections, selected randomly [47]. The Ramachandran plot was produced by Procheck version 3.0 [48].

	FM197R/WM115F	FM197R
Data-collection statistics		
Resolution range	26.4–2.3 Å	16.5–2.55 Å
R_{merge}	11.0% (53.0%)	9.1% (39.0%)
No. of unique reflections	90 855	67 571
Completeness	94.5% (71.2%)	93.6% (97.1%)
Refinement statistics		
No. of selected reflections	89 987	67 383
R-Factor	17.4%	20.2%
Free-R factor	20.0%	22.2%
Average B-factor		
Overall	42.9 Å ²	34.1 Å ²
Main chain	41.3 Å ²	31.6 Å ²
Side chain and waters	44.2 Å ²	35.0 Å ²
Geometry		
RMSD from ideality		
Bonds	0.014 Å	0.007 Å
Angles	2.130°	1.498°
Ramachandran plot		
Most favoured regions	91.8%	92.1%
Additional allowed areas	7.8%	7.2%
Generously allowed areas	0.4%	0.7%
Disallowed areas	0.0%	0.0%
Co-ordinate error	0.16 Å*	0.35 Å†
Model		
No. of protein residues	823	823
No. of cofactors	4 BChl, 2 BPhe, 2 Ubi, 1 Fe, 1 Spo	4 BChl, 2 BPhe, 2 Ubi, 1 Fe, 1 Spo
No. of waters	212	101
No. of detergent molecules	7	6
No. of phosphates	2	1

* Co-ordinate error was estimated by Cruickshank's DPI [49].

† Co-ordinate error was estimated by the Luzzati plot [50].

RESULTS AND DISCUSSION

Mutagenesis

Residue Phe M197 is positioned close to the acetyl carbonyl substituent group of the P_M BChl. The donation of a hydrogen bond to this carbonyl group has been demonstrated in reaction centres with the mutations Phe M197 to His and Phe M197 to Tyr [13–15]. In the present study, His, Tyr, Asp, Arg and Lys changes were made at the M197 position in order to examine other candidates for hydrogen-bond donation, and to study the effects of both basic and acidic amino acids on the BChls of P. The mutated M subunit genes were expressed in a strain of *Rb. sphaeroides* that lacks the antenna pigment–protein complexes that surround the reaction centre in the membrane [17].

The BChl and BPhe cofactors of the reaction centre give rise to strong absorbance bands in the near-infrared region of the spectrum. Although the attribution of these bands to individual cofactors is a complex issue, a good first-order approximation is that the band at 756 nm in the absorbance spectrum at room temperature of the wild-type reaction centre is attributable to the reaction centre BPhe (termed the H Q_y band), the band at 804 nm is attributable to the accessory BChls with a smaller contribution from the P BChls (termed the B Q_y band), whereas the band at 868 nm is attributable to the P BChls (termed the P Q_y band). The spectra of the FM197H, FM197D and FM197Y

reaction centres were similar to that of the wild-type reaction centre (Figure 1a), with the exception of a small (2–3 nm) red shift (in FM197Y) or blue shift (in FM197H and FM197D) of the P Q_y band, relative to the position in the wild-type reaction centre. In contrast, the FM197R and FM197K reaction centres exhibited a 14–15 nm blue shift of the P Q_y band, and there was also a small (1–2 nm) blue shift of the B Q_y band in these mutants. Possible origins of these shifts are discussed below.

Presence of a new hydrogen bond

The mutant reaction centres were screened for the presence of a hydrogen bond between the altered M197 residue and the acetyl carbonyl group of P_M by near-infrared FT-Raman. With excitation at 1064 nm, the 1600–1700 cm^{−1} region of the FT-Raman spectrum of ascorbate-reduced reaction centres is dominated by contributions from the acetyl and keto carbonyl groups of the P_L and P_M BChls (Figure 1b). This is because the 1064-nm excitation light is in pre-resonance with the P Q_y band, and these carbonyl groups are conjugated to the π electron system of the BChls that give rise to this band. As can be seen in Figure 1(b), the FT-Raman spectrum of purified wild-type reaction centres contains a contribution at approx. 1653 cm^{−1} (indicated by the arrow), which has been attributed to the acetyl carbonyl group of P_M [29]. This group does not form a hydrogen-

Figure 1 Spectroscopy of the mutant reaction centres

(a) Room-temperature absorption spectra of membrane-bound reaction centres. The maximum of the P_Q absorbance band is indicated by the vertical line. (b) FT-Raman spectra of purified reaction centres. Where resolved, the stretching frequency of the acetyl carbonyl of P_M is indicated by an arrow.

bond interaction with the surrounding protein. A second band at approx. 1620 cm⁻¹ has been attributed to the acetyl carbonyl group of P_L that is hydrogen-bonded to His L168 [29], the symmetry-related residue to Phe M197. In the FM197H mutant reaction centre, the band at 1653 cm⁻¹ is strongly reduced and a new band appears as a shoulder at approx. 1630 cm⁻¹ (indicated by the arrow), indicating the donation of a hydrogen bond to the acetyl carbonyl group of P_M by the new His at the M197 position

(Figure 1b). In the FM197Y reaction centre a large part of the band at 1653 cm⁻¹ is replaced by a new band at approx. 1635 cm⁻¹, indicating a weaker hydrogen bond between P_M and Tyr M197 (Figure 1b). Both of these results are consistent with previously published data on these mutant reaction centres [13–15], and act as positive controls for the formation of a new hydrogen bond. In the case of the FM197D reaction centre, which has not been reported on previously, evidence was also seen for a weak hydrogen bond with a band at 1638 cm⁻¹ in a spectrum that was otherwise similar to that of the FM197Y reaction centre (Figure 1b).

The strength of the hydrogen bonds formed following these mutations can be determined from the shift of the stretching frequency of the acetyl carbonyl group, from the empirical Badger-type relation [30]:

$$\frac{\Delta v_{\text{C=O}}}{v_{\text{C=O}}} = -K_{\text{C=O}} H_{\text{hb}}$$

where $\Delta v_{\text{C=O}}$ is the change in stretching frequency of the acetyl carbonyl group on the formation of a hydrogen bond, $v_{\text{C=O}}$ is the fundamental stretching frequency of the acetyl carbonyl group (1653 cm⁻¹ for the non-hydrogen-bonded acetyl carbonyl of P_M), $K_{\text{C=O}}$ is a proportionality coefficient with the value $4 \times 10^{-3} \text{ mol} \cdot \text{kcal}^{-1}$ [30] and H_{hb} is the energy of the hydrogen bond in kcal·mol⁻¹ (1 cal = 4.184 J). Using this equation, the strengths of the hydrogen bonds formed in the FM197H, FM197Y and FM197D reaction centres were calculated to be 3.5, 3.0 and 2.3 kcal·mol⁻¹ respectively.

The FT-Raman spectra of the FM197R and FM197K reaction centres were similar to one another, and were more difficult to interpret than those of the His, Tyr and Asp mutants. Although, as in the case of the latter reaction centres, there was a decrease in intensity of the band at 1653 cm⁻¹, there was no clear evidence for a new band in the region between 1620 and 1650 cm⁻¹ in the spectra of the FM197R and FM197K reaction centres (Figure 1b). The simplest interpretation of this result is that the stretching mode of the acetyl carbonyl group of P_M no longer contributes to the FT-Raman spectrum of P in the FM197R and FM197K mutants, or perhaps contributes only weakly. Possible reasons for this, and more information on the pattern of hydrogen bonds in the FM197R mutant, are discussed below.

***E_m* of P in the mutant reaction centres**

E_m P/P⁺ was measured by chemical titration, recording photo-oxidation of P at 542 nm on a ms timescale in membrane-bound reaction centres (see the Materials and methods section). A value of +484 mV (mean of two determinations) was obtained for the membrane-bound wild-type reaction centre, consistent with a previously determined value [31]. Titrations with membranes containing the mutant reaction centres yielded values (again the means of two determinations) of +504 mV (FM197D), +530 mV (FM197Y), +553 mV (FM197K), +562 mV (FM197R) and +603 mV (FM197H; results not shown). For each pair of titrations, the variation in the mid-point was between 6 and 15 mV. The changes in *E_m* P/P⁺ between the wild-type reaction centre and the FM197H and FM197Y reaction centres, +119 and +46 mV respectively, are in reasonable agreement with values of +125 and +31 mV obtained for purified reaction centres with these mutations [14,32]. These changes have been related to the formation of a strong hydrogen bond in the former mutant and a weaker hydrogen bond in the latter mutant [14,32]. The smallest effect on *E_m* P/P⁺ was seen in the FM197D reaction centre (+20 mV), consistent with the indication from FT-Raman spectroscopy that a weak hydrogen bond is present in this

Figure 2 Primary electron transfer at 77 K

SADS of spectrally and temporally distinct states formed during charge separation at 77 K in the wild-type (A–C) and FM197R (D–F) reaction centres. The lifetimes associated with the spectra are shown. Attribution of the spectra to states is discussed in the text.

reaction centre. The FM197K and FM197R mutants gave changes in E_m P/P⁺ relative to the wild-type reaction centre of +69 and +78 mV respectively. However, these changes do not allow a direct inference to be made concerning hydrogen bonding in these mutants.

The kinetics of primary electron transfer in the FM197R reaction centre

The effect of the FM197R mutation on primary electron transfer at 77 K was examined by ps-timescale transient absorbance spectroscopy. A series of absorbance difference spectra were recorded for the wild-type and FM197R reaction centres, covering the first 100 ps following excitation into the P Q_y band with an approx. 150 fs laser pulse. The spectra of both reaction centres (results not shown) exhibited the expected general features, with bleaching of the P Q_y band, stimulated emission from P* (manifested as an apparent additional bleach on the red side of the P Q_y band), and the development of electrochromic band shifts of the Q_y absorbance bands of the accessory BChls (at approx. 800 nm) and the BPhes (at approx. 760 nm). The bleach of the P Q_y band in the FM197R reaction centre was blue-shifted relative to that seen for the wild-type reaction centre, in line with the change in absorption spectrum.

In order to gain information on the processes that give rise to this spectral evolution, global analysis of the data was performed. The global analysis determines the minimum number of spectrally and temporally distinct components that are required to describe the experimental data. The species-associated difference spectra (SADS) obtained from a global analysis using an irreversible sequential scheme are shown in Figures 2(A)–2(C) for the wild-type reaction centre and Figures 2(D)–2(F) for the FM197R reaction centre. In the wild-type reaction centre, the SADS could be interpreted as a P* state that is created by the excitation pulse

(Figure 2A) and which decays to the radical pair state P⁺H_L[−] with a lifetime of 1.5 ps [28]. The P⁺H_L[−] state has a lifetime of 80 ps, and decays to the P⁺Q_A[−] state (Figure 2C), the lifetime of which is infinite on the timescale of the measurement. A similar scheme can be applied to the FM197R reaction centre, but the spectra indicate that the decay of the P* state is more complex than in the wild-type reaction centre. The lifetime of the species assigned as P* (Figure 2D) was 2.1 ps, similar to the 1.5 ps lifetime of this state in the wild-type reaction centre. Likewise, the lifetime of the state assigned as (mainly) P⁺H_L[−] (Figure 2E) was 94 ps in the FM197R reaction centre, similar to the 80 ps lifetime observed for the wild-type reaction centre. In contrast to results with the wild-type reaction centre, however, the spectrum of the 94-ps component in the FM197R reaction centre showed some contribution from P*-stimulated emission, on the red side of the bleach of the P Q_y band. This indicates a significant heterogeneity in the rate of decay of P* in the FM197R reaction centre. In the bulk of the reaction-centre population, P* decays with a lifetime similar to that observed in the wild-type reaction centre, but in a sub-population (approx. 20%) of FM197R reaction centres the P* state decays more slowly, on a timescale of several tens of ps. Possible origins of this heterogeneity are discussed below.

Crystallographic analysis of the FM197R mutant reaction centre

To provide additional information on the effects of the FM197R mutation, the structure of the FM197R reaction centre was determined by X-ray crystallography. In a recent publication, we described the X-ray structure of a double FM197R/YM177F mutant at an effective isotropic resolution of 2.55 Å [16]. In the present study, structures for a single FM197R mutant and a double FM197R/WM115F mutant were determined to resolutions of 2.6 and 2.3 Å respectively. The basic features of the structural change seen in the vicinity of the Arg M197 residue

Figure 3 Main structural changes in the FM197R/WM115F reaction centre

(a) Stereo view showing a superposition of the structure of the FM197R/WM115F reaction centre (yellow) and the wild-type reaction centre (red), focusing on P_L , P_M , B_L , residue M197 and nearby water molecules (1–3). Waters 1 and 2 take up part of the volume occupied by Phe M197 in the wild-type reaction centre. The introduction of waters 1 and 2 is accompanied by movement of water 3, and an out-of-plane rotation of the acetyl carbonyl group of P_M . (b) Stereo view showing the side chains of residues Arg M197, His M202 and Ser L158, the porphyrin rings of BChls P_L , P_M and B_L , and waters 1, 2 and 3 (see text). For contrast, the carbon atoms of the BChls are shown in yellow, and the carbon atoms of the amino acids are in grey. Crystallographically defined waters are shown as blue spheres. To highlight their locations, the oxygens (red) of the acetyl carbonyl group of P_M and the keto carbonyl group of B_L are enlarged.

were the same in all three mutant reaction centres, and have been discussed in detail elsewhere [16]. In this report, we will focus on how the observed changes in structure may explain the optical and electrochemical properties of the FM197R reaction centre, and on new information gleaned from the 2.3 Å resolution data for the FM197R/WM115F reaction centre, which is the structure that is described in detail below. As reported elsewhere [33], the structural changes accompanying the YM177F and WM115F mutations (these residues are in the carotenoid-binding pocket) are minimal, and do not appear to affect the structure of the reaction centre beyond the immediate vicinity of the M177 and M115 positions, respectively. In the case of the Trp-to-Phe mutation at the M115 position, the cavity created in the protein interior is filled by two new water molecules that are in suitable positions to form hydrogen-bond interactions with the surrounding protein [33].

Changes in the structure of the reaction centre caused by the FM197R mutation

The most dramatic aspect of the structural changes that accompanied the mutation of Phe M197 to Arg in both the FM197R/WM115F and FM197R reaction centres was a re-orientation of the side-chain of the M197 residue (Figure 3a; yellow structural model), such that it adopts a position quite different to that of the native Phe residue (Figure 3a; red structural model). Arg M197 forms a probable salt-bridge interaction with residue Asp L155 that is located at the periplasmic surface of the protein [16]. This reorientation was possible because of a cleft that extends from the surface of the protein inwards towards the M197 residue [16]. The reorientation created

a cavity in the interior of the protein in the immediate vicinity of the acetyl carbonyl group of P_M , which undergoes a net 20° rotation relative to the plane of the BChl macrocycle [16]. This rotation, which is evident from the superimposed structures in Figure 3(a), shortens the distance between the oxygen of this carbonyl group and the adjacent 5a methyl group of the B_L BChl, from 3.8 Å in the wild-type reaction centre to 3.2 Å in the FM197R/WM115F mutant.

The approx. 20° out-of-plane rotation observed for the acetyl group of P_M might provide an explanation for the absence of a contribution of the acetyl carbonyl group of P_M from the FT-Raman spectrum of the FM197R reaction centre (Figure 1b). As discussed above, only those carbonyl groups that are conjugated to the π electron system of the P BChls contribute to the FT-Raman spectrum of P [29]. An out-of-plane rotation of the acetyl group would be expected to weaken the degree of conjugation of this group with the π electron system of the P BChls.

Turning to the absorbance properties of P, in our previous report [16] we discussed the possibility that the pronounced blue shift of the P Q_y band in the FM197R reaction centre may also be the consequence of the observed out-of-plane rotation of the acetyl group of P_M , consistent with the calculations of Fajer and co-workers [34,35]. It is also worth noting that the replacement of Phe M197 with smaller residues (His and Glu) produced a small blue shift of the P Q_y band while the change to Tyr, a slightly larger residue than Phe, produced a small red shift of this band (Figure 1a). These changes likewise could reflect small rotations of the acetyl carbonyl group of P_M , following a change in the volume of the residue at the M197 position. However, it should be pointed out that there is some disagreement on the predicted effect of an out-of-plane rotation of this group on the

optical properties of P, and that the absorbance properties of a dimeric species such as P are sensitive to a number of parameters, including very small changes (approx. 0.1 Å) in spacing between the two BChls of the dimer [34–36]. Clearly, crystallographic data are needed on a number of mutant reaction centres with shifts in the position of the P Q_y band before any causal relationship can be established.

With regard to the redox properties of P, one possible explanation for the approx. 80 mV increase in E_m P/P⁺ caused by the FM197R mutation is that it arises from the formation of a hydrogen bond between the acetyl carbonyl of P_M and a water molecule, the location and interactions of which are discussed in the next section. This would be consistent with the trend of an increased E_m P/P⁺ observed in mutant reaction centres where the number of hydrogen bonds to the BChls of P is increased [14,32]. However, it might be the case that a weakening of the conjugation between this acetyl group and the π electron system of P, caused by the observed out-of-plane rotation, would minimize the effect of adding a new hydrogen bond. In addition, it is also possible that a decrease in the extent of conjugation of the π electron system arising from deconjugation of the carbonyl group might, in itself, have an effect on the redox properties of P [37]. At present, therefore, it is not possible to ascribe the observed increase in E_m P/P⁺ to one particular cause.

Evidence for two new buried water molecules in the FM197R reaction centre

The 2.3 Å resolution data on the FM197R/WM115F reaction centre revealed the presence of two new water molecules that partially fill the cavity created by the reorientation of Arg M197. The first of these waters (labelled 1 in Figure 3b) was also identified in the structure of the FM197R reaction centre (results not shown), and in the structure of the FM197R/YM177F reaction centre reported previously [16]. It is located within hydrogen-bonding distance (2.8 Å) of one arm of the rotated acetyl group of P_M, which we have assigned to the carbonyl bond [16]. In addition, water 1 is also within hydrogen-bonding distance of two further water molecules. One of these is a second new water (labelled 2 in Figure 3b), some 2.7 Å distant, that makes further possible connections with the surrounding protein via the oxygen of the side chain of Ser L158 (shown in Figure 3b, at the bottom left), which rotates to accommodate this interaction, and the backbone oxygen of Leu L154 (not shown). Water 2 was not identified with sufficient certainty in the lower-resolution structure of the FM197R/YM177F reaction centre, according to the criteria used for the identification of water molecules given in the Materials and methods section, and so was not described in our previous report [16].

The remaining possible connection of water 1 is to a third water molecule (labelled 3 in Figure 3b) that is also present in the structure of the wild-type reaction centre [3]. In the wild-type reaction centre, water 3 is within hydrogen-bonding distance (2.8 Å) of the oxygen of the keto carbonyl group of the accessory BChl B_L. It is also within hydrogen-bonding distance of the ND1 nitrogen of His M202 (2.9 Å; shown in Figure 3b, on the middle right), the backbone nitrogen of Gly M203 (3.4 Å; not shown) and the backbone carbonyl oxygen of Phe M197 (3.6 Å; not shown).

Modelling of water 3 in the FM197R/YM177F, FM197R and FM197R/WM115F reaction centres

In the electron-density map of the wild-type reaction centre, water 3 is modelled into the centre of a roughly spherical

electron-density feature located close to the B_L BChl (Figure 4a). This places water 3 approx. 2.8 Å from the oxygen of the keto carbonyl group of this BChl. The electron density corresponding to this water was also roughly spherical in the electron-density maps of the FM197R and FM197R/YM177F reaction centres (Figures 4c and 4d, respectively). After modelling, water 3 was placed 3.6 and 3.3 Å from the keto oxygen of the B_L BChl in the structures of the FM197R and FM197R/YM177F reaction centres, respectively. On comparing both structures with that of the wild-type reaction centre, water 3 in the mutant complexes gave the appearance of having moved towards the new water 1 (which is also shown in Figures 4b, 4c and 4d), suggesting an interaction between water 3 and water 1.

In the better-quality electron-density maps of the FM197R/WM115F reaction centre (Figure 4b), the electron density corresponding to water 3 was in a similar position to the equivalent density in the maps of the FM197R and FM197R/YM177F mutants, but it had a distinctly elongated shape (Figure 4b). In the final structural model, water 3 was modelled at the centre of this density (red sphere), placing it 3.1 Å from the oxygen of the keto carbonyl group of B_L. Modelling water 3 into either arm of the elongated density (white spheres) altered this distance to 2.9 and 3.3 Å. A possible explanation for this observation is that water 3 can adopt more than one position.

Implications of the location and potential interactions of waters 1 and 3

In the structural models of the three reaction centres with the FM197R mutation, water 3 gave the appearance of having moved towards the new water 1, with the result that it was located 3.5 Å (FM197R), 4.2 Å (FM197R/YM177F) and 3.9 Å (FM197R/WM115F) from water 1 (Figure 4). In considering the variation in these values, it should be remembered that the expected co-ordinate error for structures at this resolution (2.3–2.6 Å) is in the order of 0.3–0.5 Å, and so the significance of this variation between mutant structures is borderline.

The placement of waters 1 and 3 within hydrogen-bonding distance of one another, and within hydrogen-bond distance of the acetyl carbonyl of P_M and the keto carbonyl of B_L, respectively, raises an intriguing possibility. A feature of the design of the reaction centre is that there are no direct through-bond links between the π electron system of the P BChls and that of the B_L BChl, which is widely thought to be the first acceptor of electrons during light-driven electron transfer in the bacterial reaction centre. In the three reaction centres containing the FM197R mutation described in this study, a connection between waters 1 and 3 might provide such a link.

Factors that can alter the rate of primary electron transfer in a mutant reaction centre

The effects of mutagenesis on the rate of primary electron transfer (k_{et}) in the reaction centre are usually discussed in terms of the equations that describe non-adiabatic electron transfer. As reviewed in [7,8], within this framework the rate of electron transfer between a weakly coupled donor and acceptor redox centre in a protein is dependent on three parameters. The first, ΔG° , is the difference in free energy between the reactant and product state (i.e. the driving force of the reaction), and will be sensitive to a mutation that affects the redox properties of the electron donor or acceptor. The second is λ , the reorganization energy, which is defined as the free energy required to distort the nuclear configuration of the reactant state into that of the product state without electron transfer. The reorganization

Figure 4 REFMAC $2mF_o - DF_c$ maps showing the electron density corresponding to water 3

For the FM197R/WM115F reaction centre (**b**), water 3 is either modelled into the middle of the relevant density (red sphere), or into either arm of the density (white spheres; see text). Also shown are the electron density and fitted structures for the B_L BChl (on the left in each panel), residue His M202 (on the right in each panel), part of the P_M BChl (bottom right in each panel) and, for the mutant reaction centres, water 1.

energy, which includes the energy required to reorient solvent dipoles, is sensitive to the polarity of the medium around and between the donor and acceptor redox centres, and so may change if this polarity changes. The third parameter that governs the rate of electron transfer is the electronic coupling, V_R , between the donor and acceptor states, which decreases with the edge-to-edge distance (R) between donor and acceptor molecule. The dependence of the electronic coupling on the distance between donor and acceptor is modulated by the factor β , which describes the effect of the intervening medium on propagation of the electronic wavefunction.

In principle, mutagenesis can affect the rate of electron transfer through the electronic coupling term in two ways. The first is to bring about a change in the distance, R , between the donor and acceptor. The second is to change the electron-tunnelling properties of the medium between the redox cofactors (i.e. change β), such that it becomes a better or worse medium through which to propagate the electronic wavefunction. In considering this possibility, it should be noted that considerable discussion has surrounded the relevance of the composition of the medium between redox centres in determining the rate of electron transfer [7,8,38]. Much of this discussion has centred around whether the protein medium should be viewed as an organic glass, with the detailed structure of the medium being unimportant, or whether discrete pathways for electron tunnelling play a key role in determining the rate of electron transfer. Such pathways consist of connected covalent bonds and hydrogen bonds, together with

occasional through-space jumps, with a different value of β for each type of connection. In the view where the detailed structure of the medium is unimportant, β has a fixed intermediate value corresponding to a weighted average of β values for tunnelling through different sorts of medium (i.e. through-space or through-bond).

Changes in structure caused by the FM197R mutation, and their effects on electron transfer

From the preceding section, it is clear that the rate of a reaction such as primary electron transfer is dependent on a number of parameters, all of which can be affected by mutagenesis. Unfortunately, most of these parameters cannot be measured directly, and so in most analyses a number of simplifying assumptions have to be made. As an illustration, in experiments using a series of mutations to examine the relationship between ΔG° and k_{et} [39,40], it has been assumed that the measured change in $E_m P/P^+$ equates to the change in ΔG° (i.e. $E_m B_L/B_L^-$ does not change or changes in a constant way). It has also been assumed that λ , β and R are not affected by the mutations.

The combination of spectroscopic and crystallographic data on the FM197R reaction centre presented in this report indicates several ways in which the Phe-to-Arg mutation, and the associated structural changes, could affect the parameters that govern the rate of primary electron transfer. Two observations suggest that the driving force for this reaction, ΔG° , is affected by

the FM197R mutation. The first of these is the measured 78 mV increase in $E_m P/P^+$, which would be expected to cause a decrease in the ΔG° for primary electron transfer by raising the free energy of the $P^+B_L^-$ radical pair state. The second is the 15 nm blue shift of the P Q_y band. If this shift is accompanied by a corresponding shift of the zero-zero transition of the P band, this translates into an approx. 30 meV increase in the free energy of the P^* state, which will partially offset the decrease in ΔG° for primary electron transfer caused by the increase in $E_m P/P^+$. The net effect of these opposing changes would therefore be to decrease ΔG° for primary electron transfer by approx. 50 mV.

Assuming that other rate-governing parameters are not altered by a mutation, a decrease in ΔG° would be expected to slow the rate of primary electron transfer. In broad agreement with this expectation, a slowing of the rate of P^* decay has been observed in mutant reaction centres in which a single additional hydrogen bond to P produces an increase in $E_m P/P^+$ [14,41]. In the wild-type reaction centre, the lifetime of the P^* state at room temperature is approx. 3.5 ps. In the FM197Y reaction centre, where $E_m P/P^+$ is raised by 31 mV, the lifetime of the P^* state at room temperature is increased to 6 ps in the mutant [14]. Similarly, in LL131H and LM160H mutant reaction centres a new hydrogen bond is donated to the keto carbonyls of P_L and P_M , raising $E_m P/P^+$ by 80 and 55 mV respectively. The lifetime of the P^* state at room temperature in these mutants was increased to 12.2 and 5.7 ps respectively [41].

In the wild-type reaction centre, the lifetime of the P^* state decreases as the temperature is lowered (to approx. 1.5 ps at 77 K). One interpretation of this is that the energetics of primary electron transfer in the wild-type reaction centre are optimized, and reside in the region where $-\Delta G^\circ = \lambda$. An expected effect of decreasing ΔG° in a mutant reaction centre is therefore to cause primary electron transfer to become thermally activated, further slowing the reaction at low temperature. In the present study, the P^* states in the bulk of the FM197R reaction centres decayed with a lifetime of 2.1 ps at 77 K, similar to the 1.5 ps lifetime obtained for the wild-type reaction centre. In a sub-population of FM197R reaction centres, however, the P^* state decayed with a lifetime of several tens of ps, which is more consistent with the expected effects of decreasing ΔG° . These findings suggest that, at least in part of the population of FM197R reaction centres, a change in a parameter other than ΔG° also affects the rate of primary electron transfer, and offsets the slowing of this reaction that is expected from a decrease in the driving force.

The results of the crystallographic analysis suggest two possible sources of this effect. The first is a change in λ brought about as a result of the changes in the polarity of the environment of the P and B_L BChls. As can be seen from Figure 3, the effect of the Phe-to-Arg mutation at M197 is to replace a hydrophobic Phe residue with two waters. However, increasing the polarity of the environment in this interface region might be expected to increase λ , whereas to counteract the effect of a decrease in ΔG° a decrease in λ would be required.

The second possibility is that the changes in structure seen in the interface region between P and B_L have an influence on the electronic coupling between the two. In the wild-type reaction centre, the closest approaches between the macrocycles of P_L/P_M and B_L are through-space gaps between carbon 10 of ring V of P_L and the carbon 4a of ring II of B_L (3.7 Å), and between the acetyl carbonyl group of ring I of P_M and the 5a methyl group of ring III of B_L (3.8 Å; see Figure 5a). The out-of-plane rotation of the acetyl carbonyl of P_M , which in the wild-type reaction centre forms part of the π electron system of the P BChls, brings the carbonyl oxygen some 0.6 Å closer to the macrocycle of B_L , which significantly decreases the length of the vacuum through

which the electron would have to tunnel from P to B_L (Figure 5). If these closest approaches have a strong influence on the rate of electron transfer, this rotation might be expected to bring about an acceleration of the rate. However, another factor that has to be considered is the possibility that the out-of-plane rotation deconjugates the carbonyl group from the π electron system of the P BChls. This deconjugation would counteract the potential benefits of bringing the oxygen of the acetyl carbonyl of P_M closer to the macrocycle of B_L , by increasing the distance over which electron tunnelling has to occur to include the C2–C2a and C2a=O bonds of the acetyl group.

A final possibility is that waters 1 and 3 provide a direct through-bond link between P_M and B_L . As yet we do not have proof of this, because although it is possible to propose the presence of hydrogen bonds from the positions of atoms in the X-ray data, the data at the present resolution cannot prove the existence of hydrogen bonds. However, in considering this possibility it is worth noting that although a 'hydrogen-bond bridge' would provide a more convoluted route for electron tunnelling between P_M and B_L than the closest approach described in the last paragraph, the fact that it involves through-bond rather than through-vacuum electron tunnelling would make it a competitive route. Tunnelling from the oxygen of the acetyl carbonyl of P_M to carbon 5 of B_L ring III (the nearest atom of the π electron system of B_L) would involve a through-space jump of 3.2 Å between the acetyl oxygen and the C5a methyl carbon of B_L , and through-bond tunnelling over approx. 1.5 Å across the C5a–C5 bond (see Figure 5). In contrast, tunnelling from the oxygen of the acetyl carbonyl of P_M to the oxygen of the keto carbonyl of B_L , via waters 1 and 3, would involve three hydrogen bonds totalling 9.8 Å in length. However, because a hydrogen bond provides a medium that is approx. three times more effective for electron tunnelling than a vacuum [42], then the two routes would be comparable in terms of their effectiveness. In other words, the greater electron tunnelling distance associated with the 'hydrogen-bond route' when compared with the 'through-vacuum route' would be offset by more favourable values of β associated with the former.

Possible sources of heterogeneity in the kinetics of P^* decay in the FM197R reaction centre

Time-resolved spectroscopy shows that, at 77 K, decay of the P^* state in the FM197R reaction centre shows two principal characteristics. First, decay of the bulk of the P^* state takes place with a lifetime similar to that seen in the wild-type reaction centre. The discussion in the last section explores how this may be achieved when, at face value, the increase in $E_m P/P^+$ determined for this mutant would suggest a decrease in the driving force for primary electron transfer. The most likely explanation is a change in one or more additional parameters that either counteracts the effect on ΔG° of increasing $E_m P/P^+$, or a change in λ and/or V_R that compensates for the decrease in ΔG° .

The second feature of P^* decay in the FM197R reaction centre is its pronounced multi-exponential character, with the P^* state having a lifetime of several tens of ps in a significant fraction of the reaction-centre population. In fact, in recent years time-resolved absorption and fluorescence measurements with high temporal resolution have revealed that the decay of the P^* state in the wild-type reaction centre is multi-exponential, particularly at room temperature (reviewed in [5]). Two principal explanations have been put forward to account for this multi-exponential behaviour. The first is that it is caused by 'static heterogeneity', the 'slow' P^* lifetimes originating from a minority of reaction

Figure 5 View of the region of contact between P_L/P_M and B_L , and the postulated hydrogen-bond bridge in the FM197R/WM115F reaction centre involving waters 1 and 3

(a) Possible routes for electron tunnelling are indicated by the small magenta spheres, and distances between atoms are given in italics (in Å; figures in parentheses refer to analogous measurements made on the wild-type reaction centre). Carbon atoms specified in the text are labelled (grey), as are rings II and V of B_L and ring V of P_L . (b) Stereo representation of (a).

centres in the 'slow' tail of a distribution of lifetimes. The energetic basis for this distribution could be inhomogeneity in one or more rate-determining parameters such as λ , ΔG^0 or V_R . The principal alternative explanation is that the multiple lifetimes represent a time-dependent energetic relaxation of the $P^+H_L^-$ intermediate due to solvation of the radical pair by the protein environment, which causes a time-dependent increase in the free-energy gap between P^* and $P^+H_L^-$. This energetic relaxation of $P^+H_L^-$ is probed in the concentration profile of P^* due to some amount of thermal repopulation of P^* from $P^+H_L^-$ (i.e. a reversal of the primary electron-transfer reaction).

In the FM197R reaction centre, P^* decay at 77 K was strongly multi-exponential. One explanation for this could be that the changes in structure that result from the mutation cause an

enhancement of static heterogeneity in the reaction-centre population. In the last section, we highlighted a number of possible ways in which the structural changes caused by the Phe-to-Arg mutation could affect ΔG^0 , λ or V_R in such a way as to achieve a lifetime of 2.1 ps for P^* in the bulk of the reaction-centre population. Conceivably, some variation in these structural changes could bring about the sizeable population of reaction centres in which decay of the P^* is slow. Examples would be variation in the precise conformation of the acetyl carbonyl of P_M , or in the integrity of the putative hydrogen-bond bridge connecting the acetyl carbonyl of P_M to the keto carbonyl of B_L . Another possibility is that the mutation changes the relaxation properties of the radical pair states $P^+B_L^-$ and $P^+H_L^-$, such that more thermal repopulation of the P^* state is possible in the

FM197R reaction centre at 77 K than in the wild-type complex at this temperature.

Integrity of the hydrogen-bond bridge

Perhaps the most intriguing feature of the changes in structure caused by mutation of Phe M197 to Arg is the inclusion of waters 1 and 2 in the cavity created by reorientation of the M197 residue (Figure 3), and the possibility that water 1 may complete a through-bond connection between P_M and B_L (Figure 5). Although the X-ray crystal structures of the FM197R/WM115F, FM197R/YM177F and FM197R mutants show that waters 1 and 3 are in suitable positions to interact with each other and the adjacent carbonyl groups (Figure 4), the X-ray data does not provide proof that hydrogen-bond connections between these groups exist. Having said this, the fact that we can see these waters in the X-ray structure indicates that they are fixed in space, presumably because they engage in hydrogen-bond interactions with neighbouring groups. Although the length of this hydrogen-bond bridge is too great to provide a 'fast track' for primary electron transfer, in principle it could provide a route for electron tunnelling that would be comparable with the shortest through-space routes between P_L/P_M and B_L . It is therefore worth considering the question of whether or not this connection is intact in the FM197R reaction centre.

Considering first the question of a possible hydrogen-bond interaction between water 1 and the acetyl carbonyl of P_M , FT-Raman spectroscopy did not provide any information on the interactions of this acetyl group (see above). The best evidence in support of a hydrogen-bond interaction is provided by the 78-mV increase in $E_m P/P^+$ in the FM197R reaction centre, which would be consistent with the formation of a moderate-strength hydrogen bond between the acetyl carbonyl of P_M and the adjacent water.

The best evidence that water 1 is connected to water 3 comes from the shift in the position of the latter caused by the FM197R mutation (Figures 3a and 4). This change in geometry essentially involved movement of water 3 laterally with respect to this carbonyl group, and towards the newly introduced water 1.

Finally, we turn to the question of whether water 3 is hydrogen-bonded to the keto carbonyl of B_L . There is some disagreement over this point in the literature. In the recent high-resolution X-ray crystal structures of both the *Rb. sphaeroides* [3] and *Rps. viridis* [1] wild-type reaction centres it was proposed that, because of its position, water 3 is hydrogen-bonded to the adjacent keto carbonyl group of the B_L BChl. This assignment is supported by the resonance Raman measurements of Robert and Lutz [43], who observed a downshift of a Raman band that was attributed to the keto carbonyl of B_L from 1689 to 1675 cm^{-1} on the formation of P^+ . The interpretation of this observation was that a weak hydrogen bond is formed between this carbonyl group and the surrounding protein on oxidation of P. Robert and Lutz [43] further suggested that because free-from-interaction keto carbonyl groups can have a stretching frequency as high as 1710 cm^{-1} , the data could also be consistent with the strengthening of an existing weak hydrogen bond between the keto carbonyl group and the surrounding protein following oxidation of P. On the basis of the X-ray crystal structure, the only possible donor of this hydrogen bond would be water 3 [44,45].

Recently, however, Czarnecki et al. [46] have pointed out that the stretching frequencies of the keto carbonyl groups of B_L and B_M in resonance Raman spectra are in the 1686–1693 cm^{-1} region. This is similar to the frequency obtained for this group in isolated BChl in a non-hydrogen-bonding solvent, and as a result these workers have called into question whether the

9-keto carbonyl of B_L is in fact hydrogen-bonded to the adjacent water molecule [46], at least when P is not oxidized. This is clearly an area that requires further investigation.

An interesting point is that water 3 is also within hydrogen-bonding distance of the side-chain of His M202, which donates the axial ligand to the magnesium of the P_M BChl. Given this connection to a residue that is bonded to P, it has been suggested that oxidation of P could cause subtle changes in the structure of the protein-cofactor matrix that lead to the formation of a moderate-strength hydrogen bond between water 3 and the keto carbonyl of B_L , or possibly the strengthening of a pre-existing weak hydrogen bond between the two [44,45]. A small movement of water 3 on oxidation of P might be sufficient to produce this effect, and in this context the possibility discussed above, that water 3 may be able to adopt more than one position, is intriguing.

To summarize, therefore, there are indications that the structural changes consequent on the FM197R mutation, which include the introduction of water 1, result in a network of hydrogen-bond interactions that connect the acetyl carbonyl of P_M with the keto carbonyl of B_L via waters 1 and 3. However, at present there is no proof that this hydrogen-bond bridge is intact, and further investigation of this is required. In the light of resonance Raman results on the bonding of the keto carbonyl of B_L to the surrounding protein [43], and the possibility that water 3 can adopt more than one conformation (see above), it also seems prudent to consider the possibility that the strength of this through-bond interaction could vary according to the redox state of the neighbouring cofactors. This underscores the principle that there is mobility in this part of the protein, and that small variations in the distances between atoms within a population of reaction centres could give rise to some of the heterogeneity in electron-transfer rates that is observed for this particular mutant, even at low temperature.

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