

Regular paper

Mutations that modify or exclude binding of the Q_A ubiquinone and carotenoid in the reaction center from *Rhodobacter sphaeroides*

Justin P. Ridge¹, Marion E. van Brederode², Matthew G. Goodwin¹, Rienk van Grondelle² & Michael R. Jones^{1,*}

¹*Krebs Institute for Biomolecular Research and Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2UH, UK;* ²*Department of Physics and Astronomy, Free University of Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands;* **Author for correspondence and/or reprints (fax: +44-114-2728697; e-mail: m.jones@sheffield.ac.uk)*

Received 22 June 1998; accepted in revised form 21 October 1998

Key words: electron transfer, herbicide binding, membrane protein, site-directed mutagenesis, ubiquinone binding

Abstract

Three single-site mutations have been introduced at positions close to the Q_A ubiquinone in the reaction centre from *Rhodobacter sphaeroides*. Two of these mutations, Ala M260 to Trp and Ala M248 to Trp, result in a reaction centre that does not support photosynthetic growth of the bacterium, and in which electron transfer to the Q_A ubiquinone is abolished. In the reaction centre with an Ala to Trp mutation at the M260 residue, electron transfer from the primary donor to the acceptor bacteriopheophytin is not affected by the mutation, but electron transfer from the acceptor bacteriopheophytin to Q_A is not observed. The most likely basis for these effects is that the mutation produces a structural change that excludes binding of the Q_A ubiquinone. A third mutation, Leu M215 to Trp, produces a reaction centre that has an impaired capacity for supporting photosynthetic growth. The mutation changes the nature of ubiquinone binding at the Q_A site, and renders the site sensitive to quinone site inhibitors such as *o*-phenanthroline. Adopting a similar approach, in which a small residue located close to a cofactor is changed to a more bulky residue, we show that the reaction centre can be rendered carotenoid-less by the mutation Gly M71 to Leu.

Abbreviations: Bchl – bacteriochlorophyll; Bphe – bacteriopheophytin; E_m – mid-point redox potential; H_L – acceptor bacteriopheophytin; LDAO – *N*-lauryl-*N,N*-dimethylamine-*N*-oxide; P – primary donor of electrons; Q_A – primary acceptor ubiquinone; Q_B – secondary acceptor ubiquinone; RC – reaction centre; SADS – Species Associated Difference Spectra; UQ₁₀ – ubiquinone-10; UQ₀ – ubiquinone-0

Introduction

The process by which photosynthetic organisms convert the energy of light into electrochemical energy takes place in membrane-bound pigment-protein complexes termed reaction centres (RCs). The details of this energy transduction are best understood in the RC from *Rhodobacter (Rb.) sphaeroides*, the structure of which has been determined to atomic resolution (Al-

len et al. 1986, 1987; Chang et al. 1986; Komiya et al. 1988; Chang et al. 1991; Ermler et al. 1994a,b; Stowell et al. 1997). The redox centres of the *Rb. sphaeroides* RC are four molecules of bacteriochlorophyll (Bchl), two molecules of bacteriopheophytin (Bphe) and two molecules of ubiquinone-10 (UQ₁₀). Two of the Bchls are located close to the periplasmic face of the RC, where they form an excitonically-coupled ‘special pair’. The remaining redox centres

are arranged in two membrane-spanning branches around an axis of approximate two-fold symmetry that runs perpendicular to the plane of the membrane.

Extensive studies have revealed the route of light-driven transmembrane electron transfer in the wild type (WT) RC, although a number of details concerning the precise mechanism of individual steps in the electron transfer process remain to be clarified (Parson 1991; Fleming and van Grondelle 1994). The primary donor of electrons (P) is the first singlet excited state of the special pair of Bchls (P^*). This state drives the reduction of a Bphe (denoted H_L) located approximately half-way across the membrane in 3–4 picoseconds (ps). There is increasing evidence that the anion of the intervening Bchl (B_L^-) acts as an intermediate in the transfer of the electron from P^* to H_L (Holzapfel et al. 1989; Holzapfel et al. 1990; Arlt et al. 1993; Schmidt et al. 1994; Arlt et al. 1996a; Holzwarth and Müller 1996; Van Stokkum et al. 1997). Transfer of the electron across the membrane dielectric is completed by the movement of the electron from H_L to the Q_A ubiquinone, which is tightly bound to the RC and which operates as a one-electron carrier. Electrons are passed out of the RC by electron transfer from the Q_A ubiquinone to the Q_B ubiquinone, the latter operating as a two-electron/two-proton carrier. The Q_B ubiquinone is free to diffuse into the intramembrane space, and so shuttle electrons to the cytochrome *bc*₁ complex.

In studying the primary reactions of transmembrane electron transfer and the electronic structure of the cofactors involved, a number of techniques call for the reduction or removal of the tightly bound Q_A ubiquinone. This has the effect of blocking forward electron transfer from H_L^- , promoting charge recombination or the formation of singlet or triplet excited states of P. Reduction of Q_A can be achieved using millimolar concentrations of reagents such as sodium ascorbate, sodium dithionite or dithiothreitol, whilst removal of the Q_A ubiquinone is usually achieved by incubation of reaction centres in the presence of high concentrations of *o*-phenanthroline and *N*-lauryl-*N,N*-dimethylamine-*N*-oxide (LDAO) (Okamura et al. 1975). *o*-Phenanthroline is well known as a competitive inhibitor of ubiquinone binding at the Q_B site (Wraight and Stein 1980; Diner et al. 1984), whilst LDAO is a detergent that is commonly used (at much lower concentrations) in solubilisation, purification and crystallisation of RCs. In studies of RCs in membranes therefore, reduction of Q_A is achievable but removal of the ubiquinone is not.

In this report we describe the properties of three mutants of the *Rb. sphaeroides* RC that were designed with a view to excluding ubiquinone from the Q_A binding pocket, therefore providing an alternative means of blocking forward electron transfer from H_L^- in membrane-bound RCs. The approach taken was to replace residues in the immediate vicinity of the Q_A ubiquinone with the bulky residue tryptophan. In two cases, this simple approach appears to have generated a RC that completely lacks the Q_A ubiquinone, but which in other respects seems to be largely unaffected by the mutation. In a third case, a structural change has been brought about that appears to have altered the nature of ubiquinone binding at the Q_A site. To extend the usefulness of the RCs that lack the Q_A ubiquinone, a fourth mutation has also been designed that renders the RC carotenoid-less.

Materials and methods

Experimental material

Single site mutations were introduced using mismatch oligonucleotides and a mutagenesis system based upon the pALTER-1 plasmid (Promega). Changes in DNA sequence were restricted to the target codon. The template for mutation GM71L was plasmid pALTSP-1, which was pALTER-1 containing a 315 bp *SalI*–*PstI* fragment encompassing codons 1–83 of the *pufM* gene. The template for mutation LM215W was pALTTPC-1, which was pALTER-1 containing a 459 bp *PstI*–*SacI* fragment encompassing codons 83–235 of the *pufM* gene. The template for mutations AM248W and AM260W was plasmid pALTTCB-1, which was pALTER-1 containing a 236 bp *SacI*–*BamHI* fragment encompassing codons 234–307 of the *pufM* gene. Mutant fragments were identified by dideoxy sequencing and were cloned into plasmid pUCXB-1, which is a derivative of pUC19 containing a 1841bp *XbaI*–*BamHI* fragment encompassing *pufLM* (McAuley-Hecht et al. 1998). The *XbaI*–*BamHI* fragments containing the mutations were then shuttled into plasmid pRKEH10 (Jones et al. 1992b), which is a derivative of broad-host-range vector pRK415 containing a 6.55 kb *EcoRI*–*HindIII* fragment encoding *pufQBALMX*. The derivatives of plasmid pRKEH10 were introduced into *Rb. sphaeroides* strain DD13 (Jones et al. 1992a) by conjugative transfer, as described in (Jones et al. 1992b). Transconjugant strains were selected as described in (Jones et al. 1992a,b),

and contained RCs and the LH1 antenna complex, but lacked the LH2 antenna. Strains with an antenna-deficient (i.e. RC-only) phenotype were also constructed by shuttling the mutated *XbaI*–*Bam*HI fragments into plasmid pRKEH10D (Jones et al. 1992b), a derivative of pRKEH10 with a 300 bp deletion in the structural genes for the LH1 antenna complex (*pufBA*). Except where specified, experimental material consisted of intracytoplasmic membranes prepared using a French pressure cell from bacterial cells with an RC-only phenotype, grown under semi-aerobic conditions in the dark (Jones et al. 1994). Control strains containing WT RCs were the RC-only strain RCO2, and strain RCLH12, which has a RC⁺LH1⁺LH2⁻ phenotype (McGlynn et al. 1994).

Column purification of RCs

Mutant RCs were isolated from resuspended freshly-prepared intracytoplasmic membranes. Solid NaCl was added to the membranes to a final concentration of 1 M, the suspension was stirred briefly, and the membranes pelleted by centrifugation at approximately $250\,000 \times g$ for 2 h at 4 °C. The supernatant was decanted, and the salt-washed membranes were resuspended to a concentration of 1 absorbance unit per cm at 803 nm in 20 mM Tris/HCl (pH 8.0). LDAO (Fluka) was added to a final concentration of 0.5%, the suspension was centrifuged at $250\,000 \times g$ for 1 h, and the supernatant containing solubilised RCs was decanted. Purification of LM215W RCs was achieved by one pass of the solubilised material through a Poros anion exchange column (PerSeptive Biosystems Inc., MA, USA). Chromatographic separation was performed with a stepped gradient of increasing NaCl concentration in 20 mM Tris/HCl (pH 8.0)/0.1% LDAO buffer. Elution of bound RCs was achieved at a NaCl concentration of approximately 250 mM. Pooled RCs from the Poros column typically had an absorbance ratio $A_{280}/A_{802} = 1.5$. GM71L RCs were purified by one pass of the solubilised material through a DE52 (Whatman) anion exchange column. Chromatographic separation was performed with a stepped gradient of increasing NaCl concentration in Tris/HCl (pH 8.0)/0.1% LDAO buffer. Elution of bound RCs was achieved by washing the column with buffer containing 20 mM Tris/HCl (pH 8.0)/300 mM NaCl/0.1% LDAO. The GM71L RCs were further purified by gel filtration using a Sephadex 200 (Pharmacia) preparative grade column.

Spectroscopy

Absorbance spectra were recorded using a Beckman DU-640 spectrophotometer. Fluorescence emission spectra were recorded using a Fluorolog spectrofluorimeter (SPEX Industries Inc., NJ, USA) with samples of membranes maintained at 77 K in an Oxford Instruments DN1704 liquid nitrogen cryostat. Millisecond (ms) timescale transient absorption was recorded using a single beam spectrophotometer of local design. Excitation was provided by a pair of Xenon flashlamps (FWHM = 10 μ s). The excitation light was passed through red glass cut-off filters to ensure excitation was in the near-infrared region (>700 nm). The measuring light was passed through a monochromator and was detected using a photomultiplier which was protected from the excitation light by CS4-94 and CS4-96 filters (Corning). The output from the photomultiplier was passed through an amplifier, and analogue to digital conversion of the signal was performed using a Rapid Systems R2000 digital oscilloscope (Rapid Systems, WA, USA). Samples were housed in a glass cuvette with an optical path length of 1 cm, and where necessary anaerobic conditions were obtained by flushing the cuvette with a continuous stream of oxygen-free nitrogen. For the purposes of comparison of different samples, in all experiments purified RCs or RC-only membranes were diluted to a concentration of 0.5 μ M, using the absorbance band at 802 nm and an extinction coefficient of 288 mM^{-1} (Straley et al. 1973). In assessing the percentage of Q_A activity from measurements of photo-oxidation of P, absorbance transients were compared to the transient obtained for WT RCs or RC-only membranes, which were assumed to exhibit 100% Q_A activity.

The mid-point potential of the P/P⁺ redox couple (E_m P/P⁺) was determined by chemical titration. Samples consisted of intracytoplasmic membranes diluted to a RC concentration of approximately 0.6 μ M in 100 mM potassium phosphate buffer (pH 7.8), and housed in a glass cuvette that had been purged with a continuous stream of oxygen-free nitrogen. Redox mediators were added to the following concentrations: phenazine methosulphate, 2 μ M; phenazine ethosulphate, 2 μ M; *N,N,N',N'*-tetramethyl-1,4-phenylenediamine, 10 μ M. The ambient redox potential was adjusted by small additions of potassium ferricyanide or sodium ascorbate, and was monitored using a micro combination platinum electrode (Kent ABB). All titrations were reversible. The percentage of P in the reduced state was determined

by measuring the amplitude of the P Q_y absorbance band, at the maximum of this band in the fully reduced RC, using a Beckman DU-640 scanning spectrophotometer. The amplitude of the band was plotted as a function of redox potential, and the data was fitted with a Nernst curve with $n = 1$.

Sub-ps transient absorbance difference spectroscopy of WT and AM260W RC-only membranes was performed at 77 K using an amplified 30 Hz dye laser system (van Brederode et al. 1997a). The excitation pulse was centred at 880 nm. Data was recorded over two overlapping wavelength windows, 700–830 nm and 820–950 nm. A series of 90 spectra were recorded at increasing time intervals covering the first 300–500 ps. For the AM260W RC a time series was also recorded up to 1.5 ns. Global analysis of the data sets recorded over different wavelength regions and time intervals was performed in a simultaneous manner, as described previously (van Brederode et al. 1997a). A correction for the wavelength dependence of time zero due to group velocity dispersion was performed as described previously (van Brederode et al. 1997a).

Results

Mutant construction and steady state spectroscopy

Three mutations were introduced into the *pufM* gene that encodes the M subunit of the RC. These were Leu M215 to Trp (LM215W), Ala M248 to Trp (AM248W) and Ala M260 to Trp (AM260W). Residues Ala M248 and Ala M260 both lie within 4 Å of the ‘head group’ of the Q_A ubiquinone, with the backbone amide nitrogen of Ala M260 forming an H-bond with the distal keto oxygen of the ubiquinone head group (Figure 1A). Residue Leu M215 is located close to the isoprenoid sidechain of Q_A where it exits the main body of the RC into the membrane phase (Figure 1A). All three mutant *pufM* genes were introduced into plasmid pRKEH10D which was expressed in *Rb. sphaeroides* strain DD13, generating transconjugant strains with an antenna-deficient phenotype (see ‘Materials and methods’). Absorption spectra recorded for cells of the mutant strains showed that RCs were assembled in each case, and the level of expression was essentially the same as that of the WT RC (data not shown).

Figure 2 shows a comparison of the Q_y region of the room temperature absorption spectrum of intracytoplasmic membranes prepared from each of the

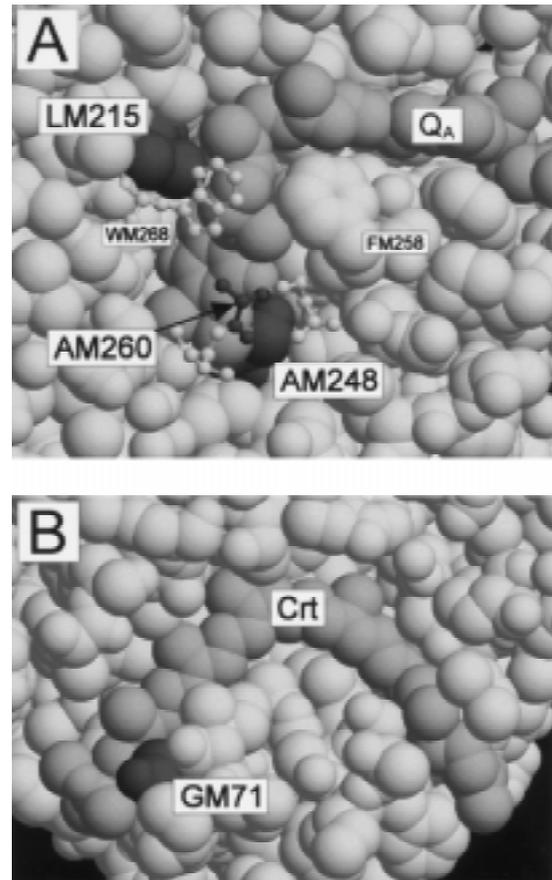


Figure 1. (A) Cut-away view of the binding pocket of the Q_A ubiquinone (mid grey). The mutated residues (dark grey) are labelled, as are residues Trp M268 and Phe M258. For clarity, residues M259–M261 and M268 are shown in ball and stick format. (B) Cut-away view of the carotenoid binding pocket, with the carotenoid shown in mid-grey. Residue Gly M71 (dark grey) is located at one end of the pocket.

mutant strains, with the spectrum of membranes containing WT RCs. In the spectrum of the WT RC, the band at 868 nm is attributable to the low energy exciton component of the Q_y transition of the P Bchls (P Q_y band), the band at 805 nm is principally attributed to the Q_y transitions of the monomeric Bchls (B Q_y band), and the band at 757 nm is attributed to the Q_y transitions of the RC Bphes (H Q_y band). The spectrum of each of the mutant RCs was broadly similar to that of the WT complex, but showed some minor changes in peak position and relative intensity. The absorbance maximum of the B Q_y band was least affected by the mutations, and so was used to normalise the spectra in Figure 2. A 2–3 nm blue-shift of the P Q_y band was observed for all of the mutant RCs, and in the AM260W and AM248W mutants this small

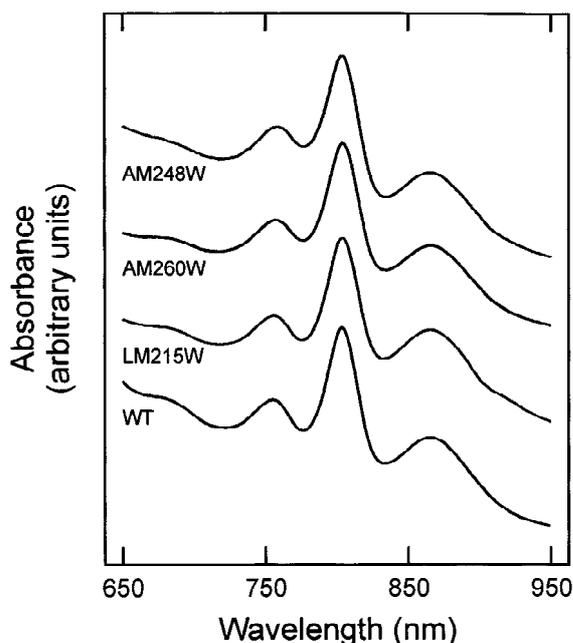


Figure 2. Room temperature absorbance spectra of intracytoplasmic membranes. Membranes were incubated in the presence of 5 mM sodium ascorbate and 25 μ M phenazine methosulphate to ensure full reduction of the P Q_y band. For comparison, spectra were normalised to approximately the same absorbance at 804 nm.

blue-shift was accompanied by a decrease in intensity of the P Q_y band relative to the B Q_y band. In the case of the AM260W RC and, in particular, the AM248W RC, there was a relative increase in intensity of the H Q_y band. This band was slightly red-shifted from the position in the WT RC, being located at 759 nm in the AM260W RC and at 760 nm in the AM248W RC.

Mutant RCs in which the rate of primary electron transfer from P* to H_L is significantly slowed exhibit fluorescence from the P* state (Shochat et al. 1994; JPR, MGG and MRJ, unpublished data). At 77 K, such RCs would exhibit an emission band centred at approximately 920 nm. To look for evidence of impairment of primary electron transfer in the three mutant RCs, fluorescence emission spectra were recorded at 77 K in the Q_y region for RC-only membranes. As for WT RC-only membranes, no emission was detected in this region (data not shown).

The *pufLM* genes for the three mutant RCs were also co-expressed with the structural genes for the LH1 antenna complex (see 'Materials and methods'). The resulting strains, which lacked the peripheral LH2 antenna complex, were grown under semi-aerobic conditions in the dark and intracytoplasmic mem-

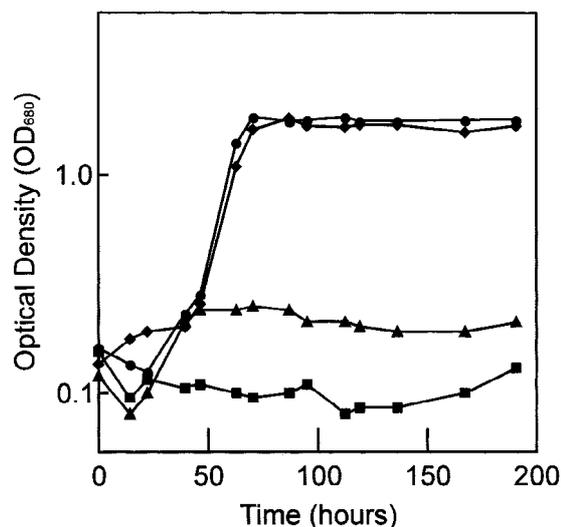


Figure 3. Photosynthetic growth of RC/LH1-containing strains. Growth was monitored by measuring OD₆₈₀ and is plotted on a log₁₀ scale. For each strain, the inoculum consisted of cells that had been grown under semi-aerobic conditions in the dark. Key: (●) WT, (◆) LM215W, (▲) AM260W, (■) AM248W.

branes were prepared. For each mutant, the absorbance spectrum of the RC/LH1 membrane was essentially identical to that of membranes from control strain RCLH12, that contains WT RCs (data not shown). From the relative height of the absorbance bands at 805 nm and 870 nm it was possible to say that the ratio of LH1 Bchls per RC in each of the mutant strains was the same as that observed in strain RCLH12 (McGlynn et al. 1994), with a value of approximately 30 LH1 Bchls per RC.

Photosynthetic growth

The capacity of the mutant RC-only and RC/LH1 strains to grow under anaerobic conditions in the light was determined using the procedure described in Fulcher et al. (1998). The AM260W and AM248W mutations abolished photosynthetic growth in both the RC-only (data not shown) and RC/LH1 background (Figure 3). In the case of the LM215W mutation, photosynthetic growth of the RC-only strain was not observed (data not shown), but the RC/LH1 strain containing this mutation did grow under photosynthetic conditions (Figure 3). The simplest interpretation of these results is that the LM215W mutation brings about some impairment in the capacity of the RC to support photosynthetic growth, the effect of which is lethal for growth in the absence of an antenna, but is

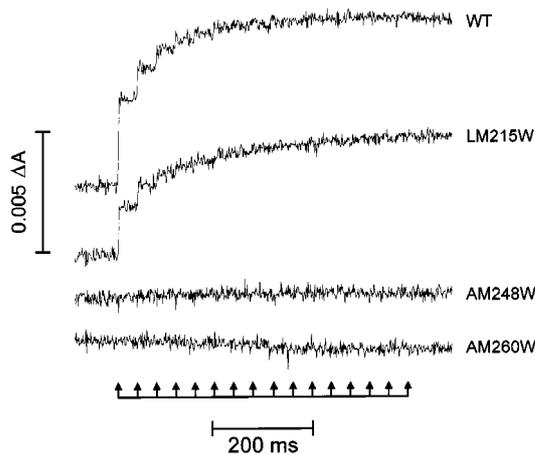


Figure 4. Flash-induced absorbance changes associated with P photo-oxidation in intracytoplasmic membranes. All samples were exposed to 16 flashes fired at 40 ms intervals. Data shown is an average of 8 traces, collected at intervals of 30 s.

not inhibitory in the presence of the antenna, probably because the LH1 complex increases the absorption cross section of the RC, thus compensating for the inefficiency of the modified RC.

Analysis of Q_A activity in the AM248W and AM260W RC

Transient absorbance spectroscopy with ms time resolution was used to look for evidence of the charge separated states $P^+Q_A^-$ or $P^+Q_B^-$. These states are formed in less than a ms after photo-excitation and, in the absence of a cytochrome donor to the RC, recombine to the neutral (PQ_AQ_B) state with time constants of approximately 80–100 ms and 1.2 s, respectively (Diner et al. 1984). Observation of photo-oxidation of P on a ms timescale is therefore a measure of Q_A activity as, in a RC where Q_A^- can not be formed, the P^+ state will not be stable on the ms time scale. Figure 4 shows the results of multi-flash excitation on the amount of P^+ formation in RC-only membranes with WT or mutant RCs, measured by monitoring the absorbance change at 542 nm (Parson and Cogdell 1975; Parson et al. 1975). Control experiments in which the absorbance change at 551–542 nm was monitored in the presence of myxothiazol showed that these RC-only membranes lacked a cytochrome donor to the RC, in line with previous observations (Jones et al. 1992b). In the case of membranes containing the WT RC, a train of sub-saturating flashes from a pair of xenon flash-lamps was capable of fully photo-oxidising P, implying the formation of the $P^+Q_A^-/P^+Q_B^-$ states

(Figure 4). Single flash measurements (not shown) yielded a time constant for charge recombination of approximately 1 s, suggesting that the predominant state formed was $P^+Q_B^-$. This is in line with what would be expected for RCs in membranes at a relatively oxidising redox potential (the ambient redox potential in a typical membrane sample was estimated to be approximately 400 mV, measured using a platinum combination electrode; see Materials and methods). At this potential the intramembrane pool of ubiquinone will be fully oxidised, and the Q_B site of the membrane-bound RC should be occupied by ubiquinone.

In contrast to findings with the WT RC, no photo-oxidation of P was detected in the case of membranes containing AM248W or AM260W RCs that had been diluted to the same RC concentration (Figure 4). This was despite the fact that, as indicated above, these RCs had a Q_Y absorbance spectrum that was very similar to that of the WT RC. The spectrum of all of the RCs examined was not affected by the kinetic measurements (data not shown), demonstrating that the failure to observe photo-oxidation of P by the train of flashes was not due to P already being oxidised, or being irreversibly oxidised by the first few flashes (the transients shown in Figure 4 are the average of 8 individual transients). Once again, difference kinetic measurements carried out at 551–542 nm in the presence of myxothiazol failed to resolve any photo-oxidation of cytochrome c_2 , ruling out the possibility that the failure to detect photo-oxidation of P in the AM248W and AM260W membranes was due to sub-ms reduction of P^+ by cytochrome c_2 (data not shown). We conclude from these results that the failure to detect photo-oxidation of P on a ms timescale in either the AM248W or AM260W RC was due to the states $P^+Q_A^-$ or $P^+Q_B^-$ not being formed by flash excitation. Results with the LM215W RC, which indicated partial occupancy of the Q_A site by a functional ubiquinone, are considered below.

Sub-ps timescale transient absorption spectroscopy of the AM260W RC

Having established that the $P^+Q_A^-$ state is not formed to any detectable extent in the AM260W and AM248W RCs, we then sought to examine the consequences for the rate of primary electron transfer and the associated spectral changes. The AM260W RC was selected for this, because its absorbance spectrum was least affected by the mutation. In WT RCs the

rate of forward electron transfer is accelerated if the temperature is lowered to 77 K (Schenck et al. 1981; Kirmaier et al. 1985; Woodbury et al. 1985; Fleming 1988), and takes place with a characteristic lifetime of approximately 1.5 ps (Woodbury et al. 1985; Fleming 1988). Decay of the state $P^+H_L^-$ to the state $P^+Q_A^-$ takes place with a lifetime of approximately 80 ps, and is accompanied by characteristic changes in absorption spectrum in the Q_y region (Kirmaier et al. 1985). In particular there is a red-shift in the isosbestic point of the band-shift of the monomeric Bchls (at around 800 nm), and the bleach of the H_L ground state band at ~ 750 nm is converted into a band-shift signal. In samples where Q_A is reduced, or absent, forward electron transfer from $P^+H_L^-$ is blocked, and the majority of the $P^+H_L^-$ state decays to the ground state (PH_L) with a lifetime of approximately 12 nanoseconds (ns) at room temperature (Parson and Cogdell 1975).

Electron transfer was monitored with sub-ps time resolution in membranes from strain RCO2 and the AM260W mutant (see Methods). The most noticeable feature of the time-resolved difference spectra of the AM260W RC was that there was very little spectral evolution after the first few ps, with the later spectra being characteristic of the $P^+H_L^-$ state (data not shown). A global analysis of the data determined that only two spectrally- and temporally-distinct components were needed to describe the data; the Species Associated Decay Spectra (SADS) of these components, which we ascribe to P^* and $P^+H_L^-$, are shown in Figure 5(A, B). Three components were required to fit equivalent data obtained for the WT RC (Figures 5C–E), ascribed to the states P^* , $P^+H_L^-$ and $P^+Q_A^-$. In the AM260W mutant the lifetime of the P^* state was 1.5 ps, essentially the same as the value determined for the WT RC. This indicates that the mutation had no significant effect of on the rate of primary electron transfer. Decay of the $P^+H_L^-$ state, which had a lifetime of 81 ps in the WT RC, was not resolved for the AM260W RC in this experiment, this state having a lifetime that was essentially infinite on the timescale of the measurement (up to 1.4 ns). The conclusion drawn from the analysis was that the AM260W RC was incapable of any forward electron transfer from H_L^- to Q_A , at least on the timescale of up to 1.4 ns, but that primary electron transfer is not affected by the mutation. The SADS of the component ascribed to $P^+H_L^-$ in the data on the WT and AM260W RC were very similar (Figures 5B, 5D).

Although in the case of the data on the AM260W RC a satisfactory fit could be obtained with just two

components, there was some indication that a third component reflecting development of the spectrum of the $P^+H_L^-$ state, with a lifetime of between 4 and 10 ps, could also be incorporated into the analysis (data not shown). One source of such a development could be relaxation of the $P^+H_L^-$ radical pair. Experiments aimed at examining this point in more detail are currently under way.

Redox potentiometry of the mutant RCs

The mid-point potential of the P/P^+ redox couple ($E_m P/P^+$) in the membrane-bound AM260W RC was measured as described in Materials and methods, and was found to have a value of +467 mV (± 10 mV). This potential was similar to the value of +484 mV (± 10 mV) determined for the membrane-bound WT RC. For the AM248W RC, $E_m P/P^+$ was +436 mV (± 10 mV). The small effect of the AM260W mutation on $E_m P/P^+$ was consistent with the finding that this mutation had little effect on the rate of primary electron transfer or the fluorescence properties of the RC. The AM248W mutation appeared to have a more significant effect on the redox properties of P, lowering the mid-point potential by several tens of millivolts.

Exclusion of the RC carotenoid

Blockage of forward electron transfer by reduction or removal of the Q_A ubiquinone results decay of the $P^+H_L^-$ state on the 10 ns timescale, partly to the ground state and partly to the triplet excited state of P, $^3P^*$ (Parson et al. 1975; Schenck et al. 1982). The $^3P^*$ state has a lifetime of approximately 50–100 microseconds at room temperature in RCs that lack carotenoid (Parson and Monger 1976), but in carotenoid-containing complexes this lifetime is reduced to approximately 30 ns as a result of quenching of the energy of the $^3P^*$ state by the RC carotenoid, the efficiency of this quenching being dependent upon temperature (Cogdell et al. 1975; Parson and Monger 1976; Frank et al. 1983; Schenck et al. 1984). As a result of this complication, most studies of recombination of the $P^+H_L^-$ state (for review see Volk et al. (1995)) have been carried out on RCs from *Rb. sphaeroides* strain R26, which has a lesion in carotenoid synthesis and yields RCs that are carotenoid deficient.

Having constructed RCs that appeared to lack Q_A function, we sought to extend their usefulness by constructing a second mutation that rendered the RC carotenoid-less. As can be seen in Figure 1B, the RC carotenoid sits in an elongated binding pocket, and

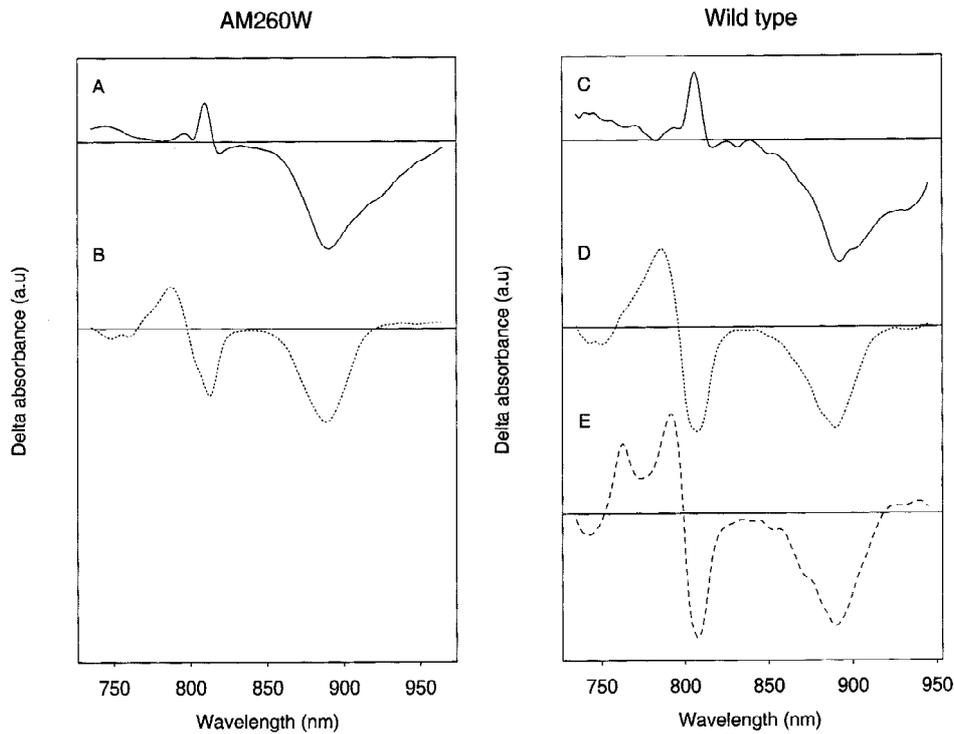


Figure 5. Results of a global analysis of transient absorbance difference spectra measured for AM260W (A, B) and WT (C–E) membrane-bound RCs. A sequential model was used with either two or three increasing lifetimes (i.e. 1→2→3). The lifetimes of the SADS are depicted in the panels.

residue Gly M71 forms part of this pocket, close to one end of the carotenoid. Gly M71 was mutated to Leu with a view to blocking off this part of the pocket, and so preventing binding of the RC carotenoid. The GM71L mutation was made singly, and in combination with the AM260W mutation (denoted GM71L/AM260W). Cells of the antenna-deficient GM71L strain and the control strain RCO2 were grown under semi-aerobic conditions in the dark. Under these conditions the RC contains spheroidenone as the principal carotenoid, and this exhibits a broad and rather featureless absorbance in the region between 450 nm and 580 nm (Schenck et al. 1984).

Cells and membranes from the antenna-deficient GM71L strain did not have the distinctive blue-green colour associated with carotenoid-less strains such as R26. Spectra of membranes from the GM71L mutant were similar to that of membranes from the control strain RCO2 (data not shown), indicating that the level of expression of the mutant RC was normal, that there were no major shifts in the position of the bacteriochlorin absorbance bands, and that the membranes also contained spheroidenone. However,

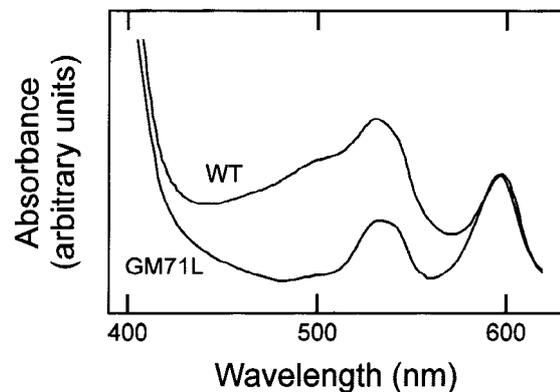


Figure 6. Room temperature absorbance spectra of purified RCs in the carotenoid absorbance region. For comparison, spectra were normalised to approximately the same absorbance at 595 nm (the Bchl Q_x band).

carotenoid absorbance in membranes from antenna-deficient strains is contributed to by free carotenoid in the membrane, in addition to the single carotenoid bound by each RC. Accordingly, GM71L RCs were solubilised using LDAO, and were purified by

anion exchange chromatography (DE52, Whatman) followed by gel filtration (Sephadex 200, Pharmacia). The purified RCs eluted from the gel filtration column had the distinctive blue-green colour and, as can be seen in Figure 6, showed considerably reduced absorption in the 450–580 nm region when compared to purified WT RCs. The spectrum of purified WT RCs was very similar to that of purified RCs from *Rb. sphaeroides* WT strain 2.4.1 grown under anaerobic/light conditions, whilst the spectrum of the purified GM71L RCs was very similar to that of purified RCs from the carotenoid-less strain R26 (Schenck et al. 1984). We ascribe this difference to the lack of any bound RC carotenoid in the mutant strain. This conclusion was confirmed by HPLC analysis which failed to detect any spheroidenone in the purified GM71L RCs.

Analysis of Q_A activity in the LM215W mutant RC

Having established that the AM260W and AM248W RCs lacked detectable Q_A activity, we then examined the LM215W RC, which has a mutation near the isoprenoid side chain of the Q_A ubiquinone. Millisecond-timescale kinetic measurements at 542 nm performed on membranes containing LM215W RCs showed that photo-oxidation of P occurred in only 60–80% of the RCs present in the sample (Figure 4). This indicated that not all of the Q_A sites in the sample were occupied by a functioning ubiquinone. The percentage of functional Q_A detected in these experiments was found to be dependent upon the age of the membranes. Whereas freshly prepared membranes containing LM215W RCs exhibited between 60 and 80% Q_A activity, membranes that had been stored for several months at -20°C totally lacked Q_A activity. The amount of functional Q_A observed did not decline significantly when freshly-prepared membranes were subjected to several daily cycles of thawing then re-freezing, nor did it decline when samples of freshly-prepared membranes were stored in the dark at 4°C or room temperature for up to one week (data not shown). The conclusion drawn from a number of experiments was that the amount of Q_A activity possessed by the LM215W RC decreased on prolonged storage of membranes, such that after several months Q_A activity was completely lost.

Q_A activity could be partially restored to such ‘aged’ membrane-bound LM215W RCs by incubating the membranes in 20 mM Tris/HCl buffer (pH 8.0) in the dark at 4°C , and adding a solution of UQ_{10} in 1%

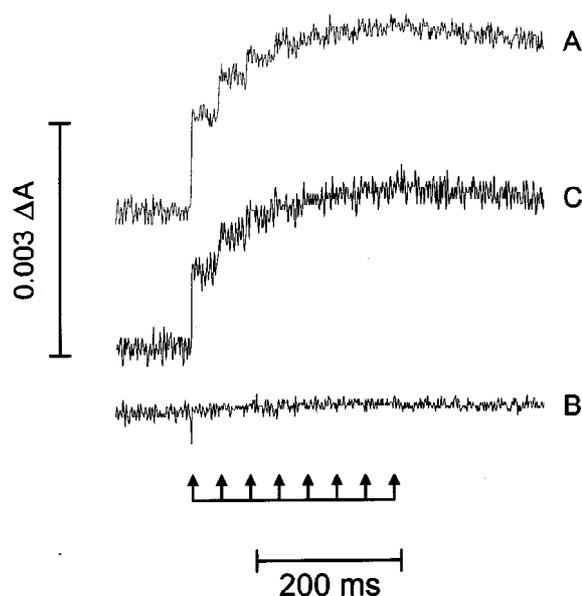


Figure 7. Flash-induced absorbance changes associated with P photo-oxidation in intracytoplasmic membranes. All samples were exposed to 8 flashes fired at 40 ms intervals. Data shown is an average of 8 traces, collected at intervals of 30 s. Key: (A) membranes containing WT RCs, (B) aged membranes containing LM215W RCs lacking Q_A function, (C) membranes from (B) with Q_A function partially restored (see text).

LDAO (Figure 7). The extent to which Q_A activity was restored was dependent on the amount of UQ_{10} added and the length of the subsequent incubation, reaching a maximum of 60% restoration of Q_A activity after incubation for 24 h in the presence of a 50-fold molar excess of ubiquinone. The concentration of ubiquinone added in this experiment was not increased beyond this point, in order to keep the concentration of added LDAO below 0.05%, and so avoid solubilisation of the LM215W RCs. To confirm that solubilisation of RCs had not occurred, membranes were pelleted by centrifugation at $250\,000 \times g$ for 2 h at 4°C , and the supernatant was checked for the presence of RCs by absorbance spectroscopy (data not shown). The supernatant was found to be essentially free of RCs. In the light of these findings with the LM215W RC, experiments were also carried out with a view to restoring Q_A activity in membranes containing AM260W and AM248W RCs, but no restoration was observed. The value of $E_m \text{ P/P}^+$ in the LM215W RC was $+477 \text{ mV}$ ($\pm 10 \text{ mV}$), essentially the same as the value of $+484 \text{ mV}$ ($\pm 10 \text{ mV}$) obtained for the WT RC.

Effect of inhibitors on ubiquinone binding by the LM215W RC

A possible cause of reduced Q_A activity in the LM215W RC is that the mutation affects the redox potential of the Q_A ubiquinone, such that 40% of the RCs contain a reduced ubiquinone at Q_A . To investigate this possibility an attempt was made to measure the rate of charge recombination from the $P^+Q_A^-$ state in a freshly-prepared sample of membrane-bound LM215W RCs (exhibiting approximately 60% functional Q_A) as a significant change in the redox properties of Q_A will affect the driving force for the reaction $P^+Q_A^- \rightarrow PQ_A$. In the WT RC, the rate of charge recombination from the $P^+Q_A^-$ state can be measured on a ms time scale from the recovery of P photo-oxidation at 542 nm following a single actinic flash (data not shown). In order to look exclusively at recombination from the $P^+Q_A^-$ state it is necessary to add an inhibitor such as *o*-phenanthroline, which blocks Q_A^- to Q_B electron transfer through competitive inhibition of the Q_B site (Wraight and Stein 1980; Diner et al. 1984).

Immediately upon the addition of 5 mM *o*-phenanthroline, all photo-oxidation of P was abolished in membrane-bound LM215W RCs (data not shown). Photo-oxidation of P was also immediately abolished in samples of 'aged' membranes where Q_A activity had previously been restored to the LM215W RC by the addition of a 50-fold molar excess of UQ₁₀ (data not shown). Experiments with lower concentrations of *o*-phenanthroline showed that the inhibitor had two effects on the signal measured at 542 nm. Increasing the concentration of inhibitor caused the expected acceleration in the rate of charge recombination, indicating inhibition of the Q_B site. However the inhibitor also caused a decrease in the initial amplitude of the signal at 542 nm, which we interpret as evidence of inhibition of the Q_A site in the LM215W RC.

In the light of this unexpected effect of *o*-phenanthroline on Q_A activity we examined the effects of terbutryn and atrazine, which are also inhibitors of the Q_B site (Diner et al. 1984). At a concentration of 5 mM, terbutryn also completely abolished Q_A activity in LM215W RCs. At a concentration of 50 μ M, atrazine caused approximately 60% inhibition of Q_A activity, but measurements at concentrations above this were hampered by precipitation in the sample.

Affinity of the Q_A site in the LM215W RC for inhibitor

Experiments with purified WT RCs from *Rb. sphaeroides* strain R26 have reported dissociation con-

stants for herbicide inhibition of the Q_B site of 3–6 μ M for terbutryn, 100–215 μ M for *o*-phenanthroline and 90–120 μ M for atrazine, the precise value depending upon the assay conditions (Stein et al. 1984). Diner et al. (1984) reported values of 1.1 μ M for terbutryn and 27–45 μ M for *o*-phenanthroline. In single flash measurements of P photo-oxidation carried out with WT RC-only membranes, we estimated dissociation constants for inhibition of the Q_B site of 13 μ M for terbutryn and 34 μ M for *o*-phenanthroline, in broad agreement with these reports. These values were determined by fitting a bi-exponential function to the decaying part (i.e. >1 ms) of the P photo-oxidation signal at 542 nm. The two exponential components were given fixed lifetimes of 100 ms and 1 s, for recombination from $P^+Q_A^-$ and $P^+Q_B^-$, respectively, and the occupancy of the Q_B site was then assumed to be equivalent to the relative amplitude of the 1 s component. In comparison, dissociation constants for terbutryn and *o*-phenanthroline inhibition of the Q_A site in RC-only membranes of the LM215W RC were estimated to be 144 μ M and 7 μ M, respectively. These values were determined by monitoring the effect of inhibitor on the amount of P that was photo-oxidised immediately after a single excitation flash. Thus, the affinity of these inhibitors for the Q_A site in the LM215W RC was similar to the affinity of the inhibitors for the Q_B site in the WT RC, with dissociation constants in the low micromolar range.

Effect of the environment of the RC on occupancy of the Q_A site

Two groups have constructed mutations at the (Trp) M252 position, as this is a conserved residue that lies directly between H_L and Q_A (Coleman and Youvan 1990; Stolz et al. 1994). In mutant RCs where residue Trp M252 had been replaced by Phe (WM252F) or Tyr (WM252Y), photo-bleaching measurements carried out on antenna-containing chromatophore membranes showed that the Q_A site was fully occupied by ubiquinone (Stolz et al. 1994). However, in that study, column purification of the mutant RC resulted in a significant decrease in the occupancy of the Q_A site, to 51% and 17% in the WM252F and WM252Y RC, respectively. This release of ubiquinone could have been due to the loss of a stabilising influence of the membrane environment, where the local concentration of ubiquinone is high, or to the loss of a more specific stabilising influence of the LH1 antenna complex, or

to the procedures involved in binding, washing and eluting RCs during column purification.

The possibility that quinone binding at Q_A in the LM215W RC could be stabilised by LH1 was examined using membranes that contained LM215W RCs plus LH1 (see above). The LH1 antenna is intimately associated with the RC in the membrane, possibly in the form of a cylinder of pigment protein that surrounds the RC in the plane of the membrane (Karrasch et al. 1995; Cogdell et al. 1996). The amount of functional Q_A in these membranes was estimated by measuring the maximum photo-oxidation of P at 542 nm on a ms timescale, all samples having been normalised to the same absorbance at 805 nm to give the same concentration of RCs. The results of this experiment were essentially the same as those obtained with RC-only membranes, in that 60% Q_A activity was retained in the LM215W RC, and both the AM248W and AM260W RC were devoid of Q_A function (data not shown).

The effects of the membrane environment and LH1 were investigated further by carrying out a crude purification of LM215W RCs from RC-only membranes, and LM215W core complexes from RC/LH1 membranes. RCs were solubilised by the addition of LDAO to membranes to a final concentration of 0.5%, and core complexes were solubilised by the addition of β -octylglucoside and deoxycholate to membranes, both to a final concentration of 15 mM (Molenaar et al. 1988). Samples were incubated on ice in the dark for 30 min, and the membrane debris was pelleted by centrifugation at 13 000 rpm for 2 min at room temperature in a MicroCentaur centrifuge. The solubilised complexes were layered on sucrose step gradients (equal volumes of 20%, 30% and 40% sucrose) and were centrifuged in a SW-41 swing-out rotor (Beckman) at 35 000 rpm and 4 °C for 18 h. Under these conditions the RCs banded on top of the 20% layer, and the core complexes banded at the 30%:40% interface.

The solubilised LM215W RCs and cores removed from the sucrose gradients were assayed, and were found to have retained their content of functional Q_A . In contrast, it was found that a more extensive purification of LM215W RC complexes using an anion exchange column (see Methods) resulted in a total loss of Q_A activity. Thus, removal of LM215W RCs from the membrane environment was insufficient to cause loss of Q_A activity, but this activity was lost as a result of the procedures involved in binding, washing and eluting LM215W RCs from the column matrix.

When the LM215W RCs removed from the sucrose gradients were washed by two successive rounds of 10-fold dilution in 20 mM Tris/HCl (pH 8.0)/0.1% LDAO followed by reconcentration in Centricon 50 concentrators (Amicon), no loss of Q_A activity was detected. However, carrying out this procedure using 20 mM Tris/HCl (pH 8.0)/0.1% LDAO/300 mM NaCl did cause a partial loss of Q_A activity (approximately 35%), suggesting that a principal factor in the loss of Q_A activity during column purification of the LM215W RC may have been the ionic strength of the buffer used to elute RCs from the column. Interestingly, when LM215W core complexes were subjected to the same salt washing, the loss of Q_A activity was less marked (<10%), suggesting that the LH1 antenna may have provided a stabilising influence. However, a more quantitative analysis of this effect was not pursued.

Q_A reconstitution in purified RCs

As described above, Q_A activity could be restored to LM215W RCs in 'aged' membranes by adding UQ₁₀, with 60% activity being obtained with a molar ratio of UQ₁₀:RC of 50:1. In the light of this, an attempt was made to reconstitute Q_A activity by adding UQ₁₀ to column purified LM215W RCs suspended at 4 °C in the dark in 20 mM Tris/HCl (pH 8.0)/0.1% LDAO to a final concentration of 0.5 μ M. No reconstitution was achieved with concentrations of UQ₁₀ up to 250 μ M.

As an alternative to UQ₁₀, reconstitution of Q_A activity was attempted under the same conditions with ubiquinone-0 (UQ₀), which lacks the isoprenoid side-chain and is water soluble. Restoration of Q_A activity in 20% of the RC population was achieved by the addition of a 100-fold molar ratio of UQ₀, rising to 60% with the addition of a 1000:1 molar ratio and 100% with 14 000:1 molar ratio. Thus, Q_A activity above the level observed for the LM215W RC in freshly-prepared membranes could be obtained by the addition of a sufficiently high concentration of UQ₀.

Discussion

Exclusion of the Q_A ubiquinone

In a number of studies of the RC, forward electron transfer from the H_L Bphe has been blocked by chemical reduction or extraction of the Q_A ubiquinone. In particular, these treatments have often been carried out when studying the characteristics of primary charge

separation using a high repetition rate laser system, in order to avoid accumulating RCs in the $P^+Q_A^-/P^+Q_B^-$ states (Woodbury et al. 1985; Breton et al. 1986; Du et al. 1992; Vos et al. 1994; Beekman et al. 1995; Woodbury et al. 1995). The treatments have also been used to study recombination dynamics of the $P^+H_L^-$ radical pair (Parson et al. 1975; Blankenship et al. 1977; Hoff et al. 1977; Michel-Beyerle et al. 1979; Chidsey et al. 1984), which gives information on free energies, reorganisation energies and electronic matrix elements relevant to both charge recombination and primary charge separation (reviewed in Volk et al. 1995). Reduction of the Q_A ubiquinone has the drawback that it introduces a negative charge within the RC, with the result that, for example, processes such as transmembrane electron transfer take place in the presence of an electric field due to Q_A^- , and possibly Q_B^- . In support of this, it has been shown that reduction of Q_A slows the rate of primary electron transfer from P^* to H_L in WT and mutant RCs (Woodbury et al. 1985; Breton et al. 1986; Beekman et al. 1995; Woodbury et al. 1995). Reduction of Q_A also introduces complications in studying the effects of external magnetic fields on the recombination dynamics of the $P^+H_L^-$ state, due to spin coupling between H_L , Q_A and the non-heme iron (Volk et al. 1995).

Extraction of the Q_A quinone is achieved by binding RCs to column material and incubating the bound RCs with LDAO and *o*-phenanthroline (Okamura et al. 1975). Presumably, the high concentration of detergent causes a disruption of the integrity of the RC, such that *o*-phenanthroline can displace the Q_A ubiquinone in a manner analogous to the competitive inhibition of ubiquinone binding at the Q_B site by this reagent. In Du et al. (1992) the rate of primary electron transfer was found to be similar in Q_A -removed and Q_A -reduced RCs, with electron transfer in the Q_A -removed RCs being slightly the slower; no direct comparison with untreated RCs was made. In studies of a mutant RC with three additional hydrogen bonds to the primary donor, Woodbury and co-workers concluded that Q_A reduction made electron transfer to H_L less favourable than in untreated RCs or RCs with Q_A removed (Woodbury et al. 1995).

The spectroscopic data presented in this report demonstrates that the AM260W and AM248W RCs no longer have a functional Q_A ubiquinone in at least 95% of the RC population (which represents the limits of detection in most of the experiments). These mutations therefore provide an alternative means of rendering the RC Q_A -deficient, facilitating studies on

membrane-bound complexes and removing the need for the addition of high concentrations of reductant. A consequence of either mutation is that the RC is no longer capable of supporting photosynthetic growth of the bacterium, even when the LH1 antenna is present. The mutations both had minor effects on the absorbance properties of the RC Bpbes and the P Bchls, and the AM248W mutation in particular also had an effect of the redox properties of P. These effects indicate that the structural changes arising from the mutations were not confined to the immediate vicinity of the Q_A site. This is perhaps not surprising, given that in each case an Ala residue had been replaced by a Trp. However, the overall structural integrity of both mutant RCs appeared to be good, each having an expression level that was comparable to that of the WT RC. In addition, time-resolved spectroscopy conducted on membrane-bound AM260W RCs shows that the kinetics of primary electron transfer from P^* to H_L , and the absorbance difference spectra of the P^* and $P^+H_L^-$ states, are not affected by the structural changes that accompany this mutation.

It should be noted that in our spectroscopic studies of the Q_A -deficient AM260W and AM248W RCs we saw no evidence for electron transfer along the inactive cofactor branch to form the state $P^+Q_B^-$.

The data presented in this study does not allow us to determine whether the Q_A ubiquinone is still associated with the RC but can no longer accept electrons from H_L , or whether we are correct in our premise that the mutations cause exclusion of the ubiquinone from the structure of the RC. Findings from a recent crystallographic analysis of the structure of the AM260W RC indicates the latter to be the case, at least for the purified complex. Preliminary analysis of X-ray diffraction data with a resolution of 3.4 Å, collected on trigonal crystals of the AM260W RC, indicates that the Q_A ubiquinone is not present in the AM260W RC (K.E. McAuley-Hecht, P.K. Fyfe, J.P. Ridge, R.J. Cogdell, N.W. Isaacs and M.R. Jones, unpublished data). In line with the basis on which the AM260W was designed, the new Trp side chain at the M260 position is seen to partially occupy the volume that is occupied by the head group of the Q_A ubiquinone in the structure of the WT RC. The protein in the immediate vicinity of the Q_A site has also undergone some rearrangement, to minimise the volume of the cavity that is created by the loss of the ubiquinone. Most of this rearrangement is associated with the residues of the loop (M256–M261) that connects the de helix of the M subunit to the E helix, and which includes Trp M260. A detailed

account of this structure, including information from data collected to higher resolution, will be presented elsewhere. Even with the benefit of crystallographic data, we cannot absolutely rule out the possibility that the membrane-bound form of the AM260W mutant still binds a non-functional ubiquinone at or near the Q_A site, and that this is lost during purification of the complex. However we feel that this is unlikely.

Modification of ubiquinone binding at the Q_A site

In the WT RC, the head group of the Q_A ubiquinone is deeply buried in the protein interior, in a binding pocket made up of mostly non-polar residues from the D, E and de helices of the M subunit. The keto oxygens of the head group H-bond with the ND1 nitrogen of the His M219 side chain and the backbone amide nitrogen of Ala M260. The first 8 carbon atoms of the isoprenoid sidechain of the ubiquinone extend to the surface of the protein, in the intra-membrane region, through a constricted channel that is formed by residues Trp M268, Leu M215, Phe M258, Met M256 and Met M218 (see Figure 1A). These residues give the appearance of clamping the isoprenoid side chain and so locking the head group of the Q_A ubiquinone in place in the interior of the protein (see Warncke et al. (1994) for an earlier discussion on this topic). In contrast the binding pocket for the Q_B ubiquinone, which is formed by the D, E and de helices of the L subunit, is a much more polar environment. In particular, residues Glu L212, Asp L213 and Ser L223, which have been implicated in protonation of the Q_B ubiquinone, replace residues Ala M248, Ala M249 and Asn M259 in the Q_A pocket. A variety of binding positions have been put forward for the Q_B ubiquinone, reflecting the functional requirement for this cofactor to be able to dissociate from the complex (see discussions in Stowell et al. (1997) and Lancaster and Michel (1997)). The channel leading from the Q_B pocket to the protein surface is also less constricted than is the case for the Q_A site, consistent with this functional requirement.

The Leu M215 to Trp mutation was designed with a view to excluding the Q_A ubiquinone by blocking the channel that accommodates the isoprenoid side-chain of the molecule. From the results presented above it would appear that the mutation was only partially successful in this, with between 60% and 80% of the WT level of Q_A activity being retained in freshly-prepared LM215W membranes, and reconstitution to a level of around 60% being achieved in aged LM215W membranes that had lost Q_A activity. The

sub-stoichiometric activity seen in freshly-prepared membranes raises the question of whether only 60–80% of the LM215W RCs have a bound ubiquinone at Q_A , or whether the site is fully occupied but a fraction of the Q_A ubiquinones are inactive in transmembrane electron transfer.

One possibility is that the change in structure at the Q_A site brings about a change in the redox properties of the Q_A ubiquinone, such that between 20% and 40% of the ubiquinone population is in the form Q_A^- . We feel this is unlikely however, assuming that the Q_A ubiquinone is in redox equilibrium with the external environment in the LM215W RC. The E_m for the Q_A/Q_A^- redox couple in the WT RC has a value in the region of -65 mV to -85 mV at pH 8.0 (Prince and Dutton 1978). The ambient redox potential in a typical sample of freshly prepared RC-only membranes appears to be much more oxidising than this. During redox titrations of membranes, following the addition of mediators but prior to the addition of oxidants or reductants, measurements with a platinum combination electrode typically indicate a redox potential of $+400$ mV. In addition, it has often been observed that following the preparation of RC-only membranes the P_{Q_y} band is partially bleached, and this is particularly noticeable in the case of RC mutants where $E_m P/P^+$ is lowered by 50–100 mV (JPR and MRJ, unpublished observations). Assuming once again that there is reasonable redox equilibrium between the RC cofactors and the bulk phase in membrane samples, these observations indicate it is unlikely that Q_A is partially reduced in the LM215W RC, unless $E_m Q_A/Q_A^-$ has increased by over 450 mV.

A second possibility is that the mutation changes the affinity of the Q_A site for ubiquinone, such that the site is only partially occupied in freshly-prepared membranes, or in ‘aged’ membranes following the addition of a 50-fold molar excess of UQ_{10} . This could come about if the newly-introduced Trp residue adopted a conformation that weakened binding of ubiquinone at the Q_A site. Unfortunately, it was not possible to test this hypothesis by increasing the concentration of added UQ_{10} in order to determine if the remaining RCs could be reconstituted with ubiquinone, due to the requirement to limit the concentration of added detergent.

The final possibility that we have considered is that there are two distinct populations of LM215W RCs, the larger of which binds ubiquinone at the Q_A site with a normal affinity, and the smaller of which does not bind the any ubiquinone at Q_A . This situation

could come about if the Trp M215 residue occupies two distinct conformations, one of which permits binding of ubiquinone at Q_A and the second of which prevents this, perhaps by adopting the conformation envisaged when the mutation was designed, with the Trp residue occupying the channel that accommodates the isoprenoid side chain of the Q_A ubiquinone.

Reconstitution of Q_A activity in purified LM215W RCs

The LM215W RC was found to lose all Q_A activity following column purification. This activity could be fully restored by adding UQ_0 , but the amount of UQ_0 that had to be added to achieve 100% Q_A activity was very high, and no reconstitution was observed with UQ_{10} , even at concentrations 10-fold greater than that successfully employed in membranes. These findings are in contrast to a number of studies in which UQ_{10} , UQ_0 or related quinones have been reconstituted into Q_A -depleted WT RCs of *Rb. sphaeroides*, where molar ratios of 10 quinones per RC or less are generally sufficient to achieve >95% reconstitution of Q_A activity (Okamura et al. 1975; Woodbury et al. 1986; Warncke et al. 1994; Labahn et al. 1995; Van Liemt et al. 1995). The results suggest that one consequence of purification of the LM215W RC is that the affinity of the Q_A site for ubiquinone is significantly reduced. At present we do not know the reasons for this, but one possibility is that on purification all of the RCs in the population adopt a conformation where Trp M215 blocks the channel that accommodates the tail of the Q_A ubiquinone in the WT RC, thus screening the binding pocket of the ubiquinone head group from the external phase. This would perhaps explain why reconstitution of Q_A activity is possible with high concentrations UQ_0 , which lacks the isoprenoid side chain, but does not occur with UQ_{10} . Crystallographic studies of the LM215W RC are planned to address this point further.

Inhibition of the Q_A site

The finding that *o*-phenanthroline, terbutryn and atrazine can inhibit the Q_A site in the membrane-bound LM215W RC is further evidence that this site is accessible to the intramembrane phase. These inhibitors are commonly used to block electron transfer from Q_A to Q_B , and their mode of action at the Q_B site has been characterised by a number of techniques, including X-ray crystallography of inhibited *Rhodospseudomonas* (*Rps.*) *viridis* RCs (Sinning 1992). Structure analysis

shows that *o*-phenanthroline essentially occupies the space taken up by the head group of the Q_B menaquinone in the *Rps. viridis* RC, and is H-bonded to His L190 (Sinning 1992). This is consistent with its role as a competitive inhibitor of ubiquinone binding at the Q_B site. Terbutryn on the other hand occupies the entrance to the Q_B site, forming possible interactions with Ser L223 and Ile L224 (Sinning 1992). The mode of action of terbutryn is more complex than that of *o*-phenanthroline (Diner et al. 1984), and it would appear that inhibition of electron transfer from Q_A^- to Q_B by terbutryn is not necessarily accompanied by displacement of the Q_B ubiquinone. Our results indicate that the affinity of the Q_A site of the LM215W RC for these inhibitors is similar to that exhibited by the Q_B site of the WT RC. No inhibition of Q_A function was achieved with either myxothiazol, an inhibitor of the ubiquinol oxidase site of the cytochrome *bc*₁ complex, or antimycin A, which is an inhibitor of the ubiquinone reductase site of the cytochrome *bc*₁ complex. A possible offshoot of planned structural studies of the LM215W RC is that it may be possible to compare the structural details of binding of these herbicides at two different ubiquinone sites within the same complex.

Construction of a carotenoid-less RC

As described in the Results, most studies of recombination of the $P^+H_L^-$ state have been carried out on Q_A -reduced or Q_A -depleted R26 RCs, as these lack the single carotenoid molecule located close to the monomeric Bchl on the inactive cofactor branch (B_M). In the WT RC, this carotenoid molecule fulfils a photo-protective role by quenching the energy of Bchl triplet states before they can react to produce singlet oxygen (Cogdell and Frank 1987; Frank 1993; Frank and Cogdell 1993). In the light of this, the Gly M71 to Leu mutation was constructed with a view to blocking one end of the elongated carotenoid binding pocket and preventing binding of the cofactor through steric hindrance. Analysis of column purified RCs showed that this strategy was successful, with no detectable spheroidenone bound to the GM71L RC. Previous experiments with the deletion strain DD13, which is devoid of both RCs and light harvesting complexes, have shown that carotenoid is synthesised and inserted into the membrane even in the absence of any of the pigment-protein complexes of the photosynthetic apparatus. The carotenoid absorbance associated with membranes from the

GM71L mutant strain is therefore ascribed to 'free' carotenoid in the membrane. However, we cannot exclude the possibility that the GM71L mutation alters the binding of the RC carotenoid in such a way that it is released from the protein upon purification of the RC. Comparative measurements of the action spectrum for photo-oxidation of P in the RCO2 and GM71L RCs were considered, in order to determine whether excitation of the RC carotenoid results in the formation of P⁺ in the latter. However, as demonstrated for the purified WT RC (Schenck et al. 1984) and for a membrane-bound YM210W mutant RC (Van Brederode et al. 1997b), the contribution of spheroidenone to this action spectrum is not particularly pronounced due to the low efficiency of singlet energy transfer from spheroidenone to P, and so this experiment was not pursued. Planned measurements of triplet formation and quenching in the AM260W and GM71L/AM260W RCs should further address the occupancy or otherwise of the carotenoid binding pocket in the membrane-bound RCs with the GM71L mutation.

Conclusions

A number of studies have shown that the bacterial RC is a robust protein that can undergo significant changes in composition and structure, making it an ideal tool for examining the degree to which new functionalities or mechanisms can be designed into a membrane-bound electron transfer protein. It has long been known that the R26 RC can assemble in the absence of the carotenoid cofactor, and a number of large scale helix swap mutations have also been successfully introduced into the complex (Robles et al. 1990; Coleman and Youvan 1993; Taguchi et al. 1996). The best characterised of these helix swap mutations resulted in RC that lacked the H_L Bphe (Robles et al. 1990). Substitution of Bphe for Bchl in the assembled complex has been achieved by mutation of the axial histidine ligands to the Bchls of P (Bylina and Youvan 1988; McDowell et al. 1991) and B_L (Arlt et al. 1996b), and the H_L Bphe has been replaced by Bchl by the introduction of such a ligand (Kirmaier et al. 1991). In addition, extensive studies have shown that the carotenoid, ubiquinone, Bphe and monomeric Bchl cofactors of the RC can be individually removed and subsequently reconstituted, and in many instances this reconstitution has been achieved with non-native cofactors (Chadwick and Frank 1986;

Gunner et al. 1986; Warncke et al. 1994; Scheer and Hartwich 1995).

The mutations constructed in this report were based upon the premise that exclusion of a particular RC cofactor can be achieved by increasing the volume of a small residue that lines the binding pocket of the cofactor. This somewhat crude but simple approach has been successful for exclusion of the Q_A ubiquinone and, in all likelihood, the carotenoid molecule. The results once again highlight the robust nature of the *Rb. sphaeroides* RC, and augur well for future attempts to carry out more extensive re-engineering of this protein.

Acknowledgements

The authors wish to thank Dr Paul Fyfe for assistance with HPLC analysis of the GM71L RC, Dr Frank van Mourik for his help with laser spectroscopy, and Dr Ivo van Stokkum for the use of software developed for global analysis of time-resolved difference spectra, and for guidance with the analysis. The authors wish to acknowledge financial support from the Biotechnology and Biological Sciences Research Council of the UK and the Dutch Foundation for Fundamental Research (NWO), through the Foundation for Earth and Lifescience (ALW).

References

- Allen JP, Feher G, Yeates TO, Rees DC, Deisenhofer J, Michel H and Huber R (1986) Structural homology of reaction centers from *Rhodospseudomonas sphaeroides* and *Rhodospseudomonas viridis* as determined by X-ray diffraction. *Proc Natl Acad Sci USA* 83: 8589–8593
- Allen JP, Feher G, Yeates TO, Komiya H and Rees DC (1987) Structure of the reaction center from *Rhodobacter sphaeroides* R-26: The cofactors. *Proc Natl Acad Sci USA* 84: 5730–5734
- Arlt T, Schmidt S, Kaiser W, Lauterwasser C, Meyer M, Scheer H and Zinth W (1993) The accessory bacteriochlorophyll: A real electron carrier in primary photosynthesis. *Proc Natl Acad Sci USA* 90: 11757–11761
- Arlt T, Bibikova M, Penzkofer H, Oesterhelt D and Zinth W (1996a) Strong acceleration of primary photosynthetic electron transfer in a mutated reaction center of *Rhodospseudomonas viridis*. *J Phys Chem* 100: 12060–12065
- Arlt T, Dohse B, Schmidt S, Wachtveitl J, Laussermair E, Zinth W and Oesterhelt D (1996b) Electron transfer dynamics of *Rhodospseudomonas viridis* reaction centers with a modified binding site for the accessory bacteriochlorophyll. *Biochemistry* 35: 9235–9244
- Beekman LMP, Visschers RW, Monshouwer R, Heer-Dawson M, Mattioli TA, McGlynn P, Hunter CN, Robert B, Van Stokkum IHM, Van Grondelle R and Jones MR (1995) Time-resolved

- and steady-state spectroscopic analysis of membrane-bound reaction centers from *Rhodobacter sphaeroides*: Comparisons with detergent-solubilized complexes. *Biochemistry* 34: 14712–14721
- Blankenship RE, Schaafsma TJ and Parson WW (1977) Magnetic field effects on radical pair intermediates in bacterial photosynthesis. *Biochim Biophys Acta* 461: 297–305
- Breton J, Martin J-L, Migus A, Antonetti A and Orszag A (1986) Femtosecond spectroscopy of excitation energy transfer and initial charge separation in the reaction center of the photosynthetic bacterium *Rhodospseudomonas sphaeroides*. In: Fleming GR and Siegman AE (eds) *Ultrafast Phenomena V*, pp 393–397. Springer-Verlag, Berlin
- Bylina EJ and Youvan DC (1988) Directed mutations affecting spectroscopic and electron transfer properties of the primary donor in the photosynthetic reaction center. *Proc Natl Acad Sci USA* 85: 7226–7230
- Chadwick BW and Frank HA (1986) ESR studies of carotenoids incorporated into reaction centers of *Rhodobacter sphaeroides* R26.1. *Biochim Biophys Acta* 851: 257–266
- Chang CH, Tiede D, Tang J, Smith U, Norris J and Schiffer M (1986) Structure of *Rhodospseudomonas sphaeroides* R-26 reaction center. *FEBS Letts* 205: 82–86
- Chang CH, El Kabbani O, Tiede D, Norris J and Schiffer M (1991) Structure of the membrane-bound protein photosynthetic reaction center from *Rhodobacter sphaeroides*. *Biochemistry* 30: 5352–5360
- Chidsey CED, Kirmaier C, Holten D and Boxer SG (1984) Magnetic field dependence of radical-pair decay kinetics and molecular triplet quantum yield in quinone-depleted reaction centers. *Biochim Biophys Acta* 766: 424–437
- Cogdell RJ and Frank HA (1987) How carotenoids function in photosynthetic bacteria. *Biochim Biophys Acta* 895: 63–79
- Cogdell RJ, Monger TA and Parson WW (1975) Carotenoid triplet states in reaction centers from *Rhodospseudomonas sphaeroides* and *Rhodospirillum rubrum*. *Biochim Biophys Acta* 408: 189–199
- Cogdell RJ, Fyfe PK, Barrett SJ, Prince SM, Freer AA, Isaacs NW, McGlynn P and Hunter CN (1996) The purple bacterial photosynthetic unit. *Photosynth Res* 48: 55–63
- Coleman WJ and Youvan DC (1990) Spectroscopic analysis of genetically-modified photosynthetic reaction centers. *Ann Rev Biophys Chem* 19: 333–367
- Coleman WJ and Youvan DC (1993) Atavistic reaction-center. *Nature* 366: 517–518
- Diner BA, Schenck CC and De Vitry C (1984) Effect of inhibitors, redox state and isoprenoid chain length on the affinity of ubiquinone for the secondary acceptor binding site in the reaction centers of photosynthetic bacteria. *Biochim Biophys Acta* 766: 9–20
- Du M, Rosenthal SJ, Xie X, Dimagno TJ, Schmidt M, Hanson DK, Schiffer M, Norris JR and Fleming GR (1992) Femtosecond spontaneous-emission studies of reaction centers from photosynthetic bacteria. *Proc Natl Acad Sci USA* 89: 8517–8521
- Ermiler U, Fritzsche G, Buchanan SK and Michel H (1994a) Structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.65 Å resolution: Cofactors and protein-cofactor interactions. *Structure* 2: 925–936
- Ermiler U, Michel H and Schiffer M (1994b) Structure and function of the photosynthetic reaction center from *Rhodobacter sphaeroides*. *J Bioenerg Biomemb* 26: 5–15
- Fleming GR and Van Grondelle R (1994) The primary steps of photosynthesis. *Physics Today* 47: 48–55
- Fleming GR, Martin JL and Breton J (1988) Rates of primary electron transfer in photosynthetic reaction centres and their mechanistic implications. *Nature* 333: 190–192
- Frank HA (1993) Carotenoids in photosynthetic bacterial reaction centers: Structure, spectroscopy and photochemistry. In: Deisenhofer J and Norris JR (eds) *The Photosynthetic Reaction Center*, Vol 2, pp 221–239. Academic Press, San Diego, CA
- Frank HA and Cogdell RJ (1993) The photochemistry and function of carotenoids in photosynthesis. In: Young AJ and Britton G (eds) *Carotenoids in Photosynthesis*, pp 252–326. Chapman and Hall, London
- Frank HA, Machnicki J and Friesner R (1983) Energy transfer between the primary donor bacteriochlorophyll and carotenoids in *Rhodospseudomonas sphaeroides*. *Photochem Photobiol* 38: 451–455
- Fulcher TK, Beatty JT and Jones MR (1998) Demonstration of the key role played by the PufX protein in the functional and structural organization of native and hybrid bacterial photosynthetic core complexes. *J Bacteriol* 180: 642–646
- Gunner MR, Robertson DE and Dutton PL (1986) Kinetic studies on the reaction center protein from *Rhodospseudomonas sphaeroides* – the temperature and free energy dependence of electron transfer between various quinones in the Q_A site and the oxidised bacteriochlorophyll dimer. *J Phys Chem* 90: 3783–3795
- Hoff AJ, Rademaker H, van Grondelle R and Duysens LNM (1977) On the magnetic field dependence of the yield of the triplet state in reaction centers of photosynthetic bacteria. *Biochim Biophys Acta* 460: 547–554
- Holzappel W, Finkele U, Kaiser W, Oesterhelt D, Scheer H, Stolz HU and Zinth W (1989) Observation of a bacteriochlorophyll anion radical during primary charge separation in a reaction center. *Chem Phys Lett* 160: 1–7
- Holzappel W, Finkele U, Kaiser W, Oesterhelt D, Scheer H, Stolz HU and Zinth W (1990) Initial electron-transfer in the reaction center from *Rhodobacter sphaeroides*. *Proc Natl Acad Sci USA* 87: 5168–5172
- Holzwarth AR and Müller MG (1996) Energetics and kinetics of radical pairs in reaction centers from *Rhodobacter sphaeroides*. A femtosecond transient absorption study. *Biochemistry* 35: 11820–11831
- Jones MR, Fowler GJS, Gibson LCD, Grief GG, Olsen JD, Crielgaard W and Hunter CN (1992a) Mutants of *Rhodobacter sphaeroides* lacking one or more pigment-protein complexes and complementation with reaction-centre, LH1, and LH2 genes. *Mol Microbiol* 6: 1173–1184
- Jones MR, Visschers RW, Van Grondelle R and Hunter CN (1992b) Construction and characterization of a mutant of *Rhodobacter sphaeroides* with the reaction center as the sole pigment-protein complex. *Biochemistry* 31: 4458–4465
- Jones MR, Heer Dawson M, Mattioli TA, Hunter CN and Robert B (1994) Site-specific mutagenesis of the reaction centre from *Rhodobacter sphaeroides* studied by Fourier transform Raman spectroscopy: Mutations at tyrosine M210 do not affect the electronic structure of the primary donor. *FEBS Lett* 339: 18–24
- Karrasch S, Bullough PA and Ghosh R (1995) The 8.5 Å projection map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits. *Embo J* 14: 631–638
- Kirmaier C, Holten D and Parson WW (1985) Temperature and detection-wavelength dependence of the picosecond electron transfer kinetics measured in *Rhodospseudomonas sphaeroides* reaction centers: Resolution of new spectral and kinetic components in the primary charge-separation process. *Biochim Biophys Acta* 810: 33–48

- Kirmaier C, Gaul D, Debey R, Holten D and Schenck CC (1991) Charge separation in a reaction center incorporating bacteriochlorophyll for photoactive bacteriopheophytin. *Science* 251: 922–927
- Komiya H, Yeates TO, Rees DC, Allen JP and Feher G (1988) Structure of the reaction center from *Rhodobacter sphaeroides* R-26 and 2.4.1: Symmetry relations and sequence comparisons between different species. *Proc Natl Acad Sci USA* 85: 9012–9016
- Labahn A, Bruce JM, Okamura MY and Feher G (1995) Direct charge recombination from $D^+Q_AQ_B^-$ to DQ_AQ_B in bacterial reaction centers from *Rhodobacter sphaeroides* containing low potential quinone in the Q_A site. *Chem Phys* 197: 355–366
- Lancaster CRD and Michel H (1997) The coupling of light-induced electron transfer and proton uptake as derived from crystal structures of reaction centres from *Rhodopseudomonas viridis* modified at the binding site of the secondary quinone, Q_B . *Structure* 5: 1339–1359
- McAuley-Hecht KE, Fyfe PK, Ridge JP, Prince SM, Hunter CN, Isaacs NW, Cogdell RJ and Jones MR (1998) Structural studies of wild-type and mutant reaction centers from an antenna-deficient strain of *Rhodobacter sphaeroides*: Monitoring the optical properties of the complex from cell to crystal. *Biochemistry* 37: 4740–4750
- McDowell LM, Gaul D, Kirmaier C, Holten D and Schenck CC (1991) Investigation into the source of electron transfer asymmetry in bacterial reaction centers. *Biochemistry* 30: 8315–8322
- McGlynn P, Hunter CN and Jones MR (1994) The *Rhodobacter sphaeroides* PufX protein is not required for photosynthetic competence in the absence of a light harvesting system. *FEBS Lett* 349: 349–353
- Michel-Beyerle ME, Scheer H, Seidlitz H, Tempus D and Haberkorn R (1979) Time-resolved magnetic field effect on triplet formation in photosynthetic reaction centers of *Rhodopseudomonas sphaeroides* R-26. *FEBS Lett* 100: 9–12
- Molenaar D, Crieelaar W and Hellingwerf KJ (1988) Characterization of protonmotive force generation in liposomes reconstituted from phosphatidylethanolamine, reaction centers with light-harvesting complexes isolated from *Rhodopseudomonas palustris*. *Biochemistry* 27: 2014–2023
- Okamura MY, Isaacson RA and Feher G (1975) Primary acceptor in bacterial photosynthesis: Obligatory role of ubiquinone in photoactive reaction centers of *Rhodopseudomonas sphaeroides*. *Proc Natl Acad Sci USA* 72: 3491–3495
- Parson WW (1991) Reaction centers. In: Scheer H (ed) *Chlorophylls*, pp 1153–1180. CRC Press, Boca Raton, FL
- Parson WW and Cogdell RJ (1975) The primary photochemical reaction of bacterial photosynthesis. *Biochim Biophys Acta* 416: 105–149
- Parson WW and Monger TG (1976) Interrelationships among excited states in bacterial reaction centers. *Brookhaven Symp Biol* 28 195–211
- Parson WW, Clayton RK and Cogdell RJ (1975) Excited states of photosynthetic reaction centers at low redox potentials. *Biochim Biophys Acta* 387: 265–278
- Robles SJ, Breton J and Youvan DC (1990) Partial symmetrization of the photosynthetic reaction center. *Science* 248: 1402–1405
- Scheer H and Hartwich G (1995) Bacterial reaction centers with modified tetrapyrrole chromophores. In: Blankenship RE, Madigan MT and Bauer CE (eds) *Anoxygenic Photosynthetic Bacteria*, pp 649–663. Kluwer Academic Publishers, Dordrecht, The Netherlands
- Schenck CC, Parson WW, Holten D, Windsor MW and Sarai A (1981) Temperature dependence of electron transfer between bacteriopheophytin and ubiquinone in protonated and deuterated reaction centers of *Rhodopseudomonas sphaeroides*. *Biophys J* 36: 479–489
- Schenck CC, Blankenship RE and Parson WW (1982) Radical-pair kinetics, triplet yields and delayed fluorescence from bacterial reaction centers. *Biochim Biophys Acta* 680: 44–59
- Schenck CC, Mathis P and Lutz M (1984) Triplet formation and triplet decay in reaction centers from the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. *Photochem Photobiol* 39: 407–417
- Schmidt S, Arlt T, Hamm P, Huber H, Nagele T, Wachtveitl J, Meyer M, Scheer H and Zinth W (1994) Energetics of the primary electron transfer reaction revealed by ultrafast spectroscopy on modified bacterial reaction centers. *Chem Phys Lett* 223: 116–120
- Shochat S, Arlt T, Francke C, Gast P, Van Noort PI, Otte SCM, Schelvis HPM, Schmidt S, Vijgenboom E, Vrieze, J, Zinth, W and Hoff AJ (1994) Spectroscopic characterization of reaction centers of the (M)Y210W mutant of the photosynthetic bacterium *Rhodobacter sphaeroides*. *Photosynth Res* 40: 55–66
- Sinning I (1992) Herbicide binding in the bacterial photosynthetic reaction center. *TIBS* 17: 150–154
- Stein RR, Castellvi AL, Bogacz JP and Wraight CA (1984) Herbicide-quinone competition in the acceptor complex of photosynthetic reaction centers from *Rhodopseudomonas sphaeroides*: a bacterial model for PS II-herbicide activity in plants. *J Cell Biochem* 24, 243–259
- Stilz HU, Finkle U, Holzapfel W, Lauterwasser C, Zinth W and Oesterhelt D (1994) Influence of m subunit Thr222 and Trp252 on quinone binding and electron transfer in *Rhodobacter sphaeroides* reaction centres. *Eur J Biochem* 223: 233–242
- Stowell MHB, McPhillips TM, Rees DC, Soltis SM, Abresch E and Feher G (1997) Light-induced structural changes in photosynthetic reaction center: Implications for mechanism of electron-proton transfer. *Science* 276: 812–816
- Straley SC, Parson WW, Mauzerall DC and Clayton RK (1973) Pigment content and molar extinction coefficients of photochemical reaction centers from *Rhodopseudomonas sphaeroides*. *Biochim Biophys Acta* 305: 597–605
- Taguchi AKW, Eastman JE, Gallo DM, Jr, Sheagley E, Xiao W and Woodbury NW (1996) Asymmetry requirements in the photosynthetic reaction center of *Rhodobacter capsulatus*. *Biochemistry* 35: 3175–3186
- van Brederode ME, Jones MR and van Grondelle (1997a) Fluorescence excitation spectra of membrane-bound photosynthetic reaction centers of *Rhodobacter sphaeroides* in which the tyrosine M210 residue is replaced by tryptophan: evidence for a new pathway of charge separation. *Chem Phys Lett* 268: 143–149
- van Brederode ME, Jones MR, van Mourik F, van Stokkum IHM and van Grondelle R (1997b) A new pathway for transmembrane electron transfer in photosynthetic reaction centers of *Rhodobacter sphaeroides* not involving the excited special pair. *Biochemistry* 36: 6855–6861
- van Liemt WBS, Boender GJ, Gast P, Hoff AJ, Lugtenburg J and de Groot HJM (1995) ^{13}C magic angle spinning NMR characterization of the functionally asymmetric Q_A binding in *Rhodobacter sphaeroides* R26 photosynthetic reaction centers using site-specific ^{13}C -labeled ubiquinone-10. *Biochemistry* 34: 10229–10236
- van Stokkum IHM, Beekman LMP, Jones MR, van Brederode ME and van Grondelle R (1997) Primary electron transfer kinetics in membrane-bound *Rhodobacter sphaeroides* reaction centers: A global and target analysis. *Biochemistry* 36: 11360–11368

- Volk M, Ogrodnik A and Michel-Beyerle M-E (1995) The recombination dynamics of the radical pair $P^+H_L^-$ in external magnetic and electric fields. In: Blankenship RE, Madigan MT and Bauer CE (eds) *Anoxygenic Photosynthetic Bacteria*, pp 595–626. Kluwer Academic Publishers, Dordrecht, The Netherlands
- Vos MH, Jones MR, Hunter CN, Breton J and Martin JL (1994) Coherent nuclear dynamics at room temperature in bacterial reaction centers. *Proc Natl Acad Sci USA* 91: 12701–12705
- Warncke K, Gunner MR, Braun BS, Gu L, Yu CA, Bruce JM and Dutton PL (1994) Influence of hydrocarbon tail structure on quinone binding and electron-transfer performance at the Q_A and Q_B sites of the photosynthetic reaction center protein. *Biochemistry* 33: 7830–7841
- Woodbury NW, Becker M, Middendorf D and Parson WW (1985) Picosecond kinetics of the initial photochemical electron-transfer reaction in bacterial photosynthetic reaction centers. *Biochemistry* 24: 7516–7521
- Woodbury NW, Parson WW, Gunner MR, Prince RC and Dutton PL (1986) Radical-pair energetics and decay mechanisms in reaction centers containing anthraquinones, naphthoquinones or benzoquinones in place of ubiquinone. *Biochim Biophys Acta* 851: 6–22
- Woodbury NW, Lin S, Lin XM, Peloquin JM, Taguchi AKW, Williams JC and Allen JP (1995) The role of reaction center excited state evolution during charge separation in a *Rhodobacter sphaeroides* mutant with an initial electron donor midpoint potential 260 mV above wild type. *Chem Phys* 197: 405–421
- Wraight CA and Stein RR (1980) Redox equilibrium in the acceptor quinone complex of isolated reaction centers and the mode of action of *o*-phenanthroline. *FEBS Lett* 113: 73–77