Photo-accumulation of the $P^+Q_B^-$ radical pair state in purple bacterial reaction centres that lack the Q_A ubiquinone

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Abstract Photo-excitation of membrane-bound *Rhodobacter sphaeroides* reaction centres containing the mutation Ala M260 to Trp (AM260W) resulted in the accumulation of a radical pair state involving the photo-oxidised primary electron donor (P). This state had a lifetime of hundreds of milliseconds and its formation was inhibited by stigmatellin. The absence of the Q_A ubiquinone in the AM260W reaction centre suggests that this long-lived radical pair state is $P^+Q_B^-$, although the exact reduction/protonation state of the Q_B quinone remains to be confirmed. The blockage of active branch (A-branch) electron transfer by the AM260W mutation implies that this $P^+Q_B^-$ state is formed by electron transfer along the so-called inactive branch (B-branch) of reaction centre cofactors. We discuss how further mutations may affect the yield of the $P^+Q_B^-$ state, including a double alanine mutation (EL212A/DL213A) that probably has a direct effect on the efficiency of the low yield electron transfer step from the anion of the B-branch bacteriopheophytin (H_B^-) to the Q_B ubiquinone.

Key words: Reaction centre; Photosynthesis; Electron transfer; Inactive branch; Mutagenesis; Spectroscopy

1. Introduction

In the reaction centres of photosynthetic organisms, light energy is used to drive a membrane-spanning electron transfer reaction [1]. This process has been most heavily studied in the reaction centre from purple photosynthetic bacteria such as *Rhodobacter sphaeroides*, where X-ray crystal structures are available at resolutions approaching 2.0 Å, and the complex is particularly amenable to spectroscopic analysis [2].

The *R. sphaeroides* reaction centre is composed of three polypeptides, termed H, L and M, that encase 10 cofactors (Fig. 1A). These are four molecules of bacteriochlorophyll (BChl), two molecules of bacteriopheophytin (BPhe), two molecules of ubiquinone, a single photoprotective carotenoid and a non-heme iron atom. The L- and M-polypeptides form a heterodimeric protein scaffold that encases the BChl, BPhe and ubiquinone cofactors [3–6]. These cofactors are arranged in two membrane-spanning branches around an axis of two-fold symmetry (see Fig. 1A) [4–7]. In the initial steps of energy transduction, light energy drives a transmembrane electron transfer from the primary electron donor (P), a pair of excitonically-coupled BChl molecules located near the periplasmic side of the membrane, to a molecule of ubiquinone (Q_A) located near the cytoplasmic side of the membrane [2,8–12]. This transmembrane electron transfer takes place on a picosecond time-scale, and involves an intervening monomeric BChl (B_A) and a molecule of bacteriopheophytin (BPhe, denoted H_A) [2,8–12]. The electron residing on the Q_A ubiquinone is passed to the Q_B cofactor binding site, where a bound ubiquinone is reduced to the ubisemiquinone [13,14]. A second light-driven transmembrane electron transfer results in further reduction and double protonation of the Q_B ubisemiquinone to form ubiquinol [13,14].

Despite its striking structural symmetry (Fig. 1A) the purple bacterial reaction centre exhibits a marked functional asymmetry, with only the so-called A-branch of cofactors between the P BChls and the Q_A ubiquinone being used to catalyse transmembrane electron transfer. The structural and energetic basis of this asymmetry has been the subject of intense interest, and in recent years there have been several attempts to affect the balance of transmembrane electron transfer along the two branches through the application of site-directed mutagenesis [15–26].

In previous work from our laboratory, carried out in conjunction with collaborating groups, it was shown that mutagenesis of residue alanine M260 to tryptophan (AM260W) results in a reaction centre that lacks the Q_A ubiquinone [27,28]. This AM260W reaction centre is incapable of supporting photosynthetic growth of the bacterium, or of carrying out forward electron transfer past the H_A BPhe on the A-branch of cofactors, although the rate of electron transfer from P to the H_A BPhe is essentially identical to that in the wild-type complex [27]. X-ray crystallography of the AM260W mutant has shown that the changes in protein structure elicited by the mutation are restricted to five amino acids that contribute to the Q_A binding pocket, and that are part of a loop of residues that connect the DE and E α-helices of the M-polypeptide [28]. The remainder of the structure, including the positions and environments of the other reaction centre cofactors, was unaffected by the AM260W mutation within the resolution of the structure (2.1 Å resolution with a coordinate error of ~0.1 Å [28]).

There has been a number of investigations into how the asymmetry of electron flow in the bacterial reaction centre

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Abbreviations: B, accessory bacteriochlorophyll; BChl, bacteriochlorophyll; BPhe or H, bacteriopheophytin; P, primary donor of electrons

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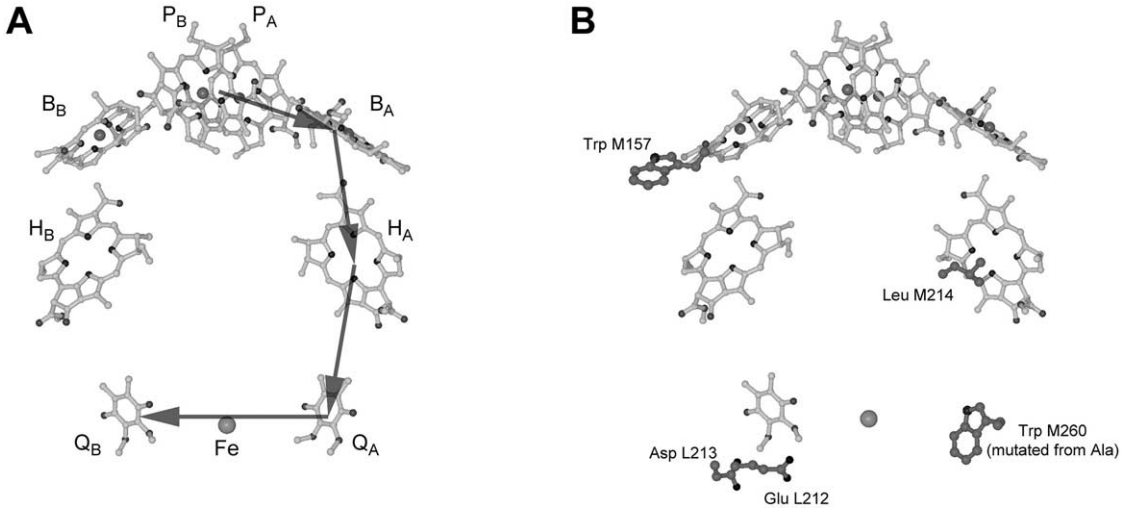

Fig. 1. Structural models showing (A) cofactor organisation in the wild-type reaction centre and (B) the AM260W reaction centre with the other residues mutated in this work highlighted. In A, the route of A-branch electron transfer is indicated by the arrows.

can be changed, concentrating mainly on the picosecond time-scale formation of the $P^+H_B^-$ radical pair in purified mutant reaction centres [15–26]. In the present report, we have used the Q_A-excluding AM260W mutation as the basis for a series of multiple mutations aimed at affecting this asymmetry. Our studies have focussed on the product of electron transfer along the full length of the B-branch of cofactors, the radical pair $P^+Q_B^-$, in reaction centres that are still bound to the bacterial membrane. We show that it is possible to photoaccumulate this state in membrane-bound Q_A-deficient reaction centres, and discuss how mutations affect the yield of this state.

2. Materials and methods

2.1. Mutations and nomenclature

The AM260W mutation was introduced into the *pufM* gene as described previously [27]. The other mutations introduced in this study were Leu M214 to His (LM214H) [15], Trp M157 to Asp (WM157D) and the double mutation Glu L212 to Ala and Asp L213 to Ala (EL212A/DL213A) [29,30] (see Fig. 1B). These mutations were generated using the QuikChange mutagenesis kit (Stratagene), either singly or in the following combinations: AM260W+EL212A/DL213A (denoted WAA), AM260W+EL212A/DL213A+LM214H (denoted WAAH), AM260W+EL212A/DL213A+LM214H+WM157D (denoted WAAHD), AM260W+LM214H+WM157D (denoted WHD).

Reaction centre *pufLM* genes containing these mutations were expressed in deletion strain DD13 [31], either with or without the *pufBA* genes that encode the core LH1 antenna complex [31]. This produced, for each combination of mutations, transconjugant strains that had a $RC^+LH1^+LH2^-$ or $RC^+LH1^-LH2^-$ phenotype (LH2 is the peripheral antenna complex). Experimental material consisted of intracytoplasmic membrane fragments prepared from cells that had been grown under semiaerobic conditions in the dark, using procedures described previously [32].

2.2. Spectroscopy and redox potentiometry

Absorbance spectra of intracytoplasmic membranes diluted in 20 mM Tris–HCl (pH 8.0) were recorded using a Beckman DU640 spectrophotometer. To ensure full reduction of the P BChls, spectra were recorded in the presence of 5 mM sodium ascorbate and 25 μM phenazine methosulphate (PMS). Values of E_m P/P^+ were determined by chemical titration of the absorbance spectrum under anaerobic conditions, as described elsewhere [33].

Millisecond (ms) time-scale transient absorption was recorded using a single beam spectrophotometer of local design. Trains of saturating actinic flashes were provided by a xenon flash-lamp (15 μF capacitor at 1000 V; 6 μs half-peak width) that was filtered with RG625 glass filters. Two light pipes (1 cm diameter) were used to deliver the excitation flashes to both sides of the sample cuvette in order to provide uniform illumination. The weak monochromatic measuring beam was detected using a photomultiplier that was protected from the excitation light by OG515 and BG39 cut-off filters. The output from the photomultiplier was passed through a current/voltage amplifier that had a time constant of 300 μs, and was digitised using a Microlink transient recorder.

Samples were housed in a glass cuvette with an optical path length of 1 cm, and consisted of antenna-deficient membranes diluted in 20 mM Tris–HCl (pH 8.0). Sodium ascorbate was added to a final concentration of 1 mM to ensure that there was no pre-oxidation of the P BChls. For the purpose of comparison of different samples, in all experiments intracytoplasmic membranes were diluted to a reaction centre concentration of approximately 1 μM, based on the extinction coefficient of 288 mM^{-1} cm^{-1} for the accessory bacteriochlorophyll (B) Q_y band [34]. After each experiment the absorbance spectrum of the sample was measured, corrected for background scatter, and the amplitude of the P Q_y band was recorded. This amplitude was then used to normalise the amplitude of the transient absorbance traces. The P Q_y band was selected for this purpose as it was least affected by the mutations studied in this report. In contrast, the H and B Q_y bands were strongly affected by the LM214H mutation in particular.

3. Results

3.1. Mutant construction, absorbance spectroscopy and redox potentiometry

Fig. 2 shows the Q_y region of absorbance spectra of antenna-deficient intracytoplasmic membranes prepared from the strains described in Section 2. For the wild-type reaction centre the band at 757 nm is attributable to the reaction centre BPhes (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), whilst the band at 869 nm is attributable to the P BChls (termed the P Q_y band). As reported previously [27], the principal effect of the AM260W mutation was to cause a small (4 nm) blue-shift of the P Q_y band (Fig. 2). This blue-shift was also seen when the AM260W mutation was combined with the double mutation EL212A/DL213A, in the mutant WAA (Fig. 2), and to varying degrees in the remaining mutants (between 2 and 5 nm). The location of Glu L212 and Asp L213 (and the other residues specified below) is shown in Fig. 1B, which is based

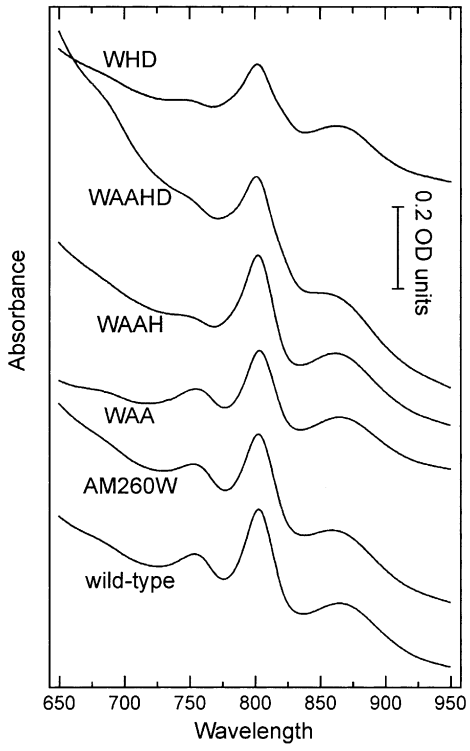

Fig. 2. Room temperature absorbance spectra for antenna-deficient intracytoplasmic membranes containing wild-type or mutant reaction centres. Spectra are offset for the purposes of comparison. Key for nomenclature of the multiple mutants: WAA, AM260W+EL212A/DL213A; WAAH, AM260W+EL212A/DL213A+LM214H; WAAHD, AM260W+EL212A/DL213A+LM214H+WM157D; WHD, AM260W+LM214H+WM157D.

on the X-ray crystal structure of the AM260W single mutant [28].

The EL212A/DL213A double mutation was originally constructed in *Rhodobacter capsulatus*, and was shown to render the reaction centre incapable of supporting photosynthetic growth by preventing protonation of the reduced Q_B ubiquinone [29,30,35]. The double alanine substitution makes the reaction centre less asymmetric at the level of the quinone binding pockets by making the Q_B binding pocket less polar, and so more like the relatively non-polar Q_A binding pocket (where the symmetry-related residues are Ala M248 and Ala M249 in *R. sphaeroides*). In the present experiments therefore, the WAA mutant lacks a Q_A ubiquinone and has a Q_B binding pocket that is reduced in polarity. The EL212A/DL213A double mutation does not affect the absorbance spectrum of the reaction centre bacteriochlorins [30], and this was also the case when it was combined with the AM260W mutation in the present work (Fig. 2; mutant WAA).

The three mutations that make up the WAA mutant were combined with the mutation Leu M214 to His, giving a quadruple mutant termed WAAH. As has been shown in extensive studies by Holten, Kirmaier and co-workers, replacement of the equivalent Leu M212 by His in the *R. capsulatus* reaction centre causes a BChl (termed β_A) to be incorporated into the reaction centre in place of the native H_A BPhe [15]. The LM212H single mutant reaction centre has a slowed rate of primary electron transfer to a state that has mixed $P^+B_A^-$/$P^+\beta_A^-$ character [16], and shows a decreased yield of electron transfer to the Q_A ubiquinone (from ~100% to ~65%) due to an increased competing decay of the mixed $P^+B_A^-$/$P^+\beta_A^-$ state to the ground state [16]. The *R. capsulatus* LM212H mutant has also been reported to carry out a small amount of B-branch electron transfer (~6–7%) to form the radical pair $P^+H_B^-$ [19,21,24], and it has been proposed on kinetic grounds that the equivalent *R. sphaeroides* LM214H mutant forms the $P^+H_B^-$ state with a yield of ~3% [24].

In the present study the WAAH mutant displayed the changes in absorbance spectrum that are characteristic of the LM214H mutation [15]. Loss of the H_A BPhe was indicated by an approximately 50% decrease in intensity of the H Q_y band, and a small (1 nm) blue-shift of the absorbance maximum of this band (Fig. 2). The presence of the new β_A BChl was signalled by an increase in absorbance in the blue wing of B Q_y band, and a small (1 nm) blue-shift of the maximum of this band (Fig. 2).

The four mutations that make up the WAAH reaction centre were then combined with the mutation Trp M157 to Asp (WM157D). This Trp residue is located adjacent to the acetyl carbonyl group of the B_B BChl (Fig. 1B), and was mutated to Asp with a view to forming a hydrogen bond interaction with the acetyl group, and so affecting the potential of the B_B/B_B^- redox couple (i.e. making B_B easier to reduce). Preliminary results from a crystallographic study of the single WM157D mutant reaction centre, using data collected to 2.6 Å resolution, indicate that the new Asp residue is within hydrogen bonding distance (3.5 Å) of this acetyl group (Potter, J., Fyfe, P.K., Goodwin, M.G. and Jones, M.R., unpublished observations).

Experiments with a WM157D single mutant have shown that this mutation causes the appearance of a distinct shoulder on the red side of the B Q_y band, around 821 nm, without affecting the position of the absorbance maximum of the B Q_y band (Goodwin, M.G. and Jones, M.R., unpublished observations). This would be consistent with an effect on the absorbance properties of the B_B BChl, which is thought to contribute mainly to the red side of the B Q_y band. The spectrum of the WAAHD reaction centre also showed this shoulder (Fig. 2), and in addition showed the absorbance changes that are characteristic of the LM214H mutation, namely the decrease of the H Q_y band, and additional intensity on the blue side of the B Q_y band. The expression level of the WAAHD mutant was somewhat lower than normal, as can be seen from the increased light scattering in the normalised spectrum shown in Fig. 2.

Finally, mutant WHD was constructed by combining the AM260W, LM214H and WM157D mutations. Its spectrum was similar to that of the WAAHD mutant apart from the contribution of background scatter, which was more like that seen for the wild-type complex (reflecting a normal expression level for the WHD reaction centre).

The effect of the different combinations of mutations on the redox properties of the primary electron donor were examined by measuring the mid-point potential of the P/P^+ redox couple (E_m P/P^+). For all of the mutant complexes described above, the value of E_m P/P^+ obtained was within 10 mV of that determined for the wild-type complex (data not shown), a difference that was of the same order as the ±10 mV error that is typical for this type of measurement. The conclusion, therefore, is that none of the combinations of mutations had a significant effect on the redox properties of the primary electron donor.

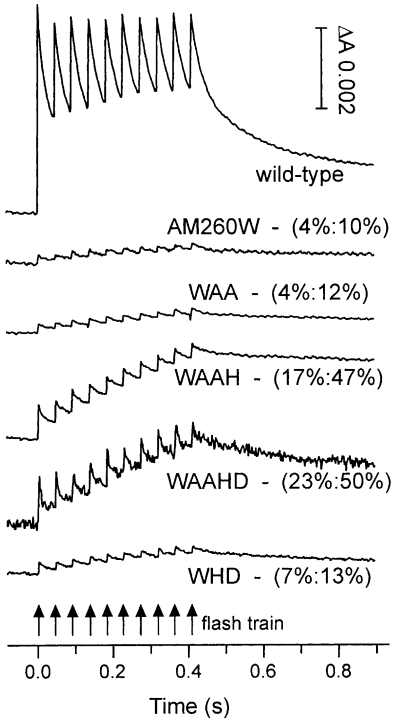

Fig. 3. P^+ formation in antenna-deficient membranes containing either wild-type or mutant reaction centres. Numbers in brackets refer to the percentage yield of the P^+-containing state in the mutant complexes after one or 10 excitation flashes, respectively (see text). For details of the nomenclature of the multiple mutants, see the legend to Fig. 2.

3.2. Transient absorbance spectroscopy

Transient absorbance spectroscopy with millisecond time resolution was used to look for evidence of transmembrane electron transfer from P to the reaction centre ubiquinone(s). In the wild-type reaction centre, photo-excitation produces the radical pair state $P^+Q_A^-$ on a picosecond time-scale. The electron is then transferred from Q_A^- to Q_B on a microsecond (μs) time-scale, forming $P^+Q_B^-$. In the absence of a cytochrome donor to the reaction centre these states are relatively long-lived, recombining to the neutral (PQ_AQ_B) state with time constants of approximately ~100 ms and ~1 s respectively [36,37]. In contrast, if forward electron transfer from the H_A BPhe to the Q_A ubiquinone is prevented by extraction or chemical reduction of this quinone, the remaining electron or energy transfer events that occur amongst the reaction centre bacteriochlorins take place on a sub-millisecond time-scale. The dominant process is recombination of the $P^+H_A^-$ radical pair, this taking place on a time-scale of 10–20 ns (see [10] for discussion). The primary donor triplet excited state can also be formed in a subpopulation of reaction centres, and has a lifetime of 30 ns or 50 μs, depending on the presence or absence of the reaction centre carotenoid [38]. Given the relatively short lifetimes of these processes, the observation of P photo-oxidation on a millisecond time-scale is therefore diagnostic of electron transfer from P to the reaction centre ubiquinones, forming the relatively long-lived $P^+Q_A^-$ and/or $P^+Q_B^-$ state.

Fig. 3 shows P^+ formation in antenna-deficient membranes containing wild-type reaction centres, measured by monitoring the absorbance changes at 542 nm [39,40] elicited by a train of 10 actinic light pulses (see Section 2). Control experiments in which the absorbance change at 551–542 nm was monitored showed that these antenna-deficient membranes lacked a cytochrome donor to the reaction centre, in line with previous observations [27]. The flash train caused full photo-oxidation of P in the wild-type reaction centres, the signal decaying during the dark time between flashes with a lifetime of hundreds of milliseconds. This pattern is consistent with that expected when flash excitation produces a mixed $P^+Q_A^-/P^+Q_B^-$ state.

Also shown in Fig. 3 are data recorded for antenna-deficient membranes containing the mutant reaction centres, normalised as described in Section 2. Membranes containing reaction centres with the single AM260W mutation also showed evidence of formation of a small amount of a P^+ state that was stable on a millisecond time-scale. The yield of this long-lived state was estimated by comparing the change in absorbance immediately following the first excitation flash with that exhibited by the same amount of wild-type reaction centres. This produced a value of approximately 4% for the AM260W mutant, with an accumulated yield of approximately 10% for this state after 10 excitation flashes.

The remaining mutants also showed evidence for photo-oxidation of P that persists on a millisecond time-scale, and the yield of this state after one and 10 flashes is shown in Fig. 3. The extent of P photo-oxidation varied between the mutants studied, and the possible reasons for this are discussed below. In particular, LM214H mutation markedly enhanced the yield of this state (WAAH compared with WAA), and the double alanine mutation also affected this yield (WAAHD compared with WHD).

3.3. Identity of the P^+-containing state formed in the mutant complexes

All of the mutant reaction centres examined in this work contain the well-characterised AM260W mutation, and are therefore expected to lack the Q_A ubiquinone [27,28]. Despite this, all of the mutant complexes were able to form a state involving P^+ that was stable on a millisecond time-scale. Giv-

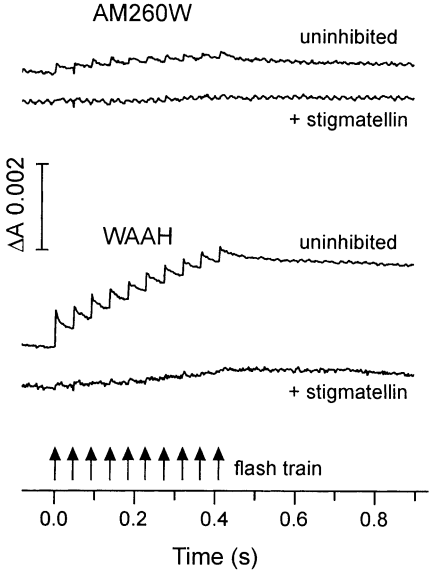

Fig. 4. Effects of stigmatellin on P^+ formation in mutants AM260W and WAAH. For details of the nomenclature of the WAAH mutant, see the legend to Fig. 2.

en the well-documented sub-millisecond lifetimes of radical pair states involving reduced monomeric BChl (e.g. $P^+B_A^-$) or reduced BPhe (e.g. $P^+H_A^-$), the implication is that the signals shown in Fig. 3 are the result of transmembrane electron transfer from P to Q_B, forming the $P^+Q_B^-$ radical pair. If this is correct, then P photo-oxidation on this time-scale should be abolished by an inhibitor of ubiquinone reduction at the Q_B site, such as the well-characterised ubiquinone analogue stigmatellin [41–43].

Fig. 4 shows the response of antenna-deficient membranes of the WAAH and AM260W mutants to the addition of 200 μM stigmatellin. For both mutants, the signal indicative of P photo-oxidation was largely abolished by this concentration of Q_B site inhibitor. Similar results were obtained with the remaining mutants (data not shown). The conclusion, therefore, is that the data shown in Fig. 3 are indeed attributable to the formation of the $P^+Q_B^-$ radical pair, following electron transfer along the B-branch of reaction centre cofactors. The residual absorbance changes in Fig. 4 are presumably due to incomplete inhibition of the Q_B site in these membrane-bound reaction centres. In the case of the wild-type reaction centre, addition of stigmatellin produced an acceleration in the rate of decay of the P^+ signal, consistent with the expected response if electron transfer from Q_A^- to Q_B is blocked, but the initial photo-oxidation was not affected (data not shown).

4. Discussion

4.1. Photo-accumulation of the $P^+Q_B^-$ state in the AM260W mutant reaction centre

In the wild-type reaction centre a train of 10 excitation flashes, each of 6 μs duration, caused photo-oxidation of the primary electron donor, and formation of a mixed $P^+Q_A^-/P^+Q_B^-$ state. As the lifetimes of these states (100 ms and 1.2 s, respectively) are much longer than the duration of the excitation flash, the experiment involves 'single turnover' excitation conditions. As can be seen from the data in Fig. 3, the first excitation flash produced full photo-oxidation of P, and subsequent flashes re-oxidised P in those reaction centres that had undergone charge recombination during the ~40 ms dark period between flashes.

In the presence of the AM260W mutation, primary electron transfer along the A-branch produces the $P^+H_A^-$ state but there is no subsequent electron transfer to Q_A [27]. The $P^+H_A^-$ radical pair has a lifetime of 10–20 ns, decaying principally by charge recombination to the ground state. This means that there is the opportunity for multiple excitation of AM260W reaction centres within the lifetime of each 6 μs excitation pulse in the experiments shown in Fig. 3. As a result, the $P^+Q_B^-$ state formed in the mutant complexes on each flash has to be considered as being photo-accumulated in response to multiple excitation, rather than being formed following a single turnover excitation event as in the wild-type reaction centre.

In membranes containing AM260W reaction centres, it was possible to photo-accumulate the $P^+Q_B^-$ state with a yield of 4% after one flash and 10% after 10 flashes. This finding is in contradiction to our previous report on this mutant, in which we were unable to resolve any P photo-oxidation in response to 16 actinic flashes of similar duration delivered at 40 ms intervals [27]. The most likely explanation of this difference is that the instrument used to collect the data in Fig. 3 had a more intense excitation flash than the instrument used in our previous study, and that the present data were associated with significantly less noise than that collected previously.

Given that each 6 μs excitation pulse was of sufficient duration to excite the AM260W reaction centre several hundred times, the observation that a long-lived $P^+Q_B^-$ state was formed in only 4% of reaction centres following the first excitation pulse suggests that the yield of electron transfer along the full length of the B-branch of cofactors was very low. As an illustration, if it is assumed that the yield of this process after a single turnover of the reaction centre population was 1%, then it can be calculated that approximately 64% of the reaction centre population would be accumulated in the $P^+Q_B^-$ state after 100 excitations. The measured amount of $P^+Q_B^-$ formed on the first flash is more consistent with a yield of B-branch electron transfer of 0.04%, assuming 100 excitation cycles, or less than 0.01% if it assumed that the reaction centre population underwent approximately 500 excitation cycles during the 6 μs pulse. This indicates that the yield of electron transfer along the full length of the B-cofactor branch is very low in this mutant and, by extension, in the wild-type reaction centre (previous spectroscopic and crystallographic studies have shown that the AM260W mutation does not have any discernible effect on the structure of the reaction centre outside the immediate environment of the Q_A binding site, or on the rate of primary electron transfer from P to H_A [27,28], and therefore it seems reasonable to assume that it does not affect the balance of electron flow along the A- and B-branches). Given estimates that the yield of $P^+H_B^-$ is a few percent in wild-type reaction centres (see [10] for a discussion), our findings suggest that the natural yield of the H_B^- to Q_B step of B-branch electron transfer is also only a few percent.

In this discussion, it has been assumed that excitation produces the $P^+Q_B^-$ radical pair. However, the exact reduction/protonation state of the Q_B ubiquinone remains to be confirmed. In particular, given the multiple excitation conditions we have employed we cannot exclude the possibility that there may be some double reduction of Q_B, if there is some reduction of P^+ by electrons derived from the medium. Although this seems unlikely, experiments are under way to examine this point in more detail. One possible way to address this would be through measurements of proton uptake using a pH indicator dye such as phenol red, but the fact that the membranes isolated from the antenna-deficient mutants used in this study are open sheets rather than sealed chromatophores would complicate the interpretation of such data. This type of membrane also precludes the use of carotenoid bandshifts to study the yield of the H_B to Q_B electron transfer step.

4.2. Effects of the LM214H mutation on photo-accumulation of the $P^+Q_B^-$ state

The yield of the $P^+Q_B^-$ state was not significantly enhanced when the double EL212A/DL213A mutation was combined with the AM260W mutation, in mutant WAA (Fig. 3). However, further addition of the LM214H mutation, to form the quadruple mutant WAAH, increased the yield of $P^+Q_B^-$ photo-accumulated after the first flash to approximately 17%. Assuming that the low efficiency of the $H_B^- \rightarrow Q_B$ electron transfer step is not altered by the LM214H mutation, this

suggests that the effect of the LM214H mutation is to increase the yield of $P^+H_B^-$ formation by approximately four-fold.

Studies of reaction centres with the LM214H mutation [15,16,24,44,45] have shown that the replacement of the H_A BPhe by BChl decreases the lifetime for the P^+I^- state to approximately 0.9 ns [24] (where P^+I^- denotes the state formed on the A-branch that is a mixture of $P^+B_A^-$ and $P^+\beta_A^-$), as compared with the 10–20 ns lifetime of the equivalent $P^+H_A^-$ state in the wild-type reaction centre. Thus the increased yield of the photo-accumulated $P^+Q_B^-$ state in the WAAH reaction centre could be due to an increased frequency of excitation of the reaction centre by the 6 μs excitation flash, brought about by the shorter A-branch radical pair lifetime.

4.3. Effects of the WM157D and EL212A/DL213A mutations

Addition of the WM157D mutation to the WAAH combination, to produce the WAAHD mutant, somewhat enhanced the amount of $P^+Q_B^-$ photo-accumulated during a single flash, and enhanced the rate at which this state decayed. One interpretation of this result is that the WM157D mutation, that is adjacent to the macrocycle of the B_B BChl, modulates the energetics of B-branch electron transfer such that both the $P^* \rightarrow P^+H_B^-$ reaction and recombination of the $P^+Q_B^-$ radical pair are accelerated. Picosecond time-scale spectroscopy should be able to clarify the effects of the WM157D mutation on B-branch electron transfer.

Removal of the double EL212A–DL213A mutation to leave the combination LM214H–AM260W–WM157D (mutant WHD) resulted in a significant decrease in the yield of $P^+Q_B^-$, from the 23% seen in the WAAHD mutant after the first excitation pulse to the 7% seen in the WHD mutant. This indicates that, at least for a reaction centre with the LM214H–AM260W–WM157D combination, the double EL212A–DL213A mutation brings about a marked increase (approximately three-fold) in the amount of $P^+Q_B^-$ formed. Inclusion of the double EL212A/DL213A mutation as part of the present study was inspired by a report by Laible and co-workers, that demonstrated that this mutation increased the yield of $P^+Q_B^-$ formation via the B-branch by approximately 50% in purified reaction centres [17]. The results on the WAAHD and WHD mutants reported in the present study are in qualitative agreement with this conclusion.

The L212 and L213 residues form part of the Q_B binding pocket, on the opposite side of the ubiquinone head-group from the nearest bacteriochlorin (the H_B BPhe), and the side chains of these amino acids are over 13 Å from the macrocycle of the H_B BPhe. In a recent report, Pokkoluri and co-workers have described the X-ray crystal structure of a *R. sphaeroides* reaction centre with the double EL212A/DL213A mutation, at a resolution of 3.1 Å [46]. The double mutation causes a rearrangement of polar residues that line the Q_B binding pocket and an expansion of the pocket. However, this expansion is restricted to sections of the protein that are on the opposite side of the pocket to the H_B BPhe, and the double EL212A/DL213A mutation has no significant effect on the structure of the protein–cofactor system in the vicinity of the six reaction centre bacteriochlorins [46].

These observations suggest that the EL212A/DL213A double mutation affects the H_B^- to Q_B step directly through an effect on the Q_B ubiquinone, rather than affecting the yield of photo-accumulated $P^+Q_B^-$ through an indirect effect (such as that proposed for the LM214H mutation, above). The reasons for this effect remain to be determined, but one obvious consideration is that the double EL212A/DL213A mutation would be expected to decrease the polarity of the Q_B binding pocket. This could alter the rate of the electron transfer from H_B^- to Q_B either by changing the redox potential of the Q_B/Q_B^- couple, and so affecting the driving force for the reaction, or by affecting the reorganisation energy for this electron transfer step. In an interesting corollary, mutagenesis of the symmetry-related residues in the Q_A binding pocket (Ala M246 and Ala M247) to Gln and Asp, respectively, has been reported to result in a 10-fold decrease in the rate of electron transfer from H_A^- to Q_A [47].

Finally, the enhancement of $P^+Q_B^-$ formation brought about by the EL212A/DL213A double mutation when put into the WHD background was not seen when this mutation was added to AM260W alone (in mutant WAA). This indicates that the effects of the double EL212A/DL213A mutation are background-specific, being larger in the mutant where the efficiency of A-branch electron transfer has been retarded. The reasons for this difference are not clear, and are the subject of ongoing work. Further combinations of mutations should clarify the effects of the EL212A/DL213A double mutation on the yield of the H_B^- to Q_B step, and our experimental set-up should also allow us to look at other ways to enhance the yield of electron transfer along the full length of the B-cofactor branch.

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