Characterization of the bonding interactions of $Q_B$ upon photoreduction via A-branch or B-branch electron transfer in mutant reaction centers from *Rhodobacter sphaeroides*

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*In *Rhodobacter sphaeroides* reaction centers (RCs) containing the mutation Ala M260 to Trp (AM260W), transmembrane electron transfer along the full-length of the A-branch of cofactors is prevented by the loss of the $Q_A$ ubiquinone, but it is possible to generate the radical pair $P^+H_A/C_0$ by A-branch electron transfer or the radical pair $P^+Q_B/C_0$ by B-branch electron transfer. In the present study, FTIR spectroscopy was used to provide direct evidence for the complete absence of the $Q_A$ ubiquinone in mutant RCs with the AM260W mutation. Light-induced FTIR difference spectroscopy of isolated RCs was also used to probe the neutral $Q_B$ and the semiquinone $Q_B/C_0$ states in two B-branch active mutants, a double AM260W–LM214H mutant, denoted WH, and a quadruple mutant, denoted WAAH, in which the AM260W, LM214H, and EL212A–DL213A mutations were combined. The data were compared to those obtained with wild-type (Wt) RCs and the double EL212A–DL213A (denoted AA) mutant which exhibit the usual A-branch electron transfer to $Q_B$. The $Q_B/C_0$ spectrum of the WH mutant is very close to that of Wt RCs indicating similar bonding interactions of $Q_B$ and $Q_B/C_0$ with the protein in both RCs. The $Q_B/C_0/Q_B$ spectra of the AA and WAAH mutants are also closely related to one another, but are very different to that of the Wt complex. Isotope-edited IR fingerprint spectra were obtained for the AA and WAAH mutants reconstituted with site-specific $^{13}$C-labeled ubiquinone. Whilst perturbations of the interactions of the semiquinone $Q_B/C_0$ with the protein are observed in the AA and WAAH mutants, the FTIR data show that the bonding interaction of neutral $Q_B$ in these two mutants are essentially the same as those for Wt RCs. Therefore, it is concluded that $Q_B$ occupies the same binding position proximal to the non-heme iron prior to reduction by either A-branch or B-branch electron transfer. © 2004 Elsevier B.V. All rights reserved.

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1. Introduction

Reaction centers (RCs) are membrane-spanning cofactor–protein complexes that perform the primary reactions in the conversion of light energy into the chemical energy of charge-separated states in photosynthetic organisms. One of the archetypal features of all RCs is the presence of dual, symmetrically arranged branches of electron-transfer cofactors [1]. Although there is some evidence that both of these branches are active in catalyzing transmembrane electron transfer in iron–sulfur-type (Type-I) RCs [2–4], it is well-established that effectively only one branch is active in pheophytin–quinone-type (Type II) complexes such as the RC of purple photosynthetic bacteria [5–8] and the Photosystem II RC of oxygenic photosynthetic organisms. The strong functional asymmetry of the Type-II RCs has remained a puzzling enigma for almost 20 years since the elucidation by X-ray crystallography of the structures of the RCs from *Rhodopseudomonas (Rps.) viridis* [9–12] and *Rhodobacter (Rb.) sphaeroides* [13–17].

In the *Rb. sphaeroides* RC the two branches of cofactors (Fig. 1A), designated A and B, start at a specialized dimer of
bacteriochlorophyll (BChl) molecules (denoted P), and extend first to two monomeric BChl molecules (B_A and B_B), then to two bacteriopheophytin (BPhe) molecules (H_A and H_B), and terminate at two ubiquinone molecules (Q_A and Q_B). The two branches are related by an axis of pseudo 2-fold symmetry that runs approximately perpendicular to the plane of the membrane, from the center of the P dimer on the periplasmic side to a non-heme iron atom located between the Q_A and Q_B ubiquinones on the cytoplasmic side. Following the absorption of a photon by P, the state P^+H_A/H_0 is formed in about 3 ps, and within 200 ps forward electron transfer proceeds to generate the radical pair P^+QA/Q_B [18–23]. On a much longer time scale (∼ 5–200 μs) the electron is passed to the secondary ubiquinone Q_B, forming the semiquinone (Q_B^−/C_0) [24,25]. Ubiquinol is generated following two successive transmembrane electron transfers and the uptake of two protons from the cytoplasmic side of the membrane, through the protein matrix [24,25]. Finally, the ubiquinol is released into the photosynthetic membrane and is replaced with an oxidized ubiquinone from the intramembrane pool.

In an attempt to understand the basis for the exclusive use of the A-branch of cofactors in the initial steps of electron transfer, extensive mutational work has been conducted in the environment of P, B_A, B_B, H_A, and H_B [26–41]. A particular feature of these studies has been use of the Leu M214 to His mutation (denoted Lm214H- and equivalent to Lm212H in Rb. capsulatus, see Fig. 1B for the location of this residue), that causes replacement of the H_A BPhe with BChl (denoted h_A) [26,27]. This replacement makes A-branch electron transfer less efficient, and leaves H_B as the only BPhe cofactor in the RC, allowing spectroscopic signals attributable to H_B reduction to be resolved. More recently, mutations have been developed that exclude or weaken ubiquinone binding at the QA site, blocking the H_A to QA electron transfer reaction, and providing a good background for investigation of the H_B to Q_B electron transfer step [28,33,34,38,39,42,43]. The location of the Ala M260 to Trp (AM260W) mutation [42,43] used to exclude binding of QA in the present work is shown in Fig. 1B.

The QA to Q_B electron transfer reaction and the coupling of electron transfer to proton transfer that accompanies the double reduction of Q_B, remain among the most challenging mechanisms to be investigated in the RC [24,25]. The latter reaction, which is of a wider biological relevance due to its occurrence in many respiratory systems, can be investigated in detail in the RC as it can be triggered with short flashes of light. It has been established that electron transfer from Q_A^− and Q_B to Q_A^+H_0.

Fig. 1. Structural models of the Rb. sphaeroides RC. (A) Cofactor organization in the Wt RC. Cofactors are shown in cpk colours, and cofactor side chains have been removed for clarity. The routes of A-branch and B-branch electron transfer are indicated by the solid and dotted arrows, respectively. (B) Cofactor organization in the AM260W RC, with Trp M260 highlighted in cyan. The positions of the other residues mutated in this work are highlighted in red, with mutations shown in brackets. (C) The distal and proximal binding positions of the Q_B ubiquinone in the Rb. sphaeroides Wt RC [66]. Hydrogen bond interactions between the carbonyl groups of the Q_B ubiquinone when in the proximal (cpk colours) and the distal (red) positions are indicated by magenta and cyan dotted lines, respectively. Also shown using green dotted lines is the connection between QA and the proximal Q_B, involving His L190, His M219 and the non-heme iron.
to Q_B is frozen out at cryogenic temperatures, suggesting that the reaction is gated by a temperature-sensitive conformational change [44–46]. Similar behavior is exhibited by the Q_A to Q_B reaction in the related Photosystem II RC [47,48]. In support of the proposal that this electron transfer step is gated, it has been shown that the rate of Q_A reduction by Q_B at room temperature is independent of the driving force for the reaction [49], at least for the main ~100 μs component seen in isolated Rb. sphaeroides RCs [50].

The gating process has been considered using both computational and experimental approaches [51–65], and a number of possible candidates for the gating conformational change have been discussed, including changes in the protonation state of residues in the Q_B pocket on formation of Q_A (see Ref. [25] for a recent review). One proposal that has drawn particular attention, made on the basis of a structural study of RC crystals cooled to cryogenic temperature either in the dark or in the light, is that a large motion of the Q_B head-group [66]. When in the proximal position (highlighted in red in Fig. 1C), the C4 backbone amides of Ile L224 and Gly L225, whilst the C4 carbonyl is hydrogen bonded to the side-chain of His L190. These hydrogen bonds are indicated by the spheres in Fig. 1C. When the ubiquinone is in the distal binding position (highlighted in red in Fig. 1C), the C4 carbonyl accepts a hydrogen bond from the backbone amide of Ile L224 whilst there is no suitable donor to the C1 carbonyl [16,66,67]. The two binding positions therefore display different hydrogen bond patterns.

Although the proposal that this distal to proximal movement of the Q_B ubiquinone gates the first Q_A to Q_B electron transfer is attractive, a number of reports have questioned whether this movement is relevant to the gating process [68–71], and the conformational changes that govern the rate of electron transfer remain the subject of active debate. Mutant RCs showing electron transfer along the full length of the B-branch in the absence of Q_A are therefore of interest because they provide an alternative means to investigate the mechanism of coupled electron and proton transfer reactions at Q_B and the nature of the gating mechanism, including the relevance of the proposed distal to proximal transition.

In a recent study [41], light-induced FTIR difference spectroscopy was used to characterize the P^+Q^- minus PQ difference spectrum (abbreviated as P^+Q^/-PQ hereafter) resulting from charge separation in wild-type (Wt) and mutant RCs from Rb. sphaeroides. These RCs perform long-lived P^+Q^- charge separation by either A-branch or B-branch electron transfer [40]. Using previously determined IR marker bands for the presence of Q_A and Q_B [72], we compared Wt RCs and complexes bearing the mutation EL212A–DL313A (denoted AA) (Fig. 1B) at the Q_B site [73–75], both of which exhibit only A-branch electron transfer to Q_B, with RCs that also contain the Q_A-excluding AM260W mutation [42,43] and the LM214H mutation described above [26,27] (Fig. 1B), which perform only B-branch electron transfer to Q_B. The double EL212A–DL313A mutation prevents reduction of Q_B beyond the semiquinone state [73]. There were no significant differences between the P^+Q^-/PQ spectra that were obtained for the A-branch-active Wt and AA RCs, and the B-branch-active counterparts with the mutations AM260W–LM214H (denoted WH) and AM260W–EL212A–DL213A–LM214H (denoted WAAH), thus suggesting that the environment of Q_B and Q_B was similar for Q_B reduction via A- or B-branch electron transfer [41]. However, a limitation of this study was that P^+Q^-/PQ spectra are dominated by P^+/P contributions [76], and give only limited information on the Q_B/Q_B components.

The present report characterizes the reduction of ubiquinone in these B-branch-active mutants in detail, through measurement of more diagnostic Q_B/Q_B FTIR difference spectra using AA, WH, and WAAH mutant RCs. We use isotope-edited difference spectroscopy [72,77], which gives precise fingerprints of the bonding interactions of the Q_B ubiquinone both before and after photoreduction. The data demonstrate that the Q_B involved in B-branch electron transfer occupies the same position as the Q_B involved in A-branch electron transfer in the Wt RC, this position corresponding to the proximal site in X-ray structural models of the RC.

2. Materials and methods

2.1. Preparation of experimental material

The procedures used in the construction of the mutant RCs were described recently for the WAAH combination [40], and the AA and WH combinations [41]. RCs were purified according to procedures described previously [78,41], and were suspended in a buffer consisting of 20 mM Tris/HCl (pH 8.0)/0.1% lauryldimethylamine oxide (LDAO).

A detailed description of the preparation of RC samples for FTIR experiments involving Q_A and Q_B is given in Ref. [79] and Ref. [77], respectively. The Q_B site was reconstituted with ubiquinone-3 (Q_3) by adding a ~10-fold excess of Q_3 [80]. The synthesis of Q_3 selectively labeled with 13C at the C1 or C4 position has been reported elsewhere [81].

2.2. FTIR spectroscopy

For both Wt and mutant RCs, the Q_A state was generated in the presence of N,N,N',N'-tetramethyl-p-phenylenedi-
amine (TMPD) (50 mM) [79], potassium ferrocyanide (250 mM), and stigmatellin (2 mM) under saturating continuous illumination, using an RG 715 cut-off filter and a water filter to prevent heating of the sample. For the mutant RCs, the $Q_B$ state was generated in the presence of potassium ferrocyanide (250 mM) and TMPD (50 mM) under saturating continuous illumination, whereas in Wt RCs the $Q_B$ state was obtained in the presence of diaminodurene (DAD, 2,3,5,6-tetramethyl-p-phenylenedia mine) (20 mM) and sodium ascorbate (10 mM) with single saturating flash excitation (Nd:YAG laser, 7 ns, 530 nm) [77]. In the latter case, ascorbate and DAD were found to be a better reductant/mediator combination for use with single flash excitation, but the choice of reductant and mediator did not affect the characteristics of the resulting FTIR difference spectrum.

Steady-state light-induced $Q_A/Q_A$ [79] and $Q_B/Q_B$ [77] FTIR difference spectra were recorded at 285 K using a Nicolet 60SX spectrometer equipped with a MCT-A detector, a KBr beam-splitter and a cryostat. Difference spectra were calculated from two data-sets each consisting of 128 scans (acquisition time: 23 s) recorded before and after illumination. For a given sample, these measurements were repeated over an approximately ~30 h period and the difference spectra were averaged. Spectral resolution was 4 cm$^{-1}$.

3. Results

3.1. Absence of the $Q_A$ ubiquinone in RCs bearing the AM260W mutation

In order to confirm the absence of a photoactive $Q_A$ ubiquinone in RCs containing the AM260W mutation, and to investigate any possible perturbations of the $Q_A$ binding site induced by the AA double mutation, the light-minus-dark FTIR difference spectrum of the photoreduction of $Q_A$ (henceforth referred to as the $Q_A/Q_A$ spectrum), was measured for stigmatellin-inhibited Wt RCs (Fig. 2a) and compared to those of stigmatellin-inhibited RCs with the AA (Fig. 2b), WH (Fig. 2c), and WAAH (Fig. 2d) mutations. In these spectra, contributions from the $Q_A$ state appear as positive bands and the unreduced $Q_A$ state as negative bands.

Whilst the $Q_A/Q_A$ spectrum of the AA mutant RCs was essentially identical to that of the Wt complex, and to spectra published previously for R26 RCs [79,82], the WH and WAAH mutant RCs give no difference spectrum. Thus, there is no indication of a contribution from the primary quinone acceptor in RCs with the AM260W mutation. Up until this point, the lack of $Q_A$ in such RCs has been based on (i) the absence of corresponding electron density in X-ray diffraction data collected for the AM260W single mutant [43], (ii) the apparent absence of forward electron transfer from $P^+H_B$ in femtosecond time scale transient absorbance data [42], and (iii) the absence or near-absence of rapid P photooxidation in millisecond time scale transient absorption measurements [40,42]. In the most recent of these reports, the small amount (~4%) of P photooxidation detected in the AM260W single mutant was attributed to B-branch electron transfer to $Q_B$, as the corresponding absorbance change was sensitive to the $Q_B$ inhibitor stigmatellin [40]. From the amplitude of the noise in the spectra displayed in Fig. 2c and d, it can be concluded that the contribution of photoactive $Q_A$ in the WH and WAAH RCs would have to be less than $10^{-3}$ of that in Wt RCs. Therefore, the present FTIR data provide a more sensitive assay of $Q_A$ function in AM260W-containing RCs, and constitute direct evidence in support of the earlier proposal that the $Q_A$ ubiquinone is excluded from RCs with this mutation [42,43].

3.2. Photoreduction of $Q_B$ upon A-branch electron transfer in the EL212A–DL213A (AA) mutant RC

Fig. 3 compares the $Q_B/Q_B$ spectra for Wt (Fig. 3a) and AA mutant RCs (Fig. 3c). In Wt RCs in the presence of
excess ubiquinone, a saturating flash generates quantitatively the state P⁺Q⁺. When a mixture of reductant (sodium ascorbate) and mediator (DAD) is added to the medium to rapidly reduce P⁺, a Q⁻/Q⁺ FTIR difference spectrum is obtained (Fig. 3a), as demonstrated previously [77, 80]. In this spectrum, the bands of the neutral Q⁺ state appears as negative contributions, whilst those of Q⁻ are positive. In Wt RCs, a highly comparable Q⁻/Q⁺ spectrum can also be generated upon excitation with very low intensity continuous illumination, albeit with a much reduced amplitude [80]. Upon increasing the intensity of the DC light, additional contributions from the ubiquinol redox state of Q⁻ appear [80]. In the case of RCs of the AA mutant, it was found that identical Q⁻/Q⁺ FTIR difference spectra could be generated either upon illumination with saturating continuous light (Fig. 3c), or upon single flash excitation (not shown). The finding that a Q⁻/Q⁺ difference spectrum was obtained with strong continuous illumination shows that formation of a stable ubiquinol from the Q⁻ state is impeded in this mutant, in agreement with kinetic studies in the visible range showing that replacement of both Glu L212 and Asp L213 with Ala prevents the transfer of the first proton and the second electron to the semiquinone Q⁻ [73].

The Q⁻/Q⁺ spectrum of the AA mutant RC (Fig. 3c) showed a number of differences to that of the Wt complex (Fig. 3a), notably in the 1500–1400 cm⁻¹ range where the semiquinone Q⁻ modes absorb [77–80], around 1675–1655 cm⁻¹ (i.e. in the amide I and side chain absorption range), around 1560–1515 cm⁻¹ (i.e. in the amide II region), and in the absorption range of protonated carboxylic groups (1770–1700 cm⁻¹). In the place of the single pronounced positive band observed at 1479 cm⁻¹ (mixed C=O and C=C Q⁻ modes) in Wt RCs (Fig. 3a), the spectrum of the AA mutant (Fig. 3c) showed two distinct peaks at 1493 and 1471 cm⁻¹. It should be noted that perturbations in the semiquinone and the amide bands that are comparable to those observed for the AA RC have been previously observed for RCs with mutations at Pro L209 [70, 71], or at Asp L213 [83]. Such changes with respect to the Wt reflect different rearrangements of the mutated protein upon Q⁺ formation, and ensuing perturbations of the semiquinone modes (as will be shown below using AA RCs reconstituted with site-specific isotope-labeled ubiquinone).

Consistent with the replacement of Glu L212 with Ala, the Q⁻/Q⁺ spectrum of the AA RC (Fig. 3c) lacked the positive band at 1727 cm⁻¹ seen in the spectrum of the Wt complex (Fig. 3a), and which has previously been attributed to the protonation of Glu L212 upon Q⁺ formation in Wt RCs [84, 85]. The differential signal at 1731–1739 cm⁻¹ in the Q⁻/Q⁺ spectrum of the AA mutant RC (Fig. 3c) has been previously observed in the spectra of Glu L212-deficient mutants [84, 86, 87], and the negative component is also seen in the spectrum of the Wt complex (Fig. 3a). The 1731–1739 cm⁻¹ signal has been tentatively related to the electrostatic influence of the electron on Q⁻ on the IR mode of the 10α-ester carbonyl of the H₂ Bphe [84, 86, 87], in a similar manner to the reported electrostatic influence of Q⁺ on the IR mode of the 10α-ester carbonyl of the H₆ Bphe [88].

### 3.3. Photoreduction of Q⁺ upon B-branch electron transfer in the WH and WAAH mutant RCs

When WH mutant RCs were illuminated with continuous light in the presence of potassium ferrocyanide and TMPD, the light-induced difference spectrum was found to contain, in addition to Q⁻/Q⁺ signals, a relatively small contribution from P⁺/P (peak to peak amplitude less than 20% of Q⁻/Q⁺), but there was no indication of formation of ubiquinol (data not shown). This indicated that the relative rates of formation and decay of the P⁺Q⁺ state are different in this mutant compared to those in Wt RCs.

In order to extract a pure Q⁻/Q⁺ spectrum without P⁺/P contributions from the composite spectrum obtained with ferrocyanide/TMPD, a pure P⁺Q⁻/PQ⁺ spectrum [41], produced with the same RCs but in the absence of reductant and mediator was subtracted from the composite spectrum. The result of an interactive subtraction between two such
spectra that minimizes all the bands characteristic of P+/P [76] is shown in Fig. 3b. An alternative method, which consisted of optimizing the balance of the three most important parameters in the build-up and decay of Qa (light intensity, concentration of redox agents, and temperature), led to an essentially identical Qb/Qa spectrum (not shown). However, this optimization process required prolonged experimentation to identify conditions that would give a pure Qb/Qb spectrum, and the exact conditions were found to be sample-specific, and so the more convenient subtraction approach was routinely used.

Most of the features in the Qb/Qb difference spectrum of the WH RC (Fig. 3b) were found to be essentially identical to those in the Wt complex (Fig. 3a). This showed that a highly comparable state was achieved in both cases, despite the fact that electron transfer proceeds via the A-branch in the Wt RC but via the B-branch in the WH mutant [41]. Some small differences were observed between the two spectra, notably at around 1657 and 1550 cm⁻¹ where additional negative peaks were seen in the spectrum of the WH mutant (Fig. 3b).

Using continuous light illumination, a pure Qb/Qb difference spectrum was obtained upon B-branch electron transfer in the WAAH quadruple mutant (Fig. 3d) which was very close to that observed in the AA double mutant upon A-branch electron transfer (Fig. 3c). As already observed for the AA mutant, the spectrum obtained with the WAAH mutant was strongly perturbed compared to that of the Wt RC, notably in the 1550–1400 and 1750–1700 cm⁻¹ regions, and at around 1680 cm⁻¹. In addition, small differences between the spectra of the WAAH and AA mutants were observed all throughout the 1700–1400 cm⁻¹ spectral range.

The small differences observed between the spectra of the AA WAAH mutants on the one hand, and of the Wt and WH RCs on the other hand, could be caused by the perturbation of the Qa site induced by the AM260W and/ or LM214H mutations, and possibly by the presence of a chloride anion in the cavity left by the exclusion of the QA ubiquinone [43]. Evidence for interactions between QA and QB sites involving hydrogen-bonded networks has been discussed in the literature [53,57,89]. Another possible source of these differences is that the location of the ubiquinone at the Qb site that undergoes photoreduction is different for A-branch (Wt, AA mutant) or B-branch (WH and WAAH mutants) electron transfer. This possibility can be probed by examining the interactions of the two carbonyl groups of the QB ubiquinone with the surrounding protein, using isotopically labeled ubiquinones [72,77] as described in the next section.

3.4. Isotope-edited IR fingerprint of QB photoreduction in RCs bearing the double AA mutation

The Qb/Qb difference spectra of AA mutant RCs in the presence of excess ubiquinone are shown in Fig. 4 for unlabeled Q3 (Fig. 4a), 13C1-labeled Q3 (Fig. 4b), 13C4-labeled Q3 (Fig. 4c), respectively. The equivalent spectra for the WAAH mutant are shown in Fig. 5 with the same colour coding. Clear effects of the labeling of the C1 and C4 carbonyls are evident, notably in the region of the C=O and C=C vibrations of the neutral quinone (1650–1600 cm⁻¹) and around 1500–1400 cm⁻¹ where the main mixed C=O/C=C modes of the semiquinone QB absorb [77,80].

These effects reflect the isotope-sensitive vibrations of QB and QB that involve displacement of the C1 and C4 atoms, and are best visualized by calculating double-difference spectra between pairs of Qb/Qb spectra recorded with RCs reconstituted with isotopically labeled or unlabeled quinones. This is achieved by performing interactive subtraction as described in previous publications [70,71,77,79,90–92]. Such spectra, in which all the IR contributions of modes of the cofactors and the protein that are affected by the QB to Qb reaction are cancelled out except for those modes that involve motion of the C1 and C4 atoms of Qb and QA, are shown for 13C1 (Figs. 4d and 5d) and 13C4 (Figs. 4e and 5e) labeling. In these double-
difference (isotopically labeled minus-unlabeled) spectra, the IR bands of the neutral unlabeled Q_B now appear with a positive sign, while the downshifted bands of the labeled quinone appear with a negative sign. All these Q_B bands are seen in the 1650–1600 cm\(^{-1}\) range. A reverse situation is found for the bands of the semiquinone Q_B in the 1500–1400 cm\(^{-1}\) range. All of the bands that are highlighted in the double-difference spectra in Figs. 4 and 5 were reproducibly observed. As discussed in previous reports \([70,71,77,79]\), the small spurious bands that sometimes appear in the regions around 1660–1650 and 1550 cm\(^{-1}\) are attributed to a poor cancellation of slightly variable signals in the protein amide I and amide II regions of the different Q_B/Q_B spectra, respectively.

The isotope-edited double-difference spectra of the AA and WAAH RCs are shown on an expanded scale in Fig. 6, where they are compared with the spectra of the Wt RC \([77]\) for \(^{13}\)C\(_1\) labeling (Fig. 6a–c) or \(^{13}\)C\(_4\) labeling (Fig. 6d–f). The features obtained for the neutral Q_B (1650–1600 cm\(^{-1}\)) in the AA (Fig. 6b,e) and WAAH (Fig. 6c,f) mutant RCs were essentially the same as those in the equivalent spectra of Wt RCs (Fig. 6a,d), demonstrating that neither the double AA mutation alone nor the combined AA and WH mutations affected the bonding pattern of the neutral ubiquinone to the protein. In contrast, comparison of the highly specific IR fingerprints of the QB anion (1500–1400 cm\(^{-1}\)) for the AA and WAAH RC upon \(^{13}\)C\(_1\) labeling (Fig. 6b,c) or \(^{13}\)C\(_4\) labeling (Fig. 6e,f) showed notable differences between the two mutants. They also displayed large changes when compared to the equivalent spectra for Wt RCs (Fig. 6a,d), indicative of different interactions of the anion with the protein in the three RCs, with the largest changes being obtained with the WAAH RC.

Dealing with the effects of \(^{13}\)C\(_1\)-labeling first (Fig. 6a–c), the Q_B band in the 1498–1490 cm\(^{-1}\) range showed significant differences in sign and relative amplitudes upon \(^{13}\)C\(_1\)-labeling in the quadruple WAAH mutant (Fig. 6c) compared to the double AA complex (Fig. 6b).\(^1\) Moreover, of Wt RCs (Fig. 6a,d), demonstrating that neither the double AA mutation alone nor the combined AA and WH mutations affected the bonding pattern of the neutral ubiquinone to the protein. In contrast, comparison of the highly specific IR fingerprints of the Q_B anion (1500–1400 cm\(^{-1}\)) for the AA and WAAH RC upon \(^{13}\)C\(_1\) labeling (Fig. 6b,c) or \(^{13}\)C\(_4\) labeling (Fig. 6e,f) showed notable differences between the two mutants. They also displayed large changes when compared to the equivalent spectra for Wt RCs (Fig. 6a,d), indicative of different interactions of the anion with the protein in the three RCs, with the largest changes being obtained with the WAAH RC.

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the unlabeled Q_B anion showed a broad negative band at 1472 cm\(^{-1}\) and a small positive signal at 1490 cm\(^{-1}\) for the AA RC (Fig. 6b), but two negative bands were apparent at 1498 and 1472 cm\(^{-1}\) in the spectrum of the WAAH complex (Fig. 6c). The positive bands for the \(^{13}\)C\(_1\)-labeled ubiquinone species were similar in the AA and WAAH spectra (at 1439–1437 cm\(^{-1}\) and 1412–1414 cm\(^{-1}\), compared to 1438 and 1415 cm\(^{-1}\) in the Wt spectrum).

In the case of \(^{13}\)C\(_4\)-labeling (Fig. 6d–f), the main positive band for the \(^{13}\)C\(_4\)-labeled Q_B anion was at 1444 cm\(^{-1}\) in the spectrum of the AA RC (Fig. 6e), comparable to the 1439 cm\(^{-1}\) band seen for the Wt complex (Fig. 6d), but was at 1410 cm\(^{-1}\) in the spectrum of the WAAH RC (Fig. 6f). Both the AA and WAAH spectra (Fig. 6e,f) showed negative bands at 1493 and \(\sim\) 1472 cm\(^{-1}\) for the unlabeled Q_B anion, compared to the main negative peak at 1479 cm\(^{-1}\) and the small positive signal at 1493 cm\(^{-1}\) for the Wt complex (Fig. 6d).

4. Discussion

The data in Fig. 2 provide direct evidence in support of the proposal that the Q_A ubiquinone is completely absent in RCs with the AM260W mutation [42,43]. The reduced ubiquinone species that can photoaccumulated in the Wt RC, the set of mutant complexes investigated in this study provide a means of comparing the bonding interactions of Q_B and Q_B\(^{\text{an}}\) upon photoreduction via the A-branch or B-branch. This also provides an alternative means of investigating the processes that accompany the first reduc-

4.1. Semiquinone (Q_B\(^{\text{an}}\)) binding in the WH, AA, and WAAH mutants: comparison with the Wt RC

The Q_B\(^{\text{an}}\)/Q_B difference spectrum of the WH mutant (Fig. 3b) was very similar to that of Wt RCs (Fig. 3a) [77], notably in the quinone absorption range (C=O mode at 1640 cm\(^{-1}\), C=C modes at 1618 cm\(^{-1}\), contributions from methoxy groups at 1285 and 1265 cm\(^{-1}\) and at 1479 cm\(^{-1}\) where a well-conserved anion band is observed. Although the isotope-edited IR fingerprint spectrum of Q_B photoreduction was not measured for this mutant, the similarity of the Q_B\(^{\text{an}}\)/Q_B difference spectra indicates that the interactions of Q_B and Q_B\(^{\text{an}}\) with the protein are well-conserved in these two RCs. Therefore, the bonding interactions of Q_B and Q_B\(^{\text{an}}\) appear to be the same for Q_B photoreduction via the A-branch of cofactors in the Wt RC, or via the B-branch in the WH mutant.

The Q_B\(^{\text{an}}\)/Q_B difference spectra of the AA (Fig. 3c) and WAAH (Fig. 3d) mutant RCs, which were closely related across the whole spectral range investigated, displayed large changes with respect to the spectra of the Wt and WH RCs (Fig. 3a,b), particularly in the semiquinone absorption range between 1400 and 1500 cm\(^{-1}\). The isotope-edited double-difference spectra of the AA (Fig. 6b,e) and WAAH (Fig. 6c,f) RCs in this region demonstrated that perturbations of the semiquinone modes occur in these two mutant RCs, as shown by changes in frequency/amplitude of the negative (unlabeled anion) and positive (labeled anion) bands. Whilst the main band of unlabeled Q_B\(^{\text{an}}\) had a maximum at 1479 cm\(^{-1}\) in the IR fingerprint spectra of the Wt RC upon either \(^{13}\)C\(_1\) (Fig. 6a) or \(^{13}\)C\(_4\) (Fig. 6d) labeling [77], a broad band at 1472 cm\(^{-1}\) with a small positive feature at 1490 cm\(^{-1}\) were seen for the AA mutant upon \(^{13}\)C\(_1\) labeling (Fig. 6b), and two negative bands at 1493 and 1472 cm\(^{-1}\) were observed for this mutant upon \(^{13}\)C\(_4\) labeling (Fig. 6e). In the case of the WAAH RC, this pattern of two negative bands was seen for both \(^{13}\)C\(_1\) and \(^{13}\)C\(_4\) labeling (Fig. 6c and f, respectively), with an additional feature at 1433 cm\(^{-1}\) for \(^{13}\)C\(_4\) labeling. Changes in the positive bands attributable to the labeled Q_B\(^{\text{an}}\) in the 1444–1410 cm\(^{-1}\) region were also observed for the AA mutant, and to a greater extent for the WAAH mutant.

Comparable perturbations of these semiquinone modes have previously been observed in isotope-edited spectra of RCs with the mutations PL209Y and PL209F [70,71]. However, the WAAH mutant showed the largest effects to be observed to date. For mutant RCs bearing the double AA mutation, the replacement of two carboxylic acid residues near Q_B (i.e. Glu L212 and Asp L213) with neutral residues is likely to induce a different charge distribution near the head-group of the Q_B ubiquinone. This would have an effect on the coupling of the C=O and C=C modes of the Q_B anion and thus on the frequency/intensity of the semiquinone bands. In addition, X-ray crystallography has shown that the double AA mutation causes a number of structural changes around the Q_B site involving backbone and side chain rearrangements near Q_B and realignment of water molecules [93], and it is conceivable that these could also contribute to a change in the charge distribution within the Q_B pocket, affecting the electronic structure of the Q_B anion. Finally, as previously discussed for another class of mutants [71], a slight displacement of the Q_B head-group in the AA and WAAH mutants relative to its position in the Wt RC could also contribute to the changes seen in the isotope-edited double difference spectra of the Q_B anion.

4.2. Bonding interactions of neutral Q_B in RCs catalyzing A- or B-branch electron transfer

The isotope-edited spectra of the AA and WAAH RCs (Fig. 6b,e and c,f) displayed a unique C=O band for the unlabeled neutral Q_B at 1641 cm\(^{-1}\) which is identical to that observed for Wt RCs from Rb. sphaeroides (Fig. 6a,d) and Rps. viridis [77]. Both carbonyls of the neutral Q_B contribute equally to this single band. In addition, the pattern of C=C modes of Q_B in AA and WAAH RCs showed a shift of the positive band at 1612–1602 cm\(^{-1}\) upon \(^{13}\)C\(_1\) labeling (Fig. 6b,c) but no effect upon \(^{13}\)C\(_4\) labeling (Fig. 6e,f). It therefore
appears that, as in the Wt RC [77], the coupling of C=O and C=O modes at the C1 and C4 positions is also not equivalent in either the AA or the WAAH mutant. This indicates that the constraints exerted by the protein at the Q6 site affect the displacement of the C1 and C4 atoms differently. More generally, the IR fingerprints for the C=O and C=O modes of Q6 in the AA and WAAH mutants were also essentially the same as those previously reported for other RCs with mutations at the Q6 site, such as SL223A [94], PL209Y [70], and PL209F [71].

As discussed previously for Wt RCs from Rb. sphaeroides and Rps. viridis [77], as well as for RCs with mutations of Pro L209 [70,71], the symmetrical hydrogen bonding pattern indicated by the unique 1641 cm⁻¹ band for the unlabeled neutral Q6 fits the description of the proximal binding site (Fig. 1C), with both carbonyl groups of the Q6 head-group engaged in equivalent hydrogen bond interactions with the surrounding protein.

As illustrated in Fig. 1C, X-ray crystallography of the Wt RC has resolved two distinct binding positions for the Q6 ubiquinone [66,67]. There is consensus that the Q6 semiquinone is located at the proximal binding site following A-branch electron transfer, and findings from FTIR spectroscopy have demonstrated that the neutral Q6 ubiquinone also occupies the proximal binding site prior to reduction [70,71,95]. Looking at the structure of the RC cofactors outlined in Fig. 1C, this seems to be a logical arrangement, as the proximal site is closer to the QA ubiquinone than the distal site.

However, as has been pointed out recently [34], the Q6 head-group would be closer to the Hb BPhe in the distal binding position than when in the proximal position (see Fig. 1C). Using the data of Stowell et al. [66] the distal Q6 is approximately 1.2 Å closer to Hb than the proximal Q6, when measured between cofactor rings and ignoring substituent groups (9.1 Å for distal, 10.3 Å for proximal). If the ring substituent groups are included, then this difference is approximately 1.7 Å (6.2 Å for distal, 7.9 Å for proximal). Given that the rate of electron transfer decreases with increasing distance between donor and acceptor, it has been speculated that B-branch electron transfer from the Hb BPhe to a distal Q6 ubiquinone could be favored over electron transfer to a proximal ubiquinone [34]. Specifically, de Boer et al. [34] have proposed that an observed 3% yield for the Hb to Q6 reaction at 20 K in a QΑ-deficient mutant could be explained if 97% of RCs have a proximal Q6 that does not accept an electron, and 3% have a distal Q6. However, the FTIR data on the WAAH and WH RCs described above show that the reduction of the Q6 ubiquinone during B-branch electron transfer takes place at the proximal binding site, with the bonding interactions of Q6 appearing to be the same for Q6 reduction via the B-branch or A-branch.

The conclusion from FTIR spectroscopy that the neutral Q6 is located in the proximal position in the AA RC is consistent with findings from X-ray crystallography of this mutant RC, that show a proximally bound Q6 ubiquinone [93]. In addition, X-ray crystallography of the single AM260W RC has also provided clear evidence for the Q6 ubiquinone being bound in the proximal site in this mutant complex [43]. It is therefore perhaps no surprise that the WAAH RC used in the present study, which combines the AM260W and AA mutations, also possesses a Q6 ubiquinone bound in the proximal position. Thus, there seems to be a good correlation between the results of FTIR spectroscopy and X-ray crystallography for these mutant complexes.

Taken together, a number of recent reports have presented compelling evidence that it is possible to reduce the Q6 ubiquinone by B-branch electron transfer in RCs where the QA ubiquinone is absent [33,34,38–41]. The driving force for the B-branch P+HBQ6 → P+HBQ6 reaction is expected to be much larger than that of the A-branch P+QAQ6 → P+QAQ6 reaction, because the B-branch donor state (P+HB) is predicted to have a much higher free energy than the A-branch donor state (P+QA) [20,39]. Although the kinetics of the P+HBQ6 to P+HBQ6 reaction have not yet been fully resolved, the first analyses of this reaction have yielded rate constants of (2–12 ns)⁻¹ [39], making the reaction much faster than the microsecond time scale of Q6 to Qb transfer, but at least 10-fold slower than the equivalent Hb to Q6 step during A-branch electron transfer. However, despite the fact that the energetics and kinetics of the Q6 reduction step during A- and B-branch electron transfer are expected to be rather different, as are the donor–acceptor geometries, the FTIR data presented above argue that the neutral Q6 ubiquinone occupies that proximal binding position prior to reduction by either A-branch or B-branch electron transfer. Changes in the IR spectroscopic fingerprint of the interactions of the semiquinone with the protein in the mutants investigated in this study appear to be an effect of the double AA mutation, and are not related to whether Q6 is formed by A- or B-branch electron transfer.

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