An investigation of slow charge separation in a Tyrosine M210 to Tryptophan mutant of the *Rhodobacter sphaeroides* reaction center by femtosecond mid-infrared spectroscopy[†]

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Energy and electron transfer in a tyrosine M210 to tryptophan (YM210W) mutant of the *Rhodobacter sphaeroides* reaction center (RC) were investigated through time-resolved visible pump/mid-infrared (mid-IR) probe spectroscopy at room temperature, with the aim to further characterize the primary charge separated states in the RC. This mutant is known to display slow and multi-exponential charge separation, and was used in earlier work to prove the existence of an alternative route for charge separation starting from the accessory bacteriochlorophyll in the active branch, B_{I} . The mutant RCs were excited at 860 nm (direct excitation of the primary donor (P) BChls (P_L/P_M)), 600 nm (unselective excitation), 805 nm (direct excitation of both accessory bacteriochlorophyll cofactors B_{I} and B_{M}) and 795 nm (direct excitation of B_L). Absorption changes associated with carbonyl (C=O) stretch vibrational modes of the cofactors and protein were recorded in the region between 1600 and 1775 $\rm cm^{-1}$, and both a sequential analysis and simultaneous target analysis of the data were performed. The decay of P* in the YM210W mutant was multi-exponential with lifetimes of 29 and 63.5 ps. The decay of $P^+B_L^-$ state was ~10 times longer in the YM210W RC than in the R-26 RC (\sim 7 ps vs. \sim 0.7 ps), and in the mid-IR difference absorption spectrum of $P^+B_L^-$ the stretching frequency of the 9-keto C=O group of B_L in the ground state was located around 1675-1680 cm⁻¹, consistent with the presence of a hydrogen bond donated by an adjacent water molecule. Excitation at 795 nm produced a small amount of B_{L}^{*} -driven charge separation, as assessed from the excitation wavelength dependence of the raw difference spectra recorded during the first few ps after excitation. This process led to the formation of $P^+B_L^-$. Only the relaxed form of the $P^+H_L^-$ radical pair was observed in the YM210W mutant, and the mid-IR difference absorption spectra of $P^+H_L^-$ and $P^+B_L^-$ showed a change in the relative amplitude of the P_L^+ and P_M^+ bands when compared to equivalent spectra for the R-26 RC. This indicates that the YM210W mutation causes an increased localization of the electron hole on the P_M half of the dimer. The absorbance difference spectrum of $P^+H_L^-$ in the R-26 RC contains a feature attributable to a Stark shift of one or more amide C=O oscillators. This feature was shifted to lower frequency by $\sim 5 \text{ cm}^{-1}$ in the YM210W RC, and consideration of the limited structural changes in this RC indicates that this feature arises from an amide C=O group in the immediate vicinity of the M210 residue, most probably that of the adjacent M209 amino acid.

Introduction

The purple bacterial reaction center (RC) is a transmembrane pigment–protein complex responsible for catalyzing lightdriven charge separation. The RC from *Rhodobacter (Rb.) sphaeroides* has been structurally characterized to atomic resolution by X-ray crystallography,^{1–3} and represents one of only a very few systems where electron transfer between redox centers can be monitored on a multitude of timescales ranging from femtoseconds (fs) to seconds. The primary charge separation in the RC involves light-driven electron transfer from a dimer of bacteriochlorophyll *a* (BChl) located near the

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^{*} Electronic supplementary information (ESI) available: Kinetic matrix and amplitude matrix for the kinetic scheme shown in Fig. 5A (Table S1); time traces for YM210W RCs and R-26 RCs (Fig. S1, S2); comparison of SADS resulting from target analysis of the four data sets recorded for YM210W RCs (Fig. S3); comparison of absorbance difference spectra measured at five selected delay times after excitation of YM210W RCs (Fig. S4). See DOI: 10.1039/ b905934b

periplasmic side of the membrane (denoted P) to a bacteriopheophytin (H_L) located close to the middle of the membrane. In the resulting $P^+H_L^-$ radical pair the cation is shared between the two BChls (termed P_L and P_M) that form the P dimer, and transfer of the electron from the excited state of P (P*) to H_L is a two-step reaction involving the intervening monomeric BChl (B_L). In WT RCs electron transfer from B_L⁻ to H_L is 3–4 times faster than the initial electron transfer from P* to B_L, with the result that the P⁺B_L⁻ radical pair does not build up to a high level and has been difficult to characterize spectroscopically.^{4–9} Formation of P⁺H_L⁻ from P* occurs with a time constant of 3–4 ps at room temperature, with subsequent electron transfer to the Q_A ubiquinone located on the cytoplasmic side of the membrane occurring with a time constant of about 220 ps.^{4–7}

Investigation of the mechanism of energy transduction in the RC has been greatly assisted by the use of site-directed mutagenesis to alter the properties of particular cofactors or change the rates of individual reactions. A large number of mutations have been reported that change the rate of the primary charge separation, and one of the strongest effects is seen when a tyrosine at position 210 on the M-polypeptide is mutated to tryptophan (denoted YM210W). Tyr M210 occupies a key location in the bacterial RC, within 4 Å of each of P_L, P_M, B_L and H_L (Fig. 1), and in the YM210W mutant the lifetime of the P* state at room temperature is extended from 3–4 ps to around 50 ps^{10–18} (and for review see ref. 5). P* decay is notably multi-exponential and extremely temperature



Fig. 1 Changes of structure in the YM210W RC. The structure of the YM210W RC²⁰ is overlaid with that of the wild-type RC.⁵⁰ Cofactors and amino acids 209–211 of the M-polypeptide are shown as sticks, with the central Mg of BChl and water molecules as spheres. For the wild-type, carbon atoms of the cofactors are shown in yellow and those of the protein in orange, with magnesium shown in pale pink and water in pale blue. For the YM210W RC carbon atoms of the cofactors are shown in cyan, with magnesium shown in magenta and water in dark blue. The P_M BChl is shown in white, and nitrogen and oxygen are shown in blue and red, respectively. The phytol side chains of the BChls and BPhes have been removed for clarity, and the figure was constructed using PyMOL.⁵¹

sensitive in the YM210W RC, slowing down by one order of magnitude upon cooling to 77 K.^{11,13,18}

The dramatic slowing of charge separation in the YM210W RC is likely to have a number of causes. Redox titrations have established that the mid-point potential of the P/P^+ couple $(E_{\rm m} P/P^+)$ is raised by ~50 mV in the YM210W RC.^{13,16} producing an increase in the free energy of $P^+B_L^-$ and $P^+H_L^$ relative to those of P* and the P ground state (which are not affected by the mutations). Furthermore, quantum-chemical calculations have indicated that the dipole of the Tyr OH group stabilizes the $P^+B_L^-$ state,¹⁹ and therefore it is possible that replacement of Tyr M210 also raises the free energy of $P^+B_L^-$ through a destabilization of B_L^- . The result is that whereas the free energy of $P^+B_L^-$ is about 450 cm⁻¹ below that of P* in the wild-type RC, in the YM210W mutant this free energy is equal to or above that of P*. In addition to this, X-ray crystallography has shown that the introduction of the bulkier Trp in the place of the native Tyr causes a small tilt of the B_L cofactor (of \sim 3 degrees) in a direction away from the P BChls²⁰ (Fig. 1). This movement affects maximally the positions of the atoms of rings III, IV and V, including the keto carbonyl of ring V (i.e. the right-hand side of the B_I BChl in Fig. 1). This is accompanied by a very small shift in the position of the PL BChl, particularly that part of the macrocycle furthest from the area of overlap with the P_M BChl, *i.e.* that part of the macrocycle that includes the 9-keto and 10a-ester C=O groups²⁰ (the left-most edge of the P_L BChl in Fig. 1). No evidence for a shift in the position of H_L was seen, or any of the M-side BChls or BPhes. As can be seen in Fig. 1 the new Trp side chain is arranged approximately co-planar to the native Tyr.

RCs with mutations to Trp and other residues at the M210 position have been used to study a number of aspects of the mechanism of the RC. In previous work from our own laboratories the strongly slowed rate of P* decay in the YM210W mutant was exploited in a study of alternative mechanisms for membrane-spanning electron transfer, in which the primary sub-picosecond charge separation reaction is $B_L^* \rightarrow P^+B_L^-$ or $B_L^* \rightarrow B_L^+H_L^-$ rather than the conventional reaction $P^* \rightarrow P^+B_L^-$ described above.^{17,18} Experiments at 77 K on YM210W RCs employing direct excitation of B_L at 795 nm showed that the $P^+B_L^-$ state was formed in ~200 fs from B_L^* , and decayed in about 6.5 ps into $P^+H_L^{-.17}$ Formation of the $P^+B_L^-$ radical pair without involving P* was also observed in wild-type RCs at 77 K.¹⁸

Investigation of the kinetics of charge separation in wild-type and mutant RCs has made extensive use of ultrafast pump-probe spectroscopy, with excitation and detection in the visible and near-infrared (IR) regions of the spectrum. A limitation of this type of spectroscopy is that it involves signals arising directly from the bacteriochlorin cofactors, and does not provide information on their protein surroundings. One way to access such information is through ultrafast visible-pump/mid-IR-probe spectroscopy, and several applications of this technique have been reported, including some studies of purple bacterial RCs (see ref. 21 for an overview of these). In this technique a mid-IR probe pulse is used to look at shifts of particular vibrational modes, such as the C=O stretch for example. Any C=O group that undergoes a shift in frequency

in response to a change in the electronic state of the RC cofactors will appear in a difference spectrum, and so the technique allows investigation of different parts of the bacteriochlorin macrocyle (BChl a has three such C=O groups—positions shown in Fig. 1), as well as protein groups in the environment of the cofactors.

In a recent report, ultrafast visible-pump/mid-IR-probe spectroscopy was used to investigate charge separation in Rb. sphaeroides R-26 RCs.²¹ This work described the mid-IR absorbance difference spectrum of PBL/P+BL-, a spectrum that is not accessible through steady state Fourier transform infrared (FTIR) difference spectroscopy, and spectral features associated with relaxation of the $P^+H_I^-$ radical pair on the ~ 20 ps time scale. In the present work we have applied this technique to the YM210W RC, to determine how the documented slowing of P* decay affects the spectral evolution in the mid-IR, to look in more detail at the spectrum of the $P^+B_I^-$ state, and to further investigate spectral features associated with the protein environment of the cofactors. We also examine whether there is an indication that the B_L*-driven charge separation documented in membranebound YM210W RCs at 77 K also takes place in purified YM210W RCs at room temperature.

Materials and methods

All experiments were performed with a femtosecond visible-pump/mid-IR-probe laser system that has been described previously.^{22,23} Instrument set-up and experimental procedures were exactly as described in detail in a recent report on R-26 RCs.²¹ Femtosecond pulses were generated using an integrated Ti:sapphire oscillator and regenerative amplifier laser system (Spectra Physics, Hurricane) that produced 800 nm, 85 fs pulses at an energy of 0.6 mJ and a repetition rate of 1 kHz. The pulses were divided into two beams by a beamsplitter, one of which provided excitation pulses and was passed through a non-collinear optical parametric amplifier (NOPA) where white light and the second harmonic were generated. The color of these excitation pulses was further extended by sum frequency generation, enabling an output tunable between 300 and 800 nm and producing 600 nm excitation pulses (fwhm = 9 nm). Excitation light at 795 nm (fwhm = 4 nm) was generated by using a 795-nm interference filter placed in the path of the fundamental laser beam (800 nm light), and the excitation light at 805 nm (fwhm = 8 nm) was produced as described previously.²¹ The 860 nm excitation light (fwhm = 10 nm) was produced using an 860-nm interference filter placed in the path of the white light seed. The pump pulses were directed through an optical delay line and a chopper operating at 500 Hz. The RCs were excited with an excitation power of 300 nJ for 600 nm and 805 nm excitation, 250 nJ for 860 nm excitation, and 200 nJ for 795 nm excitation. The polarization of the excitation pulses was set to the magic angle (54.7 degrees) with respect to the IR probe pulse using a polarization rotator placed behind the delay line.

The second part of the 800 nm light (energy 0.36 mJ) was used to pump an optical parametric generator and amplifier with a difference frequency generator (TOPAS, Light Conversion), to produce the mid-IR probe pulses that could be

varied between 2400 and 11000 nm. For each excitation wavelength experiments were repeated twice using fresh samples, with spectra being recorded at 80 different time points. The sample of $\sim 40 \ \mu$ l volume was placed in a cell comprised of two CaF windows separated by a 20 µm Teflon spacer. The cell was placed in a Lissaious scanner²² and moved in the vertical plane to ensure excitation of a different part of the sample at every laser shot. The sample holder was placed in the housing and purged with a flow of N_2 to reduce the effect of water vapor on the mid-IR pulses. After passing the sample the mid-IR probe pulses were dispersed using a spectrograph and imaged onto a 32-element HgCdTe array detector operating at 77 K that provided a spectral window of $\sim 200 \text{ cm}^{-1}$ with a spectral resolution of 6 cm^{-1} . As with previous fs mid-IR data collected for R-26 RCs a noise level of 10^{-5} OD per spectrum was obtained after 1 min of data collection, and the instrument response function was of the order of ~ 100 fs.

Four sets of experimental data were collected, with excitation at 600, 795, 800 or 860 nm. Each of these data sets was subjected to a global analysis using a kinetic scheme comprising a linear sequence of components with increasing lifetimes (model-I, see text). This resulted in a set of evolution associated difference spectra and associated lifetimes for each of the four sets of data. Each set of experimental data was then subjected to a target analysis,^{24,25} in which a kinetic scheme of excited or radical pair states was used to analyse the data (models II-IV, see text). This resulted in a set of species associated difference spectra and associated lifetimes for each of the four sets of data. The goal of the target analysis was to apply to the data a specific kinetic model consisting of a number of distinct spectroscopic states (species), in order to obtain 'pure' spectra of these species. All spectra shown in the current report display an extrapolation using a cubic-spline line fitted through the experimental data points.

The construction and X-ray crystal structure of the YM210W mutant has been described in detail,^{20,26} and the preparation of purified RCs for spectroscopy was carried out as reported previously.²¹

Results

Data acquisition and analysis

Purified YM210W RCs were excited at 860 nm and probed in the region 1773-1598 cm⁻¹, as described in the Materials and Methods section. Three other datasets were also collected with excitation at 805, 795 or 600 nm. The ESI Fig. S1[†] shows two selected time traces at 1686 cm⁻¹ (open symbols) and 1704 cm⁻¹ (filled symbols) for the YM210W RC excited at 860 nm (squares), compared with equivalent data recorded for the R-26 RC (circles) in a previous study.²¹ The time traces for the two RCs are clearly different from one another, reflecting the large differences in the kinetics of electron transfer in the two RCs. The fits through the data are the result of a global analysis of the dataset using a sequential model with increasing lifetimes (denoted model-I). Each global analysis resulted in a set of evolution associated difference spectra (EADS) for each of the four sets of experimental data collected. The first EADS represents the time zero spectrum and decays with the first



Fig. 2 EADS and concentration profiles obtained from global analysis of data recorded for YM210W RCs excited at 805 nm. The measurements were performed over a $1768-1600 \text{ cm}^{-1}$ window with a spectral resolution of 6 cm⁻¹. The analysis was performed using a sequential model with increasing lifetimes (model-I). (A) EADS for components 2–5, data points joined by a spline; (B) Concentration profiles of components 1–5.

 Table 1
 Summary of the lifetimes resulting from global analysis of the data obtained in individual experiments. Note that in addition each analysis resulted in a long lived component that was the final state formed in the experiment, the lifetime of which was not resolved

Excitation λ /nm	IR region/cm ⁻¹	Lifetimes resulting from global analysis
600	1773-1598	170 fs, 4.8 ps, 34 ps, 334 ps
805	1768-1600	150 fs, 8.7 ps, 42 ps, 225 ps
860	1773-1598	200 fs, 11 ps, 46 ps, 236 ps
795	1775–1595	300 fs, 7.0 ps, 50 ps, 223 ps

lifetime. The second EADS is formed with the first lifetime, and decays with the second lifetime, and so on (see Fig. 2B). These difference spectra therefore do not necessarily represent 'pure' states but rather reflect the spectral evolution of the system in time.²⁵ For the YM210W RC this analysis yielded five kinetic components when applied to the data recorded with 860 nm excitation, and global analysis of data recorded with excitation at 805, 795 or 600 nm using the same model-I scheme also yielded five components with broadly similar lifetimes. The lifetimes of the first four component was not resolved over the 6 ns duration of the measurement, and so the lifetime of this component does not appear in Table 1. In the global analysis this component had a lifetime of a few tens of nanoseconds.

An example set of EADS for the YM210W RC is shown in Fig. 2A. In these difference spectra the negative bands refer to ground state vibrational modes and positive bands to shifted vibrational modes in electronically excited or charge separated states. For longer-lived states in particular, such as $P^+Q_A^-$, these bands can be assigned through reference to steady-state FTIR difference spectra, as discussed previously.²¹ It should be noted that observation of the process of energy transfer is hampered in fs mid-IR spectroscopy by a component that follows the instrument response function (IRF) and describes the coherent interaction of the laser pulses with the sample. For this reason the resolved sub-picosecond components (Table 1) were not shown in any of the EADS or species

associated difference spectra (SADS) presented in this work. Also the negative signal before time zero visible in the traces presented in the ESI Fig. S1,[†] which originates from perturbed free induction decay,^{27,28} was not included in the fit. Since no reference probe pulse was used, the noise in the measured spectra (not shown) consisted mainly of so-called baseline noise, *i.e.* a flat, structure-less offset in the spectra, which was easily recognized by a singular vector decomposition of the residual matrix. For target analysis (and the time traces in the ESI Fig. S1,[†] the outer product of the first two singular vector pairs of the residual matrix (being structure-less in the time domain) was subtracted from the data, leading to reduction in the noise by a factor of two.

Evolution associated difference spectra

The general shapes of the EADS obtained for the YM210W RC were similar to those reported in previous work on the R-26 RC, where the assignments of the various positive and negative bands were discussed in depth.²¹ However, a marked difference with the data obtained for the R-26 RC was the persistence of spectral features attributable to P* on the time-scale of tens of picoseconds. This finding was consistent with the documented strong slowing of P* decay in the YM210W mutant (see above).

Four of the EADS derived from analysis of the data recorded for YM210W RCs with excitation of both accessory BChls at 805 nm are shown in Fig. 2A (the EADS of component 1, with an associated lifetime of 170 fs, is not shown (see above)). The associated lifetimes were 8.7, 42, 225 ps and a long-lived component (Table 1), and the concentration profiles of these components are shown in Fig. 2B. Given what is known about energy and electron transfer in the RC, the 170 fs component (spectrum not shown) is attributed to a mixture of B_M^* and B_L^* which then decays via energy transfer to P. The excitation pulse at 805 nm is also expected to produce some direct excitation of P via the high energy exciton component of the dimer.

The spectra of the components with associated lifetimes of 8.7 ps (Fig. 2A, red) and 42 ps (Fig. 2A, green) were generally

similar to one another, and these components are attributed to different mixtures of the P*, $P^+B_L^-$ and $P^+H_L^-$ states. The presence of spectral features attributable to P* in the 42 ps spectrum, such as the positive band at 1668 cm^{-1} (see below), is consistent with the documented slow and multi-exponential decay of P* in the YM210W RC. Features attributable to P_{L}^{+} (1714 cm^{-1}) and P_M^+ (1706 cm^{-1}) were less prominent in the 8.7 ps spectrum than in the 42 ps spectrum. Given this, the 8.7 ps component is attributed to mainly P*, whilst the 42 ps component is attributed to a mixture of P^* , $P^+B_L^-$ and $P^+H_L^-$ (the notation P_L^+ and P_M^+ refers to the oxidized state of the two BChls of the P dimer, but it should be noted that the single positive charge is shared between P_L and P_M (see below)). The spectrum of the next component (Fig. 2A, blue) is dominated by features representing the state $P^+H_L^-$, and this component decays in 225 ps into the long-lived component that is attributed to $P^+Q_A^-$ (Fig. 2B, cyan), which is the final state formed in the experiment.

Five EADS were also obtained from global analysis of the data recorded with direct excitation of the P BChls at 860 nm, selective excitation of the B_L BChl at 795 nm and non-selective excitation of all four BChls at 600 nm (data not shown). The lifetimes obtained from the global analysis are listed in Table 1. For 860 nm excitation the 200 fs component was determined by the inverse of the line-width, perturbed free induction decay and a large Stokes shift of the P* excited state, whilst for 795 nm excitation the 300 fs component represented energy transfer among the BChls in the RC. Based on the interpretation of the ground state absorbance spectrum and extensive data from the visible region pump-probe spectroscopy, it is expected that the 795 nm pump pulse should excite mainly B_L with a smaller amount of excitation of B_M , and possibly also a small amount of direct excitation of P via the high energy exciton component of the dimer (direct formation of B_M^* and P^* by the 795 nm pulse is expected to be less prevalent than when excitation was at 805 nm). Finally, excitation at 600 nm is expected to achieve non-selective excitation of all four BChls, and the 170 fs component is attributed to the processes of energy equilibration among the BChls and formation of P*. For all three excitation

wavelengths the four remaining EADS had lineshapes similar to their counterparts shown in Fig. 2, and are attributed in the same way as mainly P* (4.8–11 ps component), a mixture of P*, P⁺B_L⁻ and P⁺H_L⁻ (34–50 ps component), P⁺H_L⁻ (223–334 ps component) and P⁺Q_A⁻ (long-lived component). In comparison, in our previous work on R-26 RCs only three EADS were required to describe the spectral evolution of equivalent data obtained using either 860 or 600 nm excitation, comprising a 3.6–4.4 ps component (mainly P*), a 222–280 ps component (P⁺H_L⁻) and a long-lived component (P⁺Q_A⁻) (plus a ~100 fs component attributed to energy transfer).²¹

Determination of species associated difference spectra through target analysis

As commented above, the EADS derived from global analysis of the experimental data do not necessarily correspond to spectra of pure states, and it was clear from Fig. 2A that some of the EADS resulting from global analysis using model-I contained spectral features arising from more than one state. In particular, the 42 ps component contained features attributable to both P* and P⁺, consistent with the documented slow decay of P* in the YM210W RC.11,16,29 This indicated that model-I, which comprised a linear sequence of states with increasing lifetimes, did not provide a realistic description of evolution of the system. The most obvious reason for this is the well established observation that formation of $P^+B_L^$ from P* is followed by a more rapid transfer of the electron from B_{L}^{-} to H_{L} . This feature of charge separation in the RC would therefore be better described by a model in which the first step of electron transfer is followed by a second step with a shorter lifetime.

To account for this complication in a simple way, an inverted kinetic model (denoted model-II) was applied in a target analysis of the data recorded using 805 nm excitation (see ref. 24, 25, 29, 30 for examples of this type of analysis). Model-II still contained five steps, but the order of components 2 and 3 was reversed, such that the component with a 42 ps lifetime preceded the component with an 8.7 ps lifetime. Fig. 3 shows the difference spectra associated with species 3, 2, 4 and 5 in this inverted model (species associated difference



Fig. 3 SADS and concentration profiles obtained from a target analysis of data recorded for YM210W RCs excited at 805 nm. The analysis was performed using an inverted kinetic model (model-II). (A) SADS for components 2–5; (B) Concentration profiles of the components 1–5.

spectra—SADS). This inversion of the 42 and 8.7 ps species resulted in elimination of P* features from the SADS of the (now later) 8.7 ps species, which had a lineshape consistent with the $P^+B_L^-$ charge separated state (Fig. 3A, green). The SADS of the earlier 42 ps species had the spectral features expected for P* (Fig. 3A, red).

Although the use of the inverted kinetic model resulted in SADS that were more consistent with discrete P^* and $P^+B_L^-$ states, the amplitude of the SADS of species 2 ($P^+B_L^-$) was unrealistically large, and the concentration profile of this species was unrealistically low for an intermediate in the reaction scheme (compare the areas under the green profiles in Fig. 2B and 3B). This indicated that the description of the system offered by model-II was still inadequate, and required further refinement.

A second complicating factor in the description of charge separation in the RC is heterogeneity in decay of the P* state, a feature which is particularly prominent in the YM210W RC^{11,16,29} but also displayed by the wild-type RC. This heterogeneity has been discussed in terms of multiple static conformational states,^{10,11} conformational relaxation of P*,¹³ a Gaussian distribution of the free energy gap for primary electron transfer,^{12,31,32} additional distributions in electronic coupling and/or reorganization energy,¹⁶ additional states involving B-branch cofactors¹⁰ and dynamic solvation of the P⁺H_L⁻ state affecting thermal repopulation of P*.^{31,33} To

determine whether accounting for this pronounced heterogeneity affected the analysis, the recorded datasets were subjected to a target analysis using the kinetic scheme described in Fig. 4 (model-III). As the molecular origin(s) of the non-monoexponential decay of P* have not been identified with certainty, a model was constructed that represented the observed biphasic kinetics of P* decay in the simplest possible way. Accordingly, the P* component in model-III was split into two independent isoenergetic states, termed P_{S}^{*} and P_{F}^{*} , with significantly different decay rates $(51 \text{ ps})^{-1}$ and $(25 \text{ ps})^{-1}$ In this model the SADS of equivalent species were linked for the fast and slow populations, including those of P_S^* and P_F^* , as were the remaining decay rates. Application of model-III to the data led to a root mean square error similar to that obtained with model-I. An alternative model that lacked the $P^+B_L^-$ intermediate produced an 8% increase in the root mean square error (data not shown), showing that this component was required.

The SADS and concentration profiles obtained from the target analysis using model-III are shown in Fig. 4B. Model-III still gave rise to an anomalously high amplitude for the SADS of the $P^+B_L^-$ state (Fig. 4B, blue spectrum) when compared to the remaining SADS, and again the concentration of this component was too low (Fig. 4C, blue profile).

As, in principle, the primary reactions of charge separation are reversible, back reactions from $P^+B_L^-$ to both of the P*



Fig. 4 Target analysis applying model-III to data recorded for YM210W RCs excited at 805 nm. (A) Heterogeneous kinetic scheme employing fast (P_F^*) and slowly (P_S^*) decaying populations of P*; (B) SADS; (C) concentration profiles.



Fig. 5 Model-IV used for target analysis employing reversible initial charge separation and heterogeneous kinetics of P* decay. Green arrows and red arrows (plus associated rate constants, expressed as their reciprocal in ps) refer to forward processes and back reactions, respectively.

states were introduced, as was a slow back reaction from $P^+H_L^-$ to $P^+B_L^-$. The resulting model (model-IV) is shown in Fig. 5; the model incorporated both reversibility of the first two steps of charge separation and heterogeneity in the decay of P*. All the decay rates were free parameters, and were adjusted by varying initially introduced values until the best fit quality was achieved. The only fixed parameters were the energy differences between P^* and $P^+B_L^-$ (equal forward and backward rate constants, which correspond to 0 meV) and between $P^+B_L^-$ and $P^+H_L^-$ (ratio of forward and backward rate constants equal to 100, which corresponds to a 118 meV difference in free energy between the two states). Several free energy differences between P^{\ast} and $P^{+}B_{L}^{-}$ were investigated, but the best fit was obtained if it was assumed that the free energies of the two states were equal (for both P_F^* and P_S*). The major fraction of the total population of P* was found to decay rapidly (P_F^* - 73%), the slowly decaying fraction (Ps*) comprising 27%, and once again the SADS of equivalent states were linked.

Model-IV was used in a simultaneous target analysis of the four sets of data acquired for the YM210W RC. The resulting SADS for the data obtained with 805 nm excitation are shown in Fig. 6A, the concentration profiles of the various states are shown in Fig. 6B, and the estimated lifetimes are collated in Table 2. In Fig. 6B, solid lines show the concentration profiles of P_S^* and the $P^+B_L^-$ and $P^+H_L^-$ states that arise from this, whilst the dashed lines show the concentration profiles of P_F^* and the resulting $P^+B_L^-$ to $P^+H_L^-$ states. As can be seen in Fig. 6, introducing the back reaction from $P^+B_L^-$ to P^* state decreased the amplitude of the SADS of the $P^+B_L^-$ component (Fig. 6A) and increased its concentration (Fig. 6B, green lines). The lifetimes associated with these SADS, and the features of these spectra, are discussed in the next section.

ESI Tables S1A and B[†] show the kinetic matrix (A) and amplitude matrix (B) of the kinetic scheme presented in Fig. 5. The kinetic matrix (ESI Table S1A)[†] shows microscopic rate constants in 1/ps, which are the forward and backward rates of energy and electron transfer. The concentration profile of each species is a linear combination of exponential decays (with the lifetimes being the reciprocals of the eigenvalues of the kinetic matrix). The amplitudes of these exponential processes are



Fig. 6 SADS and concentration profiles obtained from a target analysis of data recorded for YM210W RCs excited at 805 nm, using model-IV. (A) SADS; (B) Concentration profiles.

 Table 2
 Lifetimes estimated from a target analysis using model IV, for fast- and slowly-decaying P* populations

Population	Lifetimes resulting from target analysis
P _F * (73%)	160 fs, 5.0 ps, 29 ps, 279.0 ps
P _S * (27%)	160 fs, 7.1 ps, 63.5 ps, 279.5 ps

contained in the amplitude matrix (ESI Table S1B).[†] Positive and negative amplitudes denote the decay and rise of the component, respectively, with major decays indicated in bold. The state B_L*/B_M* decays in 160 fs by energy transfer to P_F* or P_S* . In turn, the P_S* state decays mainly in ~63 ps, and the P_F* state mainly in ~29 ps, the next state to be formed being $P^+B_L^-$. The amplitude matrix is particularly complicated for the $P^+B_L^-$ and $P^+H_L^-$ species, due to the equilibrium between the two and between $P^+B_L^-$ and P^* , and the inverted kinetics of $P^+B_L^-$ (slower formation than decay). Finally, the long lived state $P^+Q_A^-$ rises in ~279 ps and the decay of this state is not resolved due to the limited duration of the measurement.

SADS for the YM210W RC

Fig. 6A shows a representative set of SADS resulting from model-IV target analysis of the data on the YM210W RC collected with 805 nm excitation. The SADS of P_S^* and P_F^* states were set in the analysis to have the same shape and are represented by the red spectrum denoted P* in Fig. 6A. This spectrum displays typical bands attributed to the 9-keto modes of P_L and P_M in the ground state at ~ 1690 cm⁻¹ (negative), which down-shifts to 1667 cm⁻¹ (positive) in the excited state. As outlined above, and listed in Table 2, the P_S^* and P_F^* states decayed to $P^+B_L^-$ with lifetimes of 63.5 and 29 ps, respectively.

The green spectrum in Fig. 6A represents the $P^+B_L^-$ state. In the YM210W RC the amount of this state was somewhat enhanced due to extension of its lifetime to 5 ps (when formed from $P_{\rm F}^*$) or 7.1 ps (when formed from $P_{\rm S}^*$). As a result it could be resolved with a higher accuracy than was possible for R-26 RCs, where it is very short-lived (0.7 ps). The spectrum was generally reproducible in the simultaneous target analysis of the four data-sets collected for the YM210W RC (see below). It contained a number of negative and positive bands attributable to the ground and cation states of P, respectively, that are also present in the SADS of the $P^+H_L^-$ and $P^+Q_A^$ states. The attribution of these features was discussed in depth in our previous report on the R-26 RC, and is summarized in ESI Table S2.[†] These attributions are not considered further here, other than to comment that there was good correspondence between the positions of these bands in the SADS in Fig. 6A and their positions in equivalent SADS for the R-26 RC described in our previous report²¹ (and see Discussion section). Rather, the remainder of this section focuses on spectral features that are characteristic of the $P^+B_L^-$ state in particular.

The SADS of $P^+B_L^-$ contained a negative band at ~1688 cm⁻¹ that is attributable to the ground state 9-keto C=O modes of P_L/P_M (Fig. 6A, green). This negative band was also present in the SADS of P*, $P^+H_L^-$ and $P^+Q_A^-$ in Fig. 6A. However, in contrast to the latter spectra, the SADS of $P^+B_L^-$ also had a prominent negative band at 1678 cm⁻¹ that can be attributed to the ground state of the 9-keto C=O of B_L. As discussed recently,²¹ this frequency is consistent with this carbonyl acting as the acceptor of a weak-to-moderate strength hydrogen bond, the donor being a water molecule.^{34,35} It is possible that the small positive band at 1662 cm⁻¹ is

attributable to the 9-keto C=O of the anion state of B_L , which would be consistent with the general picture that the stretching frequency of a keto C=O undergoes a down-shift upon reduction of a cofactor.³⁶ This said, in an FTIR difference spectrum of BChl *a* in tetrahydrofuran³⁷ the 9-keto C=O mode was observed to down-shift by 63 cm⁻¹ from 1683 cm⁻¹ in the ground state to 1620 cm⁻¹ for the anion, and so it is also conceivable that the positive band at 1612 cm⁻¹ in the P⁺B_L⁻ spectrum represents the down-shifted 9-keto of B_L⁻. These attributions will require further investigation, possibly employing a mutant RC in which the water molecule adjacent to the B_L keto C=O group is excluded, causing a frequency shift through removal of the cofactor–protein hydrogen bond.³⁵

The blue SADS in Fig. 6A represents the product of the second step of charge separation, the state $P^+H_L^-$. The SADS of this state in the YM210W RC was generally similar to the equivalent SADS for the R-26 RC,²¹ but showed a few minor differences that are discussed below. The lifetime for the $P^+H_L^-$ state was ~279 ps, irrespective of whether it was formed from P_F^* or P_S^* , and so the only consequence of this heterogeneity in P* was a small difference in the lifetime of the $P^+B_L^-$ state.

The final product of charge separation in the YM210W RC was $P^+Q_A^-$, formed in ~279 ps. The pattern of bands that is characteristic of contributions from Q_A^-/Q_A^{38-41} seen in the SADS of this state in the R-26 RC,²¹ were reproduced in the SADS of the YM210W RC (Fig. 6A, cyan and see ESI Table S2).† These included responses to Q_A reduction of a protein amide I C=O (at 1666(-)/1655(+) cm⁻¹)⁴² and a protein backbone C=O that is connected to Q_A via a hydrogen bond (at 1650(-)/1640(+) cm⁻¹).^{39,43,44}

Discussion

Femtosecond mid-IR spectroscopy provides an alternative means of examining photochemical charge separation in the RC, enabling access to information on different groups in the bacteriochlorin macrocycle as well as new information on the quinones and protein surroundings of the cofactors. One aim of the present study was to look at the reproducibility of the SADS that can be extracted from a target analysis of the data between the R-26 RC, studied in a recent report, and the YM210W RC where charge separation is slowed. Accordingly the first part of this Discussion compares SADS obtained in the present study with those obtained previously for the R-26 RC. The Discussion then goes on to consider possible structural origins of features of the data that are specific to the YM210W RC.

Comparison of SADS for the $P^{\ast}and \ P^{+}Q_{A}^{-}$ states

Fig. 7 looks at the issue of the reproducibility of the SADS extracted from target analysis of experimental data, comparing SADS for P* and P⁺Q_A⁻ obtained in the present study with equivalent spectra obtained in previous work on the R-26 RC.²¹ For P* (Fig. 7A) the spectra shown were derived from data on YM210W and R-26 RCs excited at 805 nm. The spectra are similar to one another, with the main positive band centered at 1668 cm⁻¹, and the main negative bands on either



Fig. 7 Comparison of P* and P⁺Q_A⁻ SADS. (A) P* SADS derived from data recorded with 805 nm excitation of YM210W RCs (black) and R-26 RCs (red); (B) P⁺Q_A⁻ SADS derived from data recorded with 805 nm excitation of YM210W RCs (black) and 600 nm (red) excitation of R-26 RCs.

side of this. The P* SADS derived from the remaining datasets for the YM210W and R-26 RCs also had similar lineshapes (data not shown), leading to the conclusion that the spectral signature of this state was very reproducible, with relatively little distortion, apart from the presence of some baseline uncertainty. This was despite the very different lifetimes for P* in the R-26 and YM210W RC.

Fig. 7B shows SADS corresponding to the $P^+Q_A^-$ state derived from data on YM210W RCs excited at 805 nm (black) and R-26 RCs excited at 600 nm (red). Again the spectra show a good correspondence, the positions of the multiple positive and negative bands being preserved within a few wavenumbers. The attribution of these bands has been discussed previously, and is summarized in ESI Table S2.[†]

Comparison of SADS for the P⁺H_L⁻ state

SADS of the radical pair $P^+H_L^-$ in the YM210W RC, and the relaxed form of this radical pair $((P^+H_L^-)_2)$ in Q_A-removed R-26 RCs, are compared in Fig. 8. These SADS were derived from data recorded with 805 nm excitation, and the associated lifetimes were 279 and 290 ps, respectively. The SADS for the YM210W RC differed somewhat from that of the R-26 RC, particularly in the region between 1630 and 1680 cm⁻¹ that contains multiple contributions from the 2a-acetyl C=O groups of P_L/P_L^+ , P_M/P_M^+ and the protein. In particular, the pattern of bands at 1666(+)/1656(-) cm⁻¹ in the SADS of the R-26 RC was somewhat altered in the SADS of the YM210W RC, where a down-shift of these bands of $\sim 5 \text{ cm}^{-1}$ to 1661(+)/1652(-) cm⁻¹ was observed. In WT RCs this feature has been attributed to a Stark shift of one or more amide C=O groups in response to formation of the $P^+H_L^$ radical pair, 39,42,44-46 and the observed 5 cm⁻¹ frequency shift in the YM210W RC suggests a change in the environment of this group arising from the structural changes associated with the mutation. Inspection of the X-ray crystal structure of the YM210W RC shows that the mutation has only very subtle effects on the structure of the protein component, with the biggest change in the structure of the polypeptide backbone being at the M210 residue itself (Fig. 1). The M210 amino acid forms part of the membrane-spanning D-helix of the M-polypeptide, and the alpha carbon of this residue moves

towards the center of the helix by ~0.4 Å in order to accommodate replacement of the Tyr side chain by the bulkier Trp (Fig. 1). Although the position of the backbone C=O of the M210 amino acid is not markedly affected by this flexing of the backbone, the backbone C=O of the neighbouring M209 amino acid undergoes a shift of ~0.4 Å to a position more closely aligned with the axis of the D-helix (Fig. 1). As the backbone C=O groups of the D-helix of the M-polypeptide in the region around the M210 residue lie very close to the vector that can be drawn between the P_L/P_M and H_L cofactors, it seems entirely possible that the observed 5 cm⁻¹ frequency shift of the amide I C=O in the YM210W RC is due to subtle changes in the environment of one or more of the backbone C=O groups in the immediate vicinity of the M210 residue, most probably that of the neighbouring M209 amino acid.

Turning to spectral features specific to H_L, the SADS of $(P^+H_L^-)_2$ in the R-26 RC contains a negative band at ~1687 cm⁻¹ arising from the keto C=O groups of P_L and P_M and an overlapping negative band at ~ 1675 cm⁻¹ attributed to the 9-keto C=O of H_L hydrogen bonded to Glu L104.²¹ In the SADS of $P^+H_L^-$ in the YM210W RC the latter feature appeared as a discrete band at ~1676 cm⁻¹. This seemed to originate from a $\sim 1 \text{ cm}^{-1}$ up-shift of the negative band attributed to the keto C=O of P_L/P_M to 1688 cm⁻¹, leading to improved resolution of the H_L band at 1676 cm⁻¹ as a consequence of a greater splitting of two overlapping negative components. In addition the downshift of the 1666(+)/ $1656(-) \text{ cm}^{-1}$ differential feature to $1661(+)/1652(-) \text{ cm}^{-1}$ in the YM210W RC may also have contributed to the greater prominence of the 1676 cm⁻¹ band. In view of the spectral resolution in the experiment the exact magnitudes of the up- or down-shifts of these overlapping bands should be interpreted with caution, but the net result was a reproducible change in the lineshape of the spectrum in this region.

The positive bands at 1705 cm⁻¹ and 1715 cm⁻¹ attributed to the 9-keto C=O modes of P_M^+ and P_L^+ , respectively, exhibited relative amplitudes that were somewhat different in the YM210W mutant from those observed in the R-26 RC (Fig. 8). As discussed in our previous report,²¹ in the SADS of the R-26 RC the 1715 cm⁻¹ band was more intense than the 1705 cm⁻¹ band (see Fig. 11 and ESI Fig. S1 in ref. 21). In contrast, in the YM210W RC the tendency was for the two



Fig. 8 Comparison of SADS for the $P^+H_L^-$ state derived from data recorded with 805 nm excitation of YM210W (black) and R-26 (red) RCs (with Q_A removed).

bands to have approximately equal amplitudes, or the band at 1705 cm⁻¹ to have the higher amplitude (see also Fig. 6A and the SADS of the $P^+B_L^-$ state in Fig. 9). This suggests that one of the effects of the mutation is a shift in the charge distribution within P^+ , relative to the R-26 RC.²¹ In fact the effect of the YM210W mutation on the spin density distribution within P^+ has been investigated by ENDOR spectroscopy,⁴⁷ and it was found that the magnitude of the two largest methyl groups assigned to P_L decreased by 7%. This implies an increase in the proportion of the cation localized on P_M , qualitatively consistent with our findings from fs mid-IR spectroscopy.

Comparison of SADS for the $P^+B_L^-$ state

A main aim of the present study was to further explore the mid-IR spectrum of the transient $P^+B_L^-$ state. This short-lived radical pair has eluded characterization through FTIR difference



Fig. 9 Comparison of $P^+B_L^-$ SADS resulting from target analysis of YM210W and R-26 RCs (with Q_A removed). Excitation wavelength is indicated.

spectroscopy, but the C=O stretching region of the $PB_I/P^+B_I^$ difference spectrum was described in a preliminary form in our recent report on the R-26 RC.²¹ The individual SADS determined for the $P^+B_L^-$ state in the YM210W RC are shown in Fig. 9, where they are compared with two SADS for the $P^+B_L^-$ state in R-26 RCs.²¹ The observed population of $P^+B_L^-$ was somewhat larger in the YM210W RC due to the ~10-fold slower electron transfer from B_L^- to H_L , and as a result the level of uncertainty in the $P^+B_L^-$ SADS for the YM210W RC was less than in the spectra for the R-26 RC. Bearing this in mind, there was a reasonable correspondence between the SADS from the different experiments. Particularly prominent in these spectra were bands attributable to the 9-keto C=O modes of P_L/P_M at 1688(-) cm⁻¹, P_L^+ at $1715(+) \text{ cm}^{-1}$ and P_M^+ at $1705(+) \text{ cm}^{-1}$, and the 10a-ester C=O groups of $P_{\rm I}/P_{\rm M}$ and $P_{\rm L}^+/P_{\rm M}^+$ at 1740(–)/1750(+) cm⁻¹, respectively.

The spectra of the $P^+B_L^-$ state in the YM210W mutant presented in Fig. 9 (middle and top panels) showed some variation in lineshape in the region between 1620 and 1665 cm^{-1} . These variations pertained more to the amplitude of bands in this region rather than their positions. ESI Fig. S3[†] shows all the SADS resulting from the simultaneous target analysis, using model-IV, of all four data sets collected for the YM210W RCs. It can be seen that variations in lineshape among the SADS of the $P^+B_L^-$ state were larger than those observed for the SADS of the P*, $P^+H_L^-$ and $P^+Q_A^-$ states, particularly in the region below 1665 cm^{-1} . This variation probably arises from the amplitudes of signals in this spectral region being relatively small due to the low extinction coefficient of the 2a-acetyl carbonyls, resulting in a lower signal-to-noise ratio than in the remaining part of the spectrum. In general terms the spectrum of the $P^+B_L^-$ state is expected to be the most affected by noise due to the relatively short-lifetime of this state, even in the YM210W RC.

Turning to features attributable to B_L, the broad negative feature between 1690 and 1670 cm⁻¹ present in the SADS of the $P^+B_L^-$ state in R-26 RCs (Fig. 9, bottom panel) represents the ground states of the 9-keto C=O groups of P_L/P_M and B_L . In our previous report we assigned the shoulder at $\sim 1687 \text{ cm}^$ to the combined P_L/P_M modes, based on their contribution to the spectra of $P^+H_L^-$ and $P^+Q_A^-$, and the main bleach between 1670 and 1680 cm⁻¹ to the 9 keto C=O of B_L . In the spectra of the YM210W RC (Fig. 9, middle and top panels) this part of the spectrum was more clearly resolved as a negative band at $\sim 1690 \text{ cm}^{-1}$ from $P_L P_M$ and a second negative band at ~1675–1680 cm⁻¹ from B_L . This improved resolution of the contributions of P_L/P_M and B_L could stem from (i) a small up-shift of the negative band of P_L/P_M from 1687 cm⁻¹ in the R-26 RC to 1690 cm⁻¹ in the YM210W RC, similar to that described for the SADS of $P^+H_L^-$ above, and (ii) a small shift in the frequency of the ground state C=O mode of B_L caused by a small change in environment/ hydrogen bonding. As can be seen in Fig. 1, the keto C=O is one of the substituents of the B_L macrocycle most affected by the Tyr to Trp mutation, and movement of this C=O group is likely to change the detail of its interaction with the adjacent water molecule (also shown in Fig. 1), possibly altering the frequency of the stretching mode of the C=O

group through a small change in hydrogen bond strength or electrostatic interaction.

Direct charge separation from B_L* in the YM210W RC

Excitation of the blue side of the 800 nm absorbance band of the *Rb. sphaeroides* RC involves, predominantly, selective excitation of B_L . It has been shown in previous work on the YM210W RC that at 77 K formation of B_L^* initiates a very fast process of direct charge separation not involving P* that competes with the ultrafast energy transfer from B_L^* to P.¹⁷ Visible-region pump/probe experiments on membrane-bound YM210W RCs at 77 K showed the formation of the P⁺B_L⁻ state in ~200 fs from B_L^* , and its further decay in ~6.5 ps into P⁺H_L^{-.17} In addition evidence for the involvement of a $B_L^+H_L^-$ state in an alternative pathway initiated by the reaction $B_L^* \rightarrow B_L^+H_L^-$ has been obtained.⁴⁸ The formation of a P⁺B_L⁻ radical pair without the involvement of P* was also observed in wild-type membrane-bound RCs in visible pump/probe experiments performed at 77 K.¹⁸

The observation of significant amounts of direct charge separation from B_L^* at 77 K in both wild-type and mutant RCs leads to the question of whether this alternative process also operates at room temperature. There are some indications that this may be the case. In work on R-26 RCs at room temperature, Lin and coworkers observed that less P* is formed following excitation at 760 nm (78%) or 800 nm (85%) than at 860 nm (100%).⁴⁹ This is consistent with the operation of B_L^* -driven alternative charge separation under these conditions. Furthermore, in previous work on membrane-bound wild-type RCs at room temperature our laboratories have reported that data from visible region pump-probe spectroscopy could be satisfactorily modeled by a target analysis that included a $B_L^* \to P^+B_L^-$ component with a yield of 18%.²⁴

If this alternative mechanism for charge separation does operate in purified YM210W RCs at room temperature, then there might be some indications of this process in the primary spectroscopic data. Any B_L *-driven charge separation should be maximal following excitation of (mainly) B_L at 795 nm, but much less prominent on excitation of both B_L and B_M at 805 nm, and absent on excitation of P at 860 nm. Fig. 10 compares spectra recorded 2.5 ps after excitation with three wavelengths: 795 nm vs. 860 nm (Fig. 10A) and 805 nm vs. 860 nm (Fig. 10B). At this time all energy transfer events should be complete, any ultrafast alternative charge separation should have taken place (initially $B_L^* \rightarrow P^+ B_L^-$ or $B_L^* \rightarrow B_L^+ H_L^-$) but little of the slowed $P^* \rightarrow P^+ B_L^-$ reaction will have taken place. Due to the differences in observed ΔmOD in the separate experiments the three spectra were normalized to the same amplitude of the ground state mode at 1691 cm⁻¹. In this comparison no attempt was made to correct for a possible variation in the baseline, as this would need a detailed understanding of its precise line-shape.

Issues of baseline aside, a number of observations come out of the comparison of the spectra in Fig. 10. First, none of the spectra are of a purely P* state (such as that in Fig. 7A), with all showing some indication of the presence of P^+ , most obviously the double positive peaks at ~ 1715 and 1705 cm⁻¹ attributable to the 9-keto C=O modes of P_L^+ and P_M^+ . This is in accordance with the target analysis, which predicts an 8% $P^+B_L^-$ concentration at 2.5 ps (green in Fig. 6A). Second, the main positive band at 1668 cm⁻¹, attributable to the 9-keto C=O modes of P_L and P_M in the excited state, was accompanied by shoulder at around 1676 cm^{-1} . This shoulder was seen in all of the datasets collected for the YM210W RC, but was not immediately obvious in previous data collected for the R-26 RC.²¹ However, on closer inspection of data on the latter RCs there was some hint of a component at around 1680 cm⁻¹ that influences the lineshape of EADS corresponding to the P* state between the major positive band at 1668 cm^{-1} and the major negative band at 1688 cm^{-1} . For the R-26 RC this is visible in our recent report²¹ in Fig. 2 (red EADS-3.8 ps component attributed to P*), Fig. 5 (red EADS-4.4 ps component attributed to P*) and Fig. 6 (red SADS of P*). Hence it is possible that the 1676 cm^{-1} shoulder in the spectra of the YM210W RC in Fig. 10 arises from a component that is also present in equivalent spectra of the R-26 RC, but is better resolved due to a small change in frequency.

Insight into the possible source of this change in lineshape can again be obtained from the X-ray structure of the YM210W RC.²⁰ The tilt of the B_L macrocycle is such that the oxygen of the keto carbonyl is one of the atoms that undergoes the largest change in position (~0.4 Å) and, as outlined above, it is conceivable that this could alter the strength of the hydrogen bond between this carbonyl and the adjacent water molecule, in turn modulating the stretching frequency of this C=O group. From the SADS of $P^+B_L^-$ in the YM210W RC described above the stretching frequency of



Fig. 10 Comparison of absorbance difference spectra (normalized at 1691 cm^{-1}) measured 2.5 ps after excitation of YM210W RCs at (A) 795 nm (black) and 860 nm (red) and (B) 805 nm (black) and 860 nm (red).

the keto C=O of B_L in the ground state is in the 1675–1680 cm⁻¹ region,²¹ and this mode is expected to shift to lower frequency on reduction of B_L . In addition, Robert and Lutz³⁴ have reported that the frequency of this C=O group also downshifts in response to formation of either P⁺ or the triplet excited state of P (P^T). Thus signals attributable to the keto C=O of B_L in the 1680–1660 cm⁻¹ region, arising from such changes in frequency on loss of the P ground state or charge separation, are expected to underlie the band attributable to P/P* at 1690(–)/1668(+) cm⁻¹. Given this, it seems likely that the more pronounced shoulder at 1676 cm⁻¹ in the raw early-time spectra for the YM210W mutant arises from a small change in frequency of a signal attributable to the keto C=O of B_L.

The third issue that comes out of the data presented in Fig. 10 is the increased prominence of this 1676 cm^{-1} shoulder relative to the main P* band at 1668 cm⁻¹ in the data recorded with 795 nm excitation, compared with the data recorded with 805 or 860 nm excitation. This was not simply a random effect of noise—ESI Fig. S4[†] shows spectra recorded at five delay times during the first 15 ps following excitation at 795, 805 or 860 nm, and it is clear that the increased prominence of the 1676 cm⁻¹ shoulder was a consistent feature of all spectra recorded following 795 nm excitation. The origin of this effect is not yet clear, but it could indicate (i) a simultaneous decrease in the contribution of P* and increase in the contribution of P^+ to the spectra following 795 nm excitation and/or (ii) an increased contribution to the overall signal in this region from B_L following 795 nm excitation. This behavior is consistent with the presence of some charge separation driven directly by BL*. However a deeper understanding of the attribution of the overlapping bands in this region of the spectrum is required to be certain of this.

Conclusions and outlook

The technique of femtosecond mid-IR spectroscopy has the potential of providing new information on how the protein environment of the cofactors responds to, and possibly influences, photochemical charge separation. In the present report this technique has been applied to a mutant RC of known structure in which the kinetics of primary charge separation are strongly slowed, but there is no change in the cofactor composition of the complex and no strong changes in its visible region absorbance spectrum. Absorbance difference spectra for P* and the three principal radical pair states involved in membrane-spanning electron transfer were obtained from a target analysis of the experimental data, and despite strong differences in the kinetics of charge separation there was good agreement between these spectra and equivalent spectra obtained in a previous study on R-26 RCs. In particular, data on the YM210W RC confirmed the general lineshape of the transient $P^+B_L^-$ state, and the location of the ground state absorbance band of the keto C=O of B_L at $1675-1680 \text{ cm}^{-1}$. The location of the absorbance band of the keto C=O of B_{I} remains to be determined. Other features of the experimental data and analysis indicated that the YM210W mutation elicited a change in charge distribution between the two BChls in the oxidized P dimer. An indication

that a small amount of charge separation driven directly by B_L^* operates in RCs at room temperature was found in the raw data, which is consistent with earlier observations.

One of the challenges of this type of spectroscopy is to account for the various negative and positive bands that make up the difference spectrum of each state. Some of these can be assigned with reference to steady state FTIR spectroscopy, whereas others will require investigation through isotope labeling or site-directed mutagenesis. In the present case, the most marked difference between the spectra obtained for the YM210W and R-26 RCs was a $\sim 5 \text{ cm}^{-1}$ up-shift of a feature in the SADS of $P^+H_L^-$ that has been attributed to the response of one or more amide C=O groups to the formation of $P^+H_L^-$. Given what is known about the changes in proteincofactor structure that occur in response to the YM210W mutation we conclude that this response involves one or more C=O groups in the vicinity of the M210 residue, the modified spectral response arising from small changes in geometry of one or more of these groups caused by the Tyr to Trp mutation. Future work will further investigate how crystallographically-characterised changes of structure of the protein-cofactor system affect the SADS of the various excited and radical pair states involved in charge separation, as well as probing other regions of the mid-infrared spectrum of the RC.

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