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Step-scan FTIR spectroscopy resolves the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition in *Rb. sphaeroides* R26 reaction centres

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Abstract

It is shown that step-scan Fourier transform infrared spectroscopy can be applied to resolve the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition in *Rhodobacter sphaeroides* reaction centres with a 5 μs time resolution. In the mid-infrared region (1900 – 1200 cm^{-1}), transient signals previously assigned to $Q_{A/B}$ and $Q_{A/B}^-$ vibrations, respectively (Brudler et al. 1994; Brudler et al. 1995; Breton and Nabedryk 1996), can be resolved with this new technique. In addition, the three small positive bands in the spectral region of the carboxylic C=O stretching modes of acidic amino acid side chains are also resolved at 1730, 1719 and 1704 cm^{-1}. A global fit analysis yields two exponentials with half-times of 150 μs and 1.2 ms in agreement with IR spectroscopic studies at single wavenumbers (Hienerwadel et al. 1995), in the UV/VIS and near IR (Tiede et al. 1996, Li et al. 1996). The establishement of the step-scan technique enables a new approach to elucidate the molecular mechanism of this transition.

Abbreviations: FTIR – Fourier transform infrared; H_A – intermediary electron acceptor; IR – infrared; MIR – mid-infrared; P – primary electron donor; Q_A – primary acceptor quinone; Q_B – secondary acceptor quinone; RC – reaction centre; *Rb.* – *Rhodobacter*; UQ – ubiquinone; vis – visible

Introduction

In the photosynthetic reaction centre (RC) of *Rhodobacter sphaeroides*, light excitation induces a transmembrane charge separation originating at the primary donor P (bacteriochlorophyll *a*-dimer), proceeding via H_A (bacteriopheophytin *a*) and Q_A (ubiquinone-10 = UQ_{10}) and terminating at Q_B (UQ_{10}). FTIR difference spectra ($P^+Q_A^- - PQ_A$, $P^+Q_B^- - PQ_B$, $H_A^- - H_A$, $Q_A^- - Q_A$, $Q_B^- - Q_B$) between a charge separated state stabilized by continuous illumination and the neutral ground state have been recorded to study the molecular reactions of the cofactors and the protein environment involved in the electron transfer (Gerwert 1993; Mäntele 1993; Nabedryk 1996). Specific band assignments were recently achieved for the Q_A and Q_B and the Q_A^- and Q_B^- vibrations by site-specific ^{13}C-labelling of ubiquinone and reconstitution at the Q_A and Q_B binding site, respectively (Breton et al. 1994, 1995; Brudler et al. 1994, 1995).

While steady-state FTIR difference spectroscopy monitors the molecular changes in the respective relaxed states, time-resolved IR spectroscopy must be applied to investigate the transient processes. Taking advantage of the different recombination lifetimes of the photoinduced intermediates $P^+Q_A^-$ (~100 ms) and $P^+Q_B^-$ (~1 s) a $Q_A^-Q_B - Q_AQ_B^-$ double difference spectrum was calculated using the rapid-scan FTIR technique with a 25 ms time resolution (Thibodeau et al. 1990). Direct investigation of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition which takes place in 200 μs (Vermeglio and Clayton 1977) was performed at single frequencies with tunable IR laser diodes with 0.5 μs time resolution (Hienerwadel et al. 1992; Hienerwadel et al. 1995). Stroboscopic FTIR spec-

troscopy with a time resolution of 25 μs (Souvignier and Gerwert 1992) and the step-scan FTIR technique with a time resolution of up to 10 ns (Uhmann et al. 1991; Rammelsberg et al. 1997) enable simultaneous data acquisition in the whole MIR range. Only the complete IR difference spectra allow unequivocal band assignments and thereby a complete description of the molecular changes. Burie et al. (1993) have used the step-scan method with a 10 μs time-resolution to investigate the PQ_A to $P^+Q_A^-$ and the P to ^3P transitions. Here we describe the first step-scan investigation of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition with a 5 μs time resolution. The absorbance changes of this transition are much smaller than for the $P^+Q_A^-$ transition and demand improved S/N.

Materials and methods

The experiments were performed on a vacuum FTIR spectrometer (IFS 66v, Bruker) with a globar IR source, KBr beamsplitter and MCT detector. The scanner stability was determined to be $\pm$ 1 nm (Rammelsberg et al. 1997).

The spectra were recorded between 1975 – 988 cm^{-1} with a resolution of 7 cm^{-1}; the resolution of the phase spectrum was 50 cm^{-1}. At each scanner postion 1000 time points spaced by 5 μs were collected. The reaction was started 60 μs after the scanner's stop by a laser flash (540 nm, 20 ns, ~5 mJ). The laser flashes were separated by 20 s to ensure full recombination from the charge separated state to the neutral ground state of the RC. In order to improve the S/N ratio, 24 measurements on one sample were averaged. The duration of one measurement was 1.5 h. Between the measurements, static $P^+Q_B^- - PQ_B$ difference spectra were recorded in order to control the activity of the protein. The reconstruction of the interferograms and the computation of the difference spectra were carried out with the OPUS software from Bruker. The interferograms were multiplied with the 'Norton-Beer, weak' apodization function and zero-filled by a factor of 4. Phase correction was done by the Mertz method. The resulting 1000 difference spectra were averaged on a logarithmic time scale.

The principles and experimental details of the FTIR step-scan measurement are described in Rammelsberg et al. (1997).

The kinetic analysis was performed using the 'Global Fit' program (Heßling et al. 1993) taking into account absorbance changes of the complete spectra. In order to eliminate the dominant P^+/P bands that mask the (semi) quinone and protein vibrations and to remove contributions from the $P^+Q_A^- \rightarrow PQ_A$ recombination kinetics, the first difference spectrum ($P^+Q_A^- - PQ_A$) recorded 7 μs after the laser flash was subtracted from the following difference spectra. For subtraction the spectra were normalized on the whole frequency range from 1975 to 988 cm^{-1}. A satisfactory fit resulted in a random distribution of the residuals around the zero-line, a standard deviation close to 1 (1.06) and a minor correlation of the rate constants. For the steady-state $P^+Q_A^- - PQ_A$ difference spectrum, 6400 interferograms with a spectral resolution of 4 cm^{-1} were recorded before and during continuous illumination with a halogen lamp. Therefore, the S/N is about 16 fold increased as compared to the time-resolved difference spectra.

RC protein was purified from *Rb. sphaeroides* strain R26 and reconstituted with a 20 fold excess of UQ_2 at the Q_B site (Okamura et al. 1975). 36 μl RC solution (10 mm Tris, 0.025% LDAO, 1 mm EDTA, pH 8) with OD_{802} = 20 was dried on a CaF_2 window under a gentle stream of nitrogen, rehydrated, covered with another CaF_2 window and thermostabilized at 22 °C in the spectrometer.

Results and discussion

Figure 1a shows a $P^+Q_A^- - PQ_A$ difference spectrum, recorded with the step-scan method 7 μs after the laser flash. For comparison, a $P^+Q_A^- - PQ_A$ difference spectrum generated under continuous illumination with a halogen lamp is shown in Figire 1b. Both difference spectra agree well. The noise amplitudes of the step-scan measurement and the static measurement can be compared between 1900–1800 cm^{-1}. It should be noted that the difference spectra in Figure 1 do not have to agree completely since the step- scan spectrum reflects a transient state during electron transfer and the static spectrum represents a fully relaxed state after electron transfer. In the following only signals larger than 10^{-3} absorbance units, that are clearly above the noise level, are discussed. The difference spectrum in Figure 1a reveals all the bands that are typical for the $PQ_A \rightarrow P^+Q_A^-$ transition. The difference spectra agree also nicely with the one measured in Burie et al. (1993). Some bands have been tentatively assigned in static $P^+Q_A^- - PQ_A$ and P^+ P difference spectra by comparison with model compounds (Gerwert et al. 1988; Mäntele et al. 1988; Buchanan et al.

1992; Leonhard and Mäntele 1993; Nabedryk 1996). The differential signal at 1753/1743 cm^{-1} (Figure 1a) was suggested to arrise from the 10a-ester C=O groups of P^+ and P, respectively. The positive bands at 1716 and 1706 cm^{-1} in Figure 1a (1716 and 1705 cm^{-1} in Figure 1b) are most probably due to the two 9-keto C=O groups of P^+; the negative bands at 1692 and 1683 cm^{-1} (Figure 1b) have been assigned to the 9-keto C=O groups of P (Nabedryk, 1996). The reason why the band at 1683 cm^{-1} appears only as a small shoulder (Figure 1a) and the intensities of the signals at 1692 and 1683 cm^{-1} in Figure 1a are inverse as compared to Figure 1b is not yet clear. In an another step-scan measurement these bands agree with the static difference spectra. A band at ~1665 cm^{-1} (corresponding to the band at 1669 cm^{-1} in Figure 1b), previously assigned to a 9-keto C=O group of P (Mäntele et al. 1988), more probably is due to a peptide C=O in the environment of P (Nabedryk 1996). The band at 1627 cm^{-1} in Figure 1a (1630 cm^{-1} in Figure 1b) arises partly from the coupled C=O/C=C stretching vibration of Q_A (Brudler et al. 1994). Contributions from the 2a-acetyl C=O groups of P must be also taken into account in this spectral region (Leonhard and Mäntele 1993). In addition, the positive signals at 1568, 1545, 1479, 1462, 1402 and 1296 cm^{-1} (Figure 1a) have clear correspondences in the static difference spectrum (Figure 1b) and can be attributed mainly to P^+ (Leonhard and Mäntele 1993). Between 1470–1440 cm^{-1}, Q_A^- vibrations have been identified at 1486, 1466 and 1447 cm^{-1} (see below).

Figure 2a compares the time resolved $P^+Q_A^-$ – PQ_A difference spectrum from Figure 1a 7 μs after the laser flash with a difference spectrum recorded 4.2 ms after the reaction's start (= $P^+Q_B^-$ – PQ_B; Figure 2b). Both spectra are very similar and are dominated by the P^+/P bands. This indicates that after 7 μs no reactions that lead to a redistribution of electron density on the primary donor take place. However, the S/N is not improved at 4.2 ms as expected. The noise is not determined by the detector, but due to specificities of the step-scan measurement (e.g. the scanner stability). Small differences between the two spectra are seen mainly between 1670 – 1600 cm^{-1} (C=O and C=C stretching vibrations of Q_A and Q_B) and 1500 – 1400 cm^{-1} (C···O and C···C stretching vibrations of Q_A^- and Q_B^-). To visualize more clearly these deviations, the $P^+Q_B^-$ – PQ_B spectrum was subtracted from the $P^+Q_A^-$ – PQ_A spectrum. In the resulting $Q_A^-Q_B$ – $Q_AQ_B^-$ double difference spectrum (Figure 2c), the P^+ and P bands are eliminated;

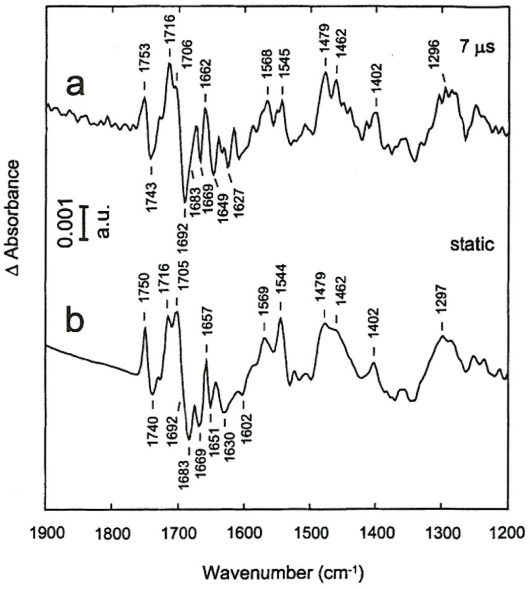

Figure 1. $P^+Q_A^-$ – PQ_A difference spectra. (a) measured with the step-scan method 7 μs after the laser flash, (b) statically measured under continuous illumination with a halogen lamp.

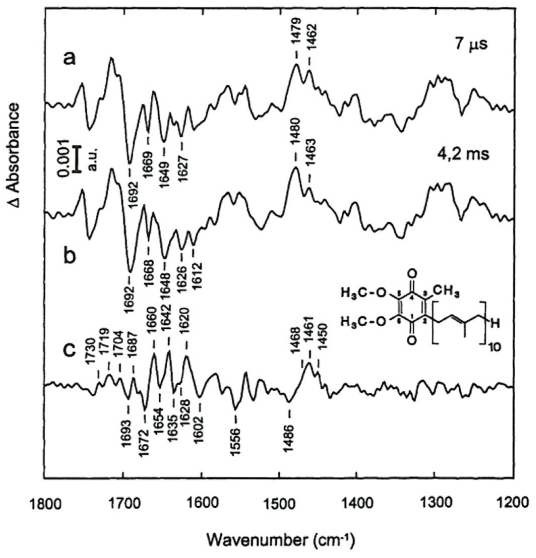

Figure 2. (a) Difference spectrum, recorded 7 μs after the laser flash: $P^+Q_A^-$ – PQ_A (b) Difference spectrum, recorded 4.2 ms after the laser flash: $P^+Q_B^-$ – PQ_B. (c) Double difference spectrum (b–a): $Q_A^-Q_B$ – $Q_AQ_B^-$. Inset: Strucural formula and IUPAC numbering of ubiquinone-10.

the positive bands belong to Q_A^- or Q_B and the negative bands to Q_B^- or Q_A. Besides the bands deriving from the (semi) quinones, vibrations of the protein backbone and of the protein's side chains contribute to the double difference spectrum. By use of site-specific ^{13}C-labelled UQ_{10} (Brudler et al. 1994, 1995) and reconstitution at the Q_A and Q_B site, respectively, the C=O and C=C stretching vibrations of Q_A and Q_B and the C⋯O and C⋯C stretching vibrations of Q_A^- and Q_B^- have been assigned. They can be found in the double difference spectrum of Figure 2c. The positive band at 1642 cm^{-1} is caused by the 1- and 4-C=O stretching vibration of Q_B. The negative band at 1628 cm^{-1} represents a coupled C=O/C=C stretching mode of Q_A. A broad positive band at 1620 cm^{-1} contains the C=C stretching vibration of Q_B. The 4-C=O group of Q_A absorbs mainly at 1602 cm^{-1}. The 1-C=O group of Q_A absorbs at 1660 cm^{-1} (Brudler et al. 1994) and should appear as a negative band in Figure 2c. Instead, a strong positive band which is not yet assigned is found at 1660 cm^{-1} in the double difference spectrum that masks the negative signal of the 1-C=O group.

Specific band assignments are more difficult to perform for the semiquinone vibrations (1500 – 1400 cm^{-1}) because the bands overlap more than for the neutral quinones. In addition, the C⋯O vibrations of the two carbonyl groups of Q_A^- are coupled, in contrast to the C=O vibrations of Q_A. Also coupling between the C⋯O and C⋯C modes is enhanced due to the increased aromatic character of the molecule (Chipman and Prebenda 1986; Brudler et al. 1994). While there is an excellent agreement for the assignments of the Q_A vibrations, different assignments have been reported for Q_A^- (compare Brudler et al. 1994 and Breton and Nabedryk 1996). It therefore should be stressed that a normal mode analysis and further site-specific isotope labelling are necessary to clarify the deviations. In the double difference spectrum of Figure 2c, three positive bands occur at 1468, 1461 and 1450 cm^{-1} which are characteristic of Q_A^- (see for example Brudler et al. 1994). The band at 1468 cm^{-1} was assigned to a predominant 4-C⋯O stretching mode and the band at 1450 cm^{-1} (corresponding most probably to the band at 1447 cm^{-1} in Brudler et al. 1994) to a C⋯C mode with a high degree of the 1- and 4-C⋯O stretching vibrations. A broad negative band with a minimum at 1486 cm^{-1} (Figure 2c) contains the 1- and 4-C⋯O stretching vibrations of Q_B^- (Brudler et al. 1995). The amplitude of this band is probably decreased by a Q_A^- band

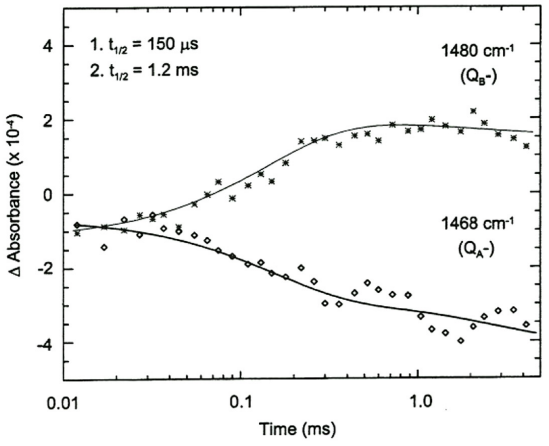

Figure 3. Kinetics at 1480 cm^{-1} (1- and 4-C⋯O stretching vibrations of Q_B^-) and 1468 cm^{-1} (4-C⋯O stretching vibration of Q_A^-).

absorbing at 1486 cm^{-1}, previously assigned to the 1-C⋯O stretching vibration of Q_A^- (Brudler et al. 1994).

Unspecific incorporation of 1'-^{15}N-labelled Trp in the RC has revealed a signal at 1553 cm^{-1} (Brudler 1996). The band at 1556 cm^{-1} (Figure 2c) therefore is (partly) assigned to Trp, most probably to Trp M252 which is in van-der-Waals contact with Q_A (Ermler et al. 1994). In the spectral region of the carboxylic C=O stretching modes of acidic amino acid side chains (1780 – 1690 cm^{-1}), three small bands at 1730, 1719 and 1704 cm^{-1} are resolved (Figure 2c). Hienerwadel et al. (1995) have described three corresponding bands at 1732, 1725 and 1706 cm^{-1}. On the basis of $^1H \rightarrow {}^2H$ exchange and a Glu L212 Gln mutant, they have assigned the band at 1725 cm^{-1} (most probably corresponding to the band at 1719 cm^{-1} in Figure 2c) to the COOH group of Glu L212.

The appearance of the typical $Q_{A/B}$ and $Q_{A/B}^-$ bands in the double difference spectrum (Figure 2c) can be taken as evidence for the correct time and amplitude resolved detection of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$transition by step-scan FTIR difference spectroscopy. For the kinetic analysis, the $P^+Q_A^- - PQ_A$ difference spectrum generated 7 after the flash was subtracted from the following difference spectra (see materials and methods). The analysis shows that a sum of two exponentials with half-times of 150 μs and 1.2 ms was sufficient for a satisfactory description at all wavenumbers. Figure 3 shows two representative kinetics, at 1480 cm^{-1}(appearance of Q_B^-) and at1468 cm^{-1} (disappearance of Q_A^-). The kinetics

show that the absorbance changes can be followed time-resolved. However, there is a small oscillation superimposed, which seems to be an artefact.

Heterogenous $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer rates have been observed by several groups in the IR and Vis spectral region (Hienerwadel et al. 1995; Tiede et al. 1995; Li et al. 1996). Hienerwadel et al. (1995) have characterized the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ transition between 1780 – 1690 cm^{-1} with a time resolution of 0.5 μs and a spectral resolution of 4 – 5 cm^{-1} with tunable diode lasers. A global fit analysis yielded half-times of 180 μs and 1.05 ms in good agreement with the published data. In contrast to the results of Hienerwadel et al. (1992), the different spectral regions representing different molecular groups can be described with the same rate constants and no hint of a different protein kinetic as compared to the chromophores is seen.

The fast rate could simply represent electron transfer and the slow rate proton transfer. But this seems unlikely because the Q_A^- band shows also the slow component. This band should not be observed during the proton uptake. In an alternative explanation the two rates could be related to different RC conformations at the Q_A and/or Q_B binding site, resulting in polyphasic kinetics for the different conformational states. In a third model the slow phase could be explained by a movement of Q_B from an inactive to an active position, as revealed recently by X-ray crystallography (Stowell et al. 1997). This model is in agreement with the finding of two different UQ_{10} positions at the Q_B site (Brudler et al. 1995). However, to discuss the results of the step-scan measurement more detailed, we have to improve the S/N ratio by averaging more samples. This experiments are in progress using an improved experimental setup with 30 ns time-resolution (Rammelsberg et al. 1997).

By establishing the step-scan technique on photosynthetic reaction centres, we opened now the door for further studies on the $Q_A^- Q_B Q_A Q_B^-$ transition in which complete mid IR spectra can be obtained in 30 ns. By use of site-specifically mutated and isotopically labeled RCs this will allow unequivocal band assignments as prerequisite for the understanding of the molecular reaction mechanisms of this transition.

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