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ARTICLE

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Investigation of ubiquinol formation in isolated photosynthetic reaction centers by rapid-scan Fourier transform IR spectroscopy

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Abstract Light-induced formation of ubiquinol-10 in *Rhodobacter sphaeroides* reaction centers was followed by rapid-scan Fourier transform IR difference spectroscopy, a technique that allows the course of the reaction to be monitored, providing simultaneously information on the redox states of cofactors and on protein response. The spectrum recorded between 4 and 29 ms after the second flash showed bands at 1,470 and 1,707 cm^{-1}, possibly due to a QH^- intermediate state. Spectra recorded at longer delay times showed a different shape, with bands at 1,388 (+) and 1,433 (+) cm^{-1} characteristic of ubiquinol [Mezzetti et al. *FEBS Lett.* 537:161–165 (2003)]. These spectra reflect the location of the ubiquinol molecule outside the Q_B binding site. This was confirmed by Fourier transform IR difference spectra recorded during and after continuous illumination in the presence of an excess of exogenous ubiquinone molecules, which revealed the process of ubiquinol formation, of ubiquinone/ubiquinol exchange at the Q_B site and between detergent micelles, and of Q_B^- and QH_2 reoxidation by external redox mediators. Kinetics analysis of the IR bands allowed us to estimate the ubiquinone/ubiquinol exchange rate between detergent micelles to approximately 1 s. The reoxidation rate of Q_B^- by external donors was found to be much lower than that of QH_2, most probably reflecting a stabilizing/protecting effect of the protein for the semiquinone form. A transient band at 1,707 cm^{-1} observed in the first scan (4–29 ms) after both the first and the second flash possibly reflects transient protonation of the side chain of a carboxylic amino acid involved in proton transfer from the cytoplasm towards the Q_B site.

Keywords Bacterial photosynthesis · Ubiquinone · Proton transfer · Electron transfer · *Rhodobacter sphaeroides*

This paper is dedicated to Professor Giovanni Giacometti in occasion of his 75th birthday.

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Introduction

Photosynthetic reaction centers (RCs) from purple bacteria are integral membrane proteins, which act as light-driven quinone reductases. Absorption of a photon induces an ultrafast charge separation between a special pair of bacteriochlorophyll a molecules, called the primary donor P, and the primary electron acceptor Q_A, a quinone molecule strongly bound to the protein. A second quinone molecule, named Q_B, accepts two electrons from Q_A in two successive photochemical events, as well as two protons from the cytoplasm through a pathway formed by amino acid side chains and water molecules. The ubiquinol thus formed is released from its binding site and replaced by an ubiquinone from the pool of quinones in the photosynthetic membrane (Feher et al. 1989). The ubiquinol is reoxidized by another membrane-bound enzyme, called the cytochrome bc_1 complex. The cyclic mechanism of all these redox reactions moves protons from the cytoplasm to the periplasm, thus acting as a proton pumping system. The proton gradient drives synthesis of ATP, which is used to power the metabolic reactions of the cell.

Quinones are key players in electron and proton transfer in many bioenergetics systems (Trumpower 1982; Mitchell and Moyle 1985). In photosynthetic RCs the reactions involving quinones can be induced by short light flashes or by continuous illumination. This makes photosynthetic RCs ideal model systems to investigate

general principles governing the kinetics and energetics of quinone redox chemistry in proteins.

Recently bacterial photosynthetic RCs have also been employed as sensor elements of highly specific bioanalytic devices to determine herbicide concentration in aqueous samples (Nakamura et al. 2003; Baldini et al. 2003; Peters et al. 1997). The mode of operation of these devices relies on the fact that several herbicides (e.g., atrazine) are competitive inhibitors of Q_B binding, displacing the quinone owing to a strong affinity for the Q_B pocket. Detailed knowledge of the functionality of the Q_B pocket is therefore particularly useful for the design of new experimental photosynthetic RC-based biosensors.

The structure of the RC from the purple non-sulfur bacterium *Rhodobacter sphaeroides* has been determined by X-ray crystallography up to 2-Å resolution (Allen et al. 1988; Ermler et al. 1994; El-Kabbani et al. 1991; Fritzsch et al. 2002). This has provided detailed knowledge of the position of pigments, cofactors and amino acids, and of their relative distance and orientation. Such a wealth of information is particularly useful in the interpretation of spectroscopic data, which can therefore give deeper insight into the molecular mechanisms of the enzyme, in particular into the coupling between electron transfer and proton transfer, which is an essential feature not only of the bacterial RC, but of all ubiquinone-binding enzymes (Mitchell and Moyle 1985).

The first electron transfer step $Q_A^- Q_B \rightarrow Q_A Q_B^-$ has been investigated by several techniques (Paddock et al. 2003; Sebban et al. 1995; Okamura et al. 2000; Wraight 2004, and references therein). It is now generally accepted that the reaction does not involve the direct protonation of Q_B^-. However, the electron transfer step is coupled to a substoichiometric proton uptake from the cytoplasm towards the interior of the protein. In addition, driving force assay methods (Graige et al. 1998) have demonstrated that this first electron transfer to Q_B is rate-limited by another step. Although different processes have been proposed as the rate-limiting step (Stowell et al. 1997; Okamura et al. 2000) the implication of a proton transfer is strongly suggested by recent studies (Paddock et al. 2003; Remy and Gerwert 2003). The second electron transfer leads to the formation of the state QH^- in less than 1 ms and is coupled to, but (under normal conditions) not limited by, proton uptake from the cytoplasm (Graige et al. 1996). Subsequently, rapid protonation of QH^- takes place and the ubiquinol formed is released from its binding site (McPherson et al. 1989).

Whereas there is no definite agreement on the detailed mechanisms of both electron transfer reactions, the pathway for proton transfer from the cytoplasmic surface towards the Q_B pocket has been identified (Paddock et al. 2003; Okamura et al. 2000). In both cases, protons are taken up from solution via two histidines located at the surface (His-H126 and His-H128) (Adelroth et al. 2000) and then transferred towards the interior of the protein through a network of hydrogen bonds involving the side chains of two aspartic acids, Asp-L210 and Asp-M17 (Fig. 1). From here proton transfer to the carbonyl groups of Q_B occurs through Asp-L213, Glu-L212 and Ser-L223 (Okamura et al. 2000).

Fourier transform (FT) IR difference spectroscopy is a powerful tool to investigate the mechanism of biochemical reactions, as it allows us to monitor simultaneously reaction-induced changes in both the protein and the cofactors (Vogel and Siebert 2000; Barth and Zscherp 2002; Mäntele 1993). A large amount of data has been obtained by steady-state FTIR difference spectroscopy on isolated RCs from *Rb. sphaeroides* (Breton and Nabedryk 1996; Mäntele 1995; Nabedryk 1996, and references therein), leading to the precise identification of IR marker bands for specific redox states of cofactors (Breton and Nabedryk 1996) and concomitant protonation of amino acid side chains (Nabedryk et al. 1995). Relying on this knowledge, we can use time-resolved FTIR difference spectroscopy to follow the time evolution of the reaction, as well as to observe transient states (Gerwert 2000).

Several kinetics FTIR and single-wavelength IR techniques have been applied to investigate the photoreduction of quinones in *Rb. sphaeroides* RCs. After the pioneering experiments of Thibodeau et al. (1990) and Hienerwadel et al. (1992, 1995), the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ reaction has recently been investigated by rapid-scan FTIR (Mezzetti et al. 2002) and step-scan FTIR (Brudler and Gerwert 1998; Remy and Gerwert 2003). Reduction of ubiquinone to ubiquinol has been investigated by rapid-scan FTIR measurements in *Rb. sphaeroides* photosynthetic membranes (Mezzetti et al. 2003). This led to the identification of IR marker bands for ubiquinol in the phospholipid membrane. However, the relatively low concentration of RCs in photosynthetic membranes has prevented the study of the molecular mechanisms of formation and release of a single ubiquinol molecule per RC, and only multiple reduction events could be followed. In the present investigation we applied rapid-scan FTIR difference spectroscopy to investigate the mechanism of ubiquinol formation in isolated *Rb. sphaeroides* RCs, where the signal-to-noise ratio is greatly improved compared with that for photosynthetic membranes, thereby making it possible to follow the process of formation of a single ubiquinol molecule per RC. This led to the identification of IR marker bands for an intermediate state (most likely QH^-) transiently formed after the second flash. Investigation under and after photoaccumulation conditions also allowed us to investigate the dynamics of ubiquinone/ubiquinol exchange at the Q_B site and between different detergent micelles.

Experimental

Sample preparation

RCs from *Rb. sphaeroides* R26 were isolated in 15 mM tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 8, 0.025% lauryl dimethylamide–*N*-oxide (LDAO), as

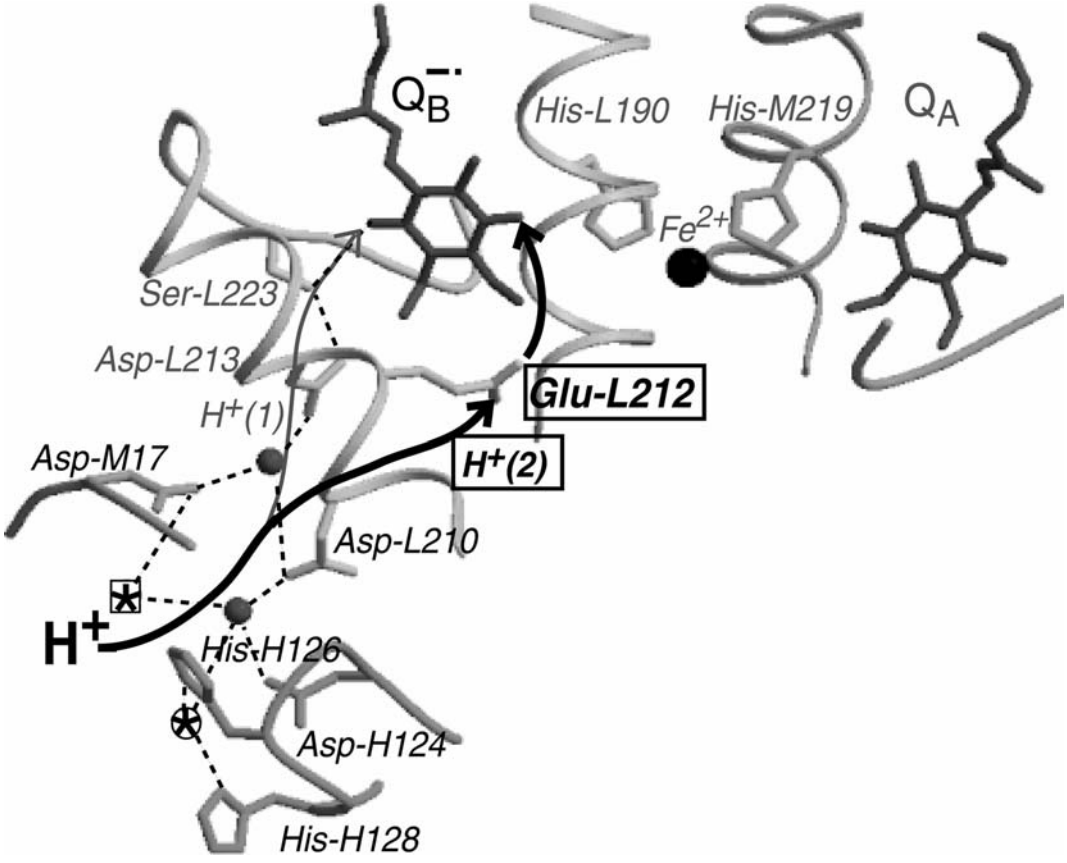

Fig. 1 Scheme of the quinone-binding site in the reaction center (*RC*) of *Rhodobacter sphaeroides* with pathways for proton transfer from the cytoplasm to Q_B. Adapted from Nabedryk et al. (2001)

described in Isaacson et al. (1995). The samples for FTIR measurements were prepared as previously described (Breton and coworkers 1991a, 1991b). Briefly, RCs were deposited on a CaF_2 window and dried under argon. Before complete dryness, the RC film was rehydrated with Tris buffer (pH 8, 100 mM). To guarantee full occupancy of the Q_B site, a fivefold excess of ubiquinone-6 (UQ_6, Sigma, St.Louis, MO, USA) was added to the RC suspension. Sodium ascorbate (10 mM) and 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD; 20 mM) were added as external redox components to ensure fast reduction of P^+. The concentrations of the redox mediators (DAD and sodium ascorbate) determine the reoxidation time of Q_BH_2 between averaging cycles and therefore were carefully chosen. A second CaF_2 window was used to squeeze the sample, in order to yield an absorbance in the amide I region of the spectrum of 0.6–0.9 a.u. Experiments at a lower temperature (268 K) were performed in order to characterize better the transient species observed after the first and the second laser flashes. In this case, the DAD/sodium ascorbate couple could not be used because of the slower rate of P^+ reduction by DAD and because of the long time (more than 5 min) necessary for complete reoxidation of QH_2 by DAD^+/sodium ascorbate at this temperature. In these experiments ferrocyanide (250 mM) and tetramethyl-*p*-phenylenediamine (TMPD; 50 mM) were used as redox mediators, buffered with Tris (pH 7, 100 mM).

FTIR difference spectroscopy experiments

Two-flash rapid-scan experiments

Rapid-scan measurements were performed using the experimental parameters as described by Mezzetti et al. (2002). A Bruker IFS 88 FTIR spectrometer equipped with a photoconductive MCT-A detector and with OPUS software was used. Measurements were performed at 281 ± 1 K or at 268 ± 1 K using a temperature-controlled N_2 cryostat. The photoreactions were triggered by a saturating flash from a frequency-doubled Nd:YAG laser (7 ns, approximately 20 mJ, Quantel, France). The experimental scheme was the following. A total of 40 interferograms were recorded in the dark and averaged. Then, a first laser flash was fired and two successive interferograms were recorded to monitor the time evolution of the system. A second flash was fired and five interferograms were recorded at increasing delay to monitor the process of ubiquinol formation. Subsequent interferograms were averaged in groups of 5. The results from 3,200 cycles (obtained on three different samples) were averaged to improve the signal-to-noise ratio. Between cycles an appropriate delay time (3 min for the samples with DAD and sodium ascorbate as

redox mediators; 30 s for the samples with ferrocyanide and TMPD as redox mediators) allowed the complete relaxation of the system owing to reoxidation of QH_2 by the external redox chemicals. The necessary relaxation time was determined by investigating the decay of the differential spectra at long times. Difference spectra at various times after the laser flash were calculated as previously described (Mezzetti et al. 2002).

Rapid-scan experiments under and after continuous illumination

To study the process of reduction of multiple ubiquinone molecules, rapid-scan FTIR spectra were recorded at 281 K under and after continuous illumination with a 250-W tungsten–halogen lamp (Oriel) equipped with a water filter and a 5% transmission neutral density filter, following the approach described by Mezzetti et al. (2003).

Kinetics analysis

To simulate the time evolution of intermediate states under and after continuous illumination a kinetics model was developed using the Gepasi 3.30 software available on the web (http://www.gepasi.org; Mendes 1997 and references therein).

Results

Two-flash rapid-scan FTIR experiments

Time-resolved rapid-scan FTIR difference spectra recorded at 281 K after one and two laser flashes are shown in Fig. 2. As expected, the spectrum recorded between 4 and 29 ms after the first flash is already characteristic of Q_B^- formation (Mezzetti et al. 2002; Breton et al. 1991a; Nabedryk et al. 1995); in particular, a positive peak at 1,479 cm^{-1} is observed, which had previously been attributed to a mixed C–C and C–O^- vibration of Q_B^- (Breton et al. 1995; Brudler et al. 1995). Negative bands given by the disappearing neutral form of Q_B are visible at 1,265, 1,288 and 1,640 cm^{-1} (Breton et al. 1991a). The bands at 1,265 and 1,288 cm^{-1} are attributed to C–O–C modes of the methoxy groups, whereas the band at 1,640 cm^{-1} is in part attributed to a Q_B C=O mode (Breton and Nabedryk 1996; Brudler et al. 1995). In the region where protonated Glu and Asp side chains absorb—between approximately 1,700 and approximately 1,770 cm^{-1} (Barth 2000 and references therein)—a positive band is observed at 1,728 cm^{-1}, due to protonation of the side chain of Glu-L212 (Nabedryk et al. 1995). A small positive band at 1,707 cm^{-1} is also visible. It is important to underline that no contributions arising from oxidation of the primary donor P are present in the spectrum, because with the chosen

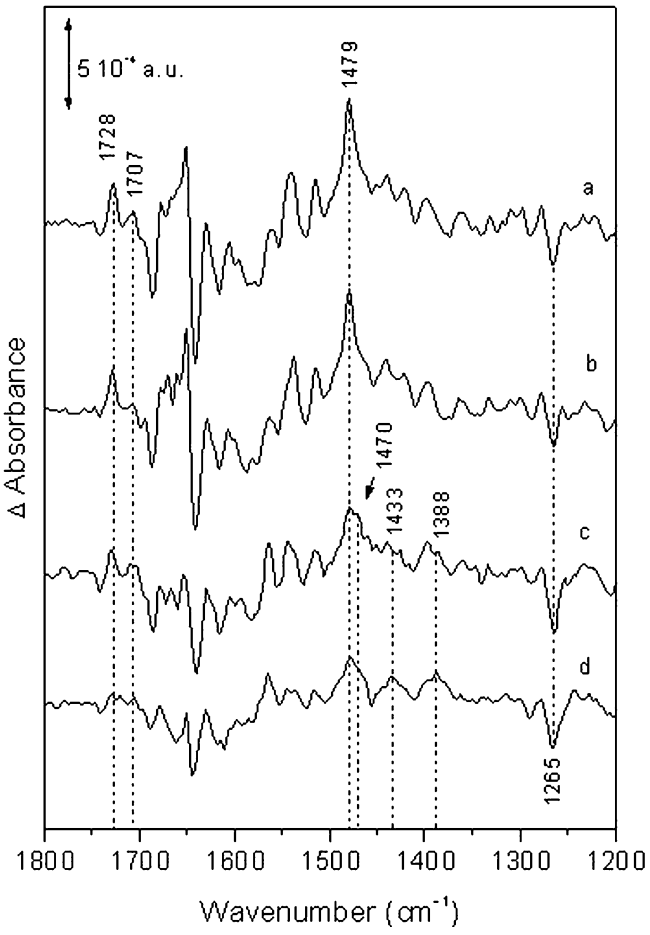

Fig. 2 Rapid-scan spectra obtained after two consecutive flashes on isolated RCs from *Rb. sphaeroides*. $T = 281$ K. Redox mediators 2,3,5,6-tetramethyl-*p*-phenylenediamine (20 mM) and sodium ascorbate (10 mM). *a* Spectrum recorded between 4 and 29 ms after the first flash. *b* Spectrum recorded between 77 and 102 ms after the first flash. *c* Spectrum recorded between 4 and 29 ms after the second flash. *d* Spectrum recorded between 77 and 102 ms after the second flash

concentrations of redox mediators P^+ is completely reduced to P within approximately 2 ms, i.e., 2 ms before beginning to record the interferogram. This can easily be assessed by the absence of the broad electronic transition at approximately 2,500 cm^{-1} characteristic of the P^+/P spectrum (Breton et al. 1992b), as well as by the absence of the characteristic sharp positive band at 1,751 cm^{-1} given by the stretching vibration of the 10*a*-ester group of P^+ (Nabedryk 1996). It is also important to point out that the IR bands in the spectrum correspond either to Q_B or to Q_B^- vibrations or to protein response after the formation of a single semiquinone molecule per RC. This is due to the properties of the laser flash used for excitation, the intensity of which is sufficiently high to ensure that a photochemical event takes place in almost all the RCs (Breton et al. 1991a) and the duration of which is sufficiently short (7 ns) to avoid two successive photochemical events from taking place

during the same laser pulse.[1] In fact, the primary donor P is inactive after its photooxidation until reduction of P^+ by DAD takes place. This, however, is a bimolecular reaction with much slower kinetics (approximately 500 μs), so P cannot be successively excited twice during the same laser pulse.

The second spectrum (recorded between 77 and 102 ms after the first flash, but still before the second flash) is very similar to the first one, except for the diminished amplitude of the 1,707 cm^{-1} positive band.

The spectrum recorded between 4 and 29 ms after the second flash has a different shape from that of the precedent spectra. The bands at 1,479 and 1,728 cm^{-1} are smaller, and new features are present, in particular a positive band at 1,470 cm^{-1} and a positive shoulder at 1,388 cm^{-1}. Smaller variations are also visible throughout the whole spectrum, for instance, in the so-called amide I and amide II regions, where the protein backbone absorbs (Fig. 2). As in the case of the spectrum recorded between 4 and 29 ms after the first flash, a small positive band at 1,707 cm^{-1} is visible. Also in this case no P^+/P contributions are present in the spectrum. The fact that this spectrum corresponds to the double reduction of a single ubiquinone molecule per RC can be assessed by looking at the intensity of the ubiquinone marker band at 1,265 cm^{-1} (Breton et al. 1991a), which remains almost unchanged compared with that in the spectrum recorded immediately after the first laser flash. As this band is a probe to assess the number of quinone molecules, which have undergone photo-induced reduction in the sample (Mezzetti et al. 2003), we can conclude that, in a first approximation, the number of reduced quinones per RC remains the same before and after the second flash. As we already demonstrated that after the first flash a single ubiquinone molecule was reduced per RC, it is possible to affirm that the spectrum recorded after the second flash corresponds to the double photo-induced reduction of a single ubiquinone molecule per RC.

The following spectrum, recorded between 77 and 102 ms after the second flash, shows further changes compared with the precedent one. The band at 1,470 cm^{-1} almost disappears, while the bands at 1,728 and 1,707 cm^{-1} further diminish in intensity. A much broader band centered at the same frequency replaces the positive peak at 1,479 cm^{-1}. In addition, a positive band characteristic of ubiquinol formation (Mezzetti et al. 2003) appears at 1,433 cm^{-1}, whereas the positive shoulder at 1,388 cm^{-1} is replaced by a positive band at the same frequency. Successive spectra recorded later after the second flashes do not show any further change with time. We can conclude that the spectrum recorded between 4 and 29 ms after the second flash contains contributions from an intermediate state formed rapidly after the absorption of the second photon, whereas the following spectra represent a state which is much stabler.

To investigate further the nature of the transient positive band at 1,707 cm^{-1} observed in the first spectrum recorded after the first and second flash, rapid-scan spectra were recorded at a lower temperature (268 K), to slow down the rate of the two Q_B reduction steps (Kleinfeld et al. 1984); consequently, observation of transient intermediate states in the reactions becomes easier. Because of the temperature effects on the kinetics of ubiquinol reoxidation by DAD^+/sodium ascorbate, a different mix of redox mediators (ferrocyanide and TMPD) was used. Although TMPD redox changes give rise to intense signals at 1,620 (−), 1,546 (+), 1,520 (−), 1,431 (+), and 1,382 (+) cm^{-1} (Breton et al 1992a), no signals arising from $TMPD^+$/TMPD are expected in the 1,650–1,750-cm^{-1} region. Therefore, peaks in this spectral region can be attributed to contributions from either the cofactors or from the protein. The rapid-scan spectra at 268 K recorded between 4 and 29 ms after the first and second flashes are shown in Fig. 3. After both the

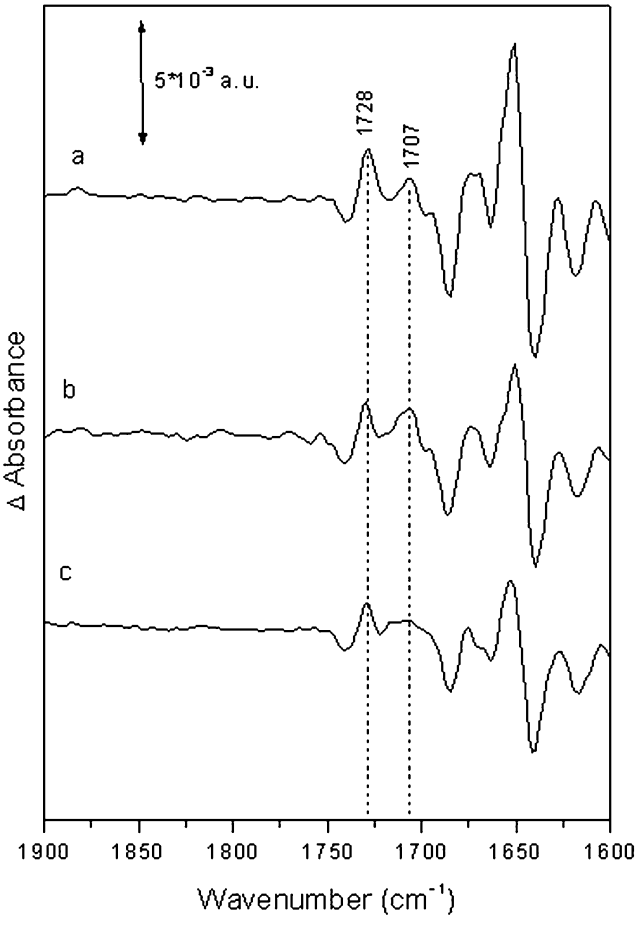

Fig. 3 Rapid-scan spectra obtained on isolated RCs from *Rb. sphaeroides*. $T = 268$ K. Redox mediators ferrocyanide (250 mM) and tetramethyl-*p*-phenylenediamine (50 mM). *a* Spectrum recorded between 4 and 29 ms after the first flash. *b* Spectrum recorded between 4 and 29 ms after the second flash. *c* Spectrum recorded between 77 and 102 ms after the second flash

[1]The energy of the laser flash (approximately 20 mJ) is, however, not chosen too high in order to avoid nonlinear, biphotonic photophysical effects.

first and the second flash, the 1,707-cm^{-1} band shows an increased amplitude compared with that in the spectrum recorded at 281 K, especially after the second flash (where its intensity is comparable to that of the 1,728-cm^{-1} band).

Rapid-scan experiments under and after continuous illumination

To investigate the dynamics of ubiquinol formation and of ubiquinone/ubiquinol exchange at the Q_B site, time-resolved rapid-scan experiments under and after continuous illumination were performed in the presence of an excess of external neutral ubiquinone. Figure 4A shows the spectra obtained at different times after the onset of continuous illumination. A strong evolution in the spectral shape is observed, reflecting a complex sequence of photochemical and redox reactions. The evolution in the concentration of the different states involved in the sequence can be easily followed through characteristic IR bands, as shown in Fig. 6a. A detailed interpretation of the mechanisms of chemical reactions is provided in the "Discussion". In the following, a simple description of the observed evolution in the concentration of the Q_A, Q_A^-, Q_B, Q_B^-, QH_2 states is given.

The negative band at 1,265 cm^{-1} is given by a C–O–C vibration of a methoxy group of neutral ubiquinone (Breton et al. 1995; Brudler et al. 1995) and its intensity is therefore a probe to assess the number of ubiquinone molecules (including Q_A and Q_B) per RC which undergo reduction (Mezzetti et al. 2003). As can be seen, at early times the intensity of the band increases, showing that several ubiquinone molecules per RC are undergoing reduction. In fact, once one Q_BH_2 molecule is formed, it leaves the RC and it is replaced by a new ubiquinone molecule from the surrounding solution (McPherson et al. 1989). Because of this exchange, the process of photo-induced reduction of ubiquinone to ubiquinol can continue. At later times (approximately 3,000 ms after the onset of illumination) a point is reached beyond which the bleaching of the 1,265-cm^{-1} band stops (Figs. 4A, 6A), showing that no further ubiquinone molecules are reduced. At early times, a Q_B^- state is also formed, as can be seen through the characteristic positive C–O^- stretching band at 1,479 cm^{-1} (Breton and Nabedryk 1996). The concentration of the Q_B^- state rises until approximately 1 s after the onset of the light, and then decreases, approaching a constant value for $t > 2.5$ s (Figs. 4A, 6A). As will be discussed in more detail in the "Discussion", the kinetics data for this band could not be fitted using simple exponentials, suggesting that several chemical equilibria involving Q_B are present. Ubiquinol formation can be followed through characteristic bands at 1,388 and 1,433 cm^{-1} (Mezzetti et al. 2003; Hellwig et al. 1999), which grow during the whole illumination period with a time constant of around 1 s. The concentration of the Q_A^- state can be followed

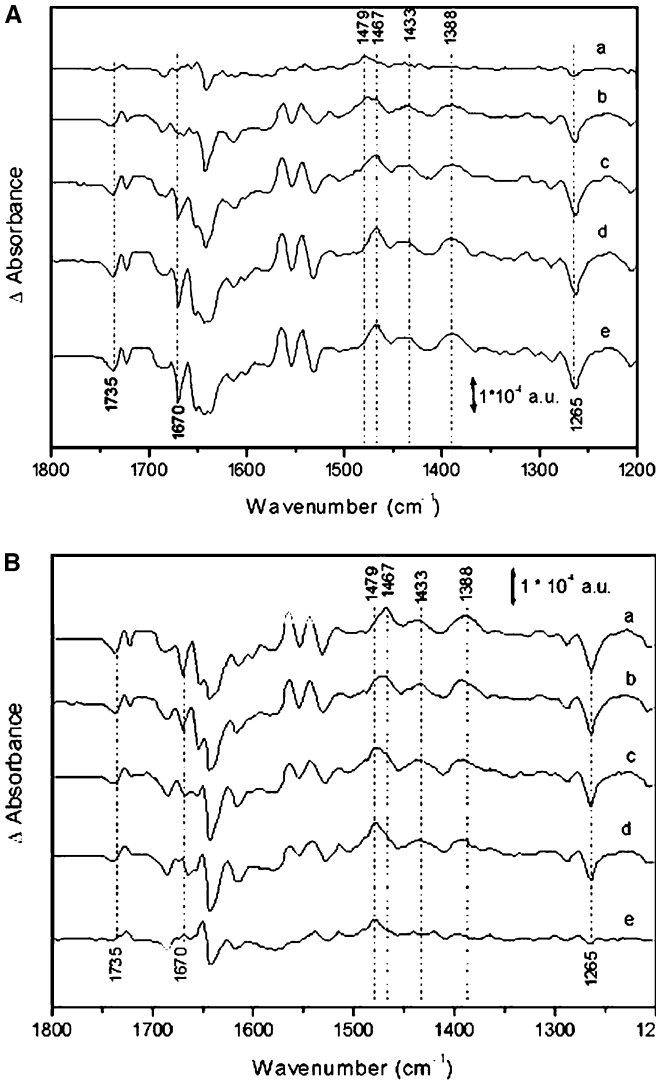

Fig. 4 **A**Rapid-scan Fourier transform (*FT*) IR spectra recorded under photoaccumulation conditions after the onset of continuous illumination. *a* Spectrum recorded between 0 and 405 ms. *b* Spectrum recorded between 868 and 1,273 ms. *c* Spectrum recorded between 1,736 and 2,141 ms. *d* Spectrum recorded between 3,038 and 3,443 ms. *e* Spectrum recorded between 3,906 and 4,311 ms. **B** Rapid-scan FTIR spectra recorded after switching off the continuous illumination. *a* Spectrum recorded between 0 and 405 ms. *b* Spectrum recorded between 434 and 839 ms. *c* Spectrum recorded between 1,736 and 2,141 ms. *d* Spectrum recorded between 6,510 and 7,349 ms. *e* Spectrum recorded between 24,353 and 28,664 ms

through characteristic bands at 1,467 (+), 1,670 (−) and 1,735 (−) cm^{-1} (Breton et al. 1991b). It increases with the illumination period with a time constant of around 2 s.

The spectra recorded after illumination again show a strong time evolution (Figs. 4B, 6B). At early times after switching off the lamp, the spectra are essentially identical to the spectra recorded at the end of the illumination period, with bands characteristic of QH_2 (1,433 (+) and 1,388 (+) cm^{-1}) and Q_A^- (1,467 (+), 1,670 (−) and 1,735 (−) cm^{-1}). The strong negative band at 1,265 cm^{-1} is, as during the illumination period, a probe

to assess the overall number of ubiquinone molecules that have undergone reaction.[2] The intensity of this band decreases, showing the reoxidation of the reduced ubiquinone (in any form) to oxidized ubiquinone. Also the concentrations of QH_2 and of Q_A^- decrease exponentially, but with different kinetics (approximately 5 s for QH_2 and approximately 1 s for Q_A). The concentration of Q_B^- has a "rise-and-fall" behavior. It increases with a time constant of approximately 1 s and decays with a time constant of approximately 20 s. The rapid, increasing phase is synchronous with the decay rate of Q_A^- and therefore reflects electron transfer from Q_A^- to Q_B. As the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer has a much higher intrinsic rate constant (Okamura et al. 2000), in this situation the reaction is clearly rate-limited by another process, faster than reoxidation of QH_2 ($\tau \sim 5$ s). A detailed kinetics model (see the "Discussion") strongly suggests its identification with a ubiquinone exchange equilibrium between micelles (Shinkarev and Wraight 1997). Furthermore, the time evolution of the spectra after illumination clearly shows that QH_2 (positive bands at 1,388 and 1,433 cm^{-1}) is reoxidized by DAD^+/sodium ascorbate to Q more rapidly than Q_B^- to Q_B (positive band at 1,479 cm^{-1}).

Discussion

The goal of this study was the investigation of the molecular mechanisms of ubiquinol formation in isolated RCs from *Rb. sphaeroides*. To this aim, we used time-resolved FTIR difference spectroscopy, which allowed us to follow very precisely the chemical evolution of the whole system because specific chemical events such as amino acid side chain protonation/deprotonation and oxidation/reduction or protonation/deprotonation of cofactors can, in principle, be monitored directly and simultaneously through specific IR marker bands. In addition, as molecular vibrations are determined by intramolecular and intermolecular interactions, time-resolved FTIR difference spectroscopy also provides some information on the structural evolution of the system with time (protein conformational changes, formation of hydrogen bonds, and internal displacement of cofactors). The rapid-scan FTIR technique was preferred to step-scan FTIR difference spectroscopy for practical reasons. The reduction of ubiquinone to ubiquinol is a two-electron reaction, involving two consecutive monoelectronic photooxidation steps of the primary donor P to P^+. This requires fast reduction of P^+ by an external donor, preventing the possibility of using a relatively fast (approximately 1 s) recombination reaction like $P^+Q_B^- \rightarrow PQ_B$ to reestablish the starting conditions. The slow reversibility of the reaction in the presence of external donors, however, hampers cycle averaging necessary to improve the signal-to-noise ratio in the step-scan mode, making step-scan FTIR experiments virtually unfeasible.

In the following, the different experimental observations presented in the "Results" section will be discussed.

Ubiquinol vibrations

In photosynthetic membranes bands at 1,491, 1,470, 1,433, 1,388 and 1,375 cm^{-1} were identified and assigned to ubiquinol-10 vibrations (Mezzetti et al. 2003). Comparable patterns have been observed for ubiquinol-10 bound to the cytochrome bc_1 complex from *Rb. capsulatus* (Baymann et al. 1999), and for ubiquinol-2 and ubiquinol-3 in water solution and in various protein environments (Zhang et al. 2002; Hellwig et al. 1999, and references therein). In isolated RCs (this work) the pattern of the bands found at long times after two saturating flashes is partially different, with broad peaks centered at 1,479, 1,433, and 1,388 cm^{-1}. As the Q_B^-/Q_B spectrum is characterized by a positive band at 1,479 cm^{-1} as the most prominent feature, it appears that Q_B^- contributions are present in the spectrum, i.e., the experimental spectra recorded after two flashes reflect ubiquinol formation but are contaminated by contributions owing to Q_B^- formation in a minor fraction of RCs. Indeed, if an appropriate subtraction to eliminate these Q_B^-/Q_B contributions is performed, a calculated difference spectrum is obtained, which shows the five bands at 1,491, 1,470, 1,433, 1,388, and 1,375 cm^{-1} observed for ubiquinol in photosynthetic membranes (Fig. 5). This evidence supports the interpretation of the spectrum observed in isolated RCs at long times after two flashes as a mixture of the QH_2/Q spectrum (77%) and the Q_B^-/Q_B spectrum (23%).

The reason for the formation of Q_B^- in a small fraction of the RCs after two flashes is not easy to understand. One possibility is that the first laser flash does not induce formation of a Q_B^- state in all RCs, so that some of them remain in the neutral Q_B state. When the second laser flash is fired, a QH_2 state is formed in all RCs already in a Q_B^- state, whereas in the remaining ones a Q_B^- state is formed. An estimate of the percentage of RCs where the first photo-induced electron transfer does not take place can be deduced from the intensity of the negative band at 1,265 cm^{-1}, which serves as a probe to assess the number of ubiquinone molecules reduced to any reduced form. Comparison of the intensity of this band in the two spectra recorded after the first and the second flash allows us to estimate, within the limitations imposed by the signal-to-noise ratio, that this percentage is in the 0–20% range. Most probably, however, the situation is more complicated and several other phenomena should also be taken into account. A point to take into account is the observation (see later) that reoxidation of Q_B^- by DAD^+/sodium ascorbate is slow compared with reoxidation of Q_BH_2. This could result in a "trapping" of some RCs in a Q_B^-

[2] With respect to the beginning of the illumination period.

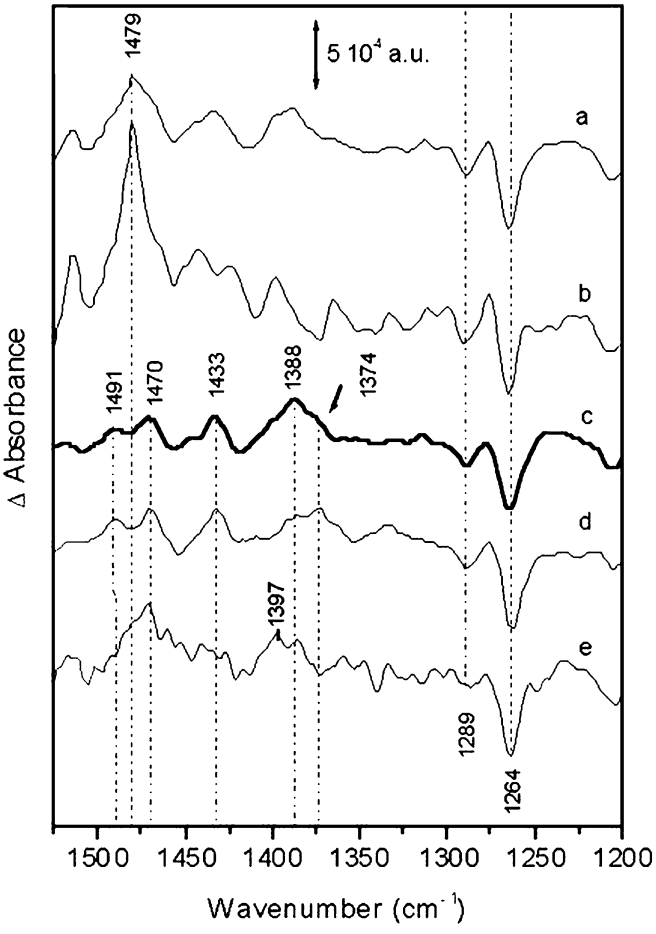

Fig. 5 Reconstruction of the QH_2 FTIR spectrum in the 1,525–1,200-cm^{-1} region. *a* Experimental spectrum obtained after two laser flashes, resulting from QH_2/Q (mainly) and Q_B^-/Q_B (partially) contributions. *b* Spectrum recorded after the first flash and reflecting only Q_B^-/Q_B contributions. *c* Spectrum calculated by subtracting the Q_B^-/Q_B contributions from spectrum a, so that it reflects only QH_2/Q contributions. The formula applied is spectrum c = spectrum a − (0.3 × spectrum b); the coefficient 0.3 was chosen to yield a resulting difference spectrum with QH_2 bands (1,491, 1,470, 1,433, 1,388, and 1,374 cm^{-1}) in agreement with those reported in the literature for QH_2 in the photosynthetic membrane (spectrum *d*, from Mezzetti et al. 2003). *e* Reconstruction of the putative (intermediate)/Q_B spectrum devoid of contributions from Q_B^-/Q_B. The formula applied is spectrum e = spectrum c of Fig. 2 − (0.3 × spectrum b)

state at the start of the averaging cycles. In addition, the presence of detergent (LDAO) micelles implies a quinone (and quinol) exchange equilibrium between them (Shinkarev and Wraight 1997; Shinkarev 1998; Agostiano et al. 1999), which further complicates the reaction system. Indeed, the kinetics analysis of time-resolved experiments under photoaccumulation conditions (see later) strongly suggests that such equilibria and their influence on light-induced ubiquinol formation have to be taken into account. It should also be noted that LDAO might entail some structural modifications at the Q_B site compared with RC in chromatophores (Trotta et al. 2002; Nagy et al. 1999).

The "ubiquinol-minus-ubiquinone" spectrum reconstructed by subtraction of the Q_B^- contribution shows an interesting feature, which is not related to ubiquinol vibrations. In fact, no bands are visible in the 1,720–1,750-cm^{-1} region (not shown), in sharp contrast with the spectra recorded upon ubiquinol formation in photosynthetic membranes, where a differential band was observed at 1,743 (-)/approximately 1,720 (+) cm^{-1} (Mezzetti et al. 2003). This differential band was tentatively assigned to a perturbation of the carbonyl modes of phospholipid head groups reflecting a different interaction of ubiquinol and ubiquinone with phospholipids. The absence of this feature in the ubiquinol-minus-ubiquinone spectrum reported here is in agreement with the proposed assignment, as isolated RC preparations do not contain phospholipids and the detergent used to solubilize the RCs (LDAO) does not have C=O groups.

Intermediate state after the second flash

As described before, the spectrum recorded between 4 and 29 ms after the second flash indicates the formation of an intermediate state. This first spectrum after the second flash already shows a band characteristic of QH_2 (1,388 cm^{-1}), which is fully formed in the later spectra. However, a positive peak at approximately 1,470 cm^{-1}, which is reproducibly observed in the first spectrum, becomes significantly weaker in the following spectra (Fig. 2). As already mentioned, a 1,470-cm^{-1} band has been assigned to ubiquinol vibrations (Mezzetti et al. 2003; Baymann et al. 1999) and identified also in the reconstructed "pure" ubiquinol-minus-ubiquinone difference spectrum described earlier. A possible interpretation to explain its increased intensity compared with the following spectra is that this band corresponds also to a Q_BH^- vibration. Indeed, FTIR spectra of electrochemically generated QH^- in solution showed a peak at 1,465 cm^{-1} (Port et al. 1995). Therefore, it appears that this spectrum does contain contributions from a state which is a precursor of the fully protonated form of double-reduced ubiquinone.

This interpretation is also supported by the observation that in the first spectrum after the second flash the band at 1,728 cm^{-1} (due to protonation of the side chain of Glu-L212) is still present, whereas it is almost absent in the following spectra (Fig. 2). This indicates that in the intermediate state the Glu-L212 side chain is still (at least partially) protonated. As Glu-L212 is believed to be the donor for the second proton to Q_BH^- to lead to the fully protonated ubiquinol (Paddock et al. 2003), the presence of the 1,728-cm^{-1} band strengthens the identification of Q_BH^- as the intermediate state.

The differences in the amide I and amide II regions observed in the comparison of the intermediate state spectrum (Fig. 2; not reported in Fig. 5) with the preceding and following spectra reflect two successive conformational changes probably related to the second

electron transfer to Q_B and to the release of Q_BH_2 from its binding pocket to the outside, respectively. A more detailed interpretation of these spectral changes is, however, not possible because the signal-to-noise ratio in these two regions is known to be poorer than in other spectral ranges, e.g., the 1,700–1,770-cm^{-1} region, where the side chains of Glu and Asp absorb.[3]

Based on the assumption that, after the second flash, QH_2 is formed in approximately 77% of the RCs whereas in 23% of the RCs a Q_B^- state is formed (see earlier), we calculated a putative FTIR spectrum of the intermediate devoid of Q_B^- contributions (Fig. 5, spectrum e). Although limited in the signal-to-noise ratio, this spectrum shows a positive peak at 1,470 cm^{-1} as its most prominent feature.

The 1,707-cm^{-1} transient band

Immediately after both the first and the second flashes a transient positive band is observed at 1,707 cm^{-1}. The region between approximately 1700 and approximately 1,770 cm^{-1} is the region where the carbonyl groups of the side chains of protonated Glu and Asp residues absorb (Barth 2000, and references therein). As both the first and the second reduction steps of Q_B are coupled to proton transfer reactions, a possible explanation is that this transient band arises from the transient protonation of one of the Asp and Glu amino acids known to be involved in the proton transfer process.[4] This interpretation is in agreement with a recent step-scan investigation carried out on *Rb. sphaeroides* RCs, which revealed that the first electron transfer reaction between Q_A and Q_B is associated with a transient signal at 1,707 cm^{-1} attributed to a transient protonation of a Glu or Asp side chain (Remy and Gerwert 2003). These authors also observed a transient signal at 1,751 cm^{-1}, which did not appear in the present measurements, possibly because of the limited time resolution. However, it should be kept in mind that in the 1,700–1,760-cm^{-1} region also ester carbonyls from bacteriochlorophylls and bacteriopheophytins absorb (Lutz and Mäntele 1991; Breton et al. 1997). Therefore, the 1,707-cm^{-1} band could, in principle, also arise from a transient electrochromic shift of one of these carbonyls as a consequence of the different charge distribution within the protein.[5]

To summarize, although the transient nature of the 1,707-cm^{-1} band and the comparison with previous works (Remy and Gerwert 2003; Hienerwadel et al. 1992) suggest that this band reflects transient protonation of a glutamic or aspartic residue, its attribution to a given residue is not possible. Detailed experiments of mutant RCs will be required to investigate in detail the protonation/deprotonation of amino acid side chains associated with the first and second reduction of Q_B.

Time evolution of spectra under and after photoaccumulation conditions

Rapid-scan FTIR measurements under and after photoaccumulation conditions allowed us to investigate several aspects related to ubiquinol formation and to ubiquinol/ubiquinone exchange at the Q_B site. First, a qualitative explanation of the time evolution of the spectra shall be given. During illumination, at early times, on average only one photon is absorbed per RC, so Q_B^- is the predominant state in the majority of the RCs. At increasing times, however, the percentage of RCs which have absorbed two photons increases, and this is reflected by the appearance of positive bands at 1,388 and 1,433 cm^{-1} characteristic of ubiquinol formation. At this stage the bleaching of the 1,265-cm^{-1} band also increases, showing that several ubiquinone molecules per RC are undergoing reduction. Eventually, a point is reached beyond which no further ubiquinone molecules are reduced. As a consequence, the Q_B binding pocket remains empty (or occupied by Q_BH_2) and the electron transfer reaction between Q_A and Q_B cannot take place, so accumulation of Q_A^- occurs in the majority of RCs.

After illumination, at early times after switching off the lamp, the situation is the same as found at the end of the illumination period, with all the exogenous quinones reduced to the quinol state and a Q_A^- state present in the majority of the RCs. In the following spectra, the bands characteristic of the Q_A^- state (1,467 (+), 1,670 (−), 1,735 (−) cm^{-1}) progressively disappear, while the 1,479-cm^{-1} band characteristic of Q_B^- appears with the same kinetics. This is due to a progressive refilling of the Q_B binding site of the RC by oxidized ubiquinone; as soon as a new ubiquinone has bound to the RC, it is reduced to Q_B^- by electron transfer from Q_A^-. QH_2 and Q_B^- are then reoxidized by DAD^+/sodium ascorbate.

A more quantitative analysis of the overall dynamics of the quinone acceptor complex (under and after illumination), however, shows that the situation is more

[3] Attempts to improve the signal-to-noise ratio by increasing the measuring time and by sample replacement were vain; in fact, the signal-to-noise ratio in these regions is 4–5 times worse than in other spectral regions. We underline that this is an intrinsic limitation of the rapid-scan FTIR difference spectroscopy technique which is able to provide time-resolved data only with a limited signal-to-noise ratio.

[4] A definite assignment of the band to a Glu or Asp protonated side chain could be given by a clear isotopic downshift of the band when comparing spectra recorded in H_2O and D_2O. Indeed, comparison of the rapid-scan FTIR difference spectra recorded in D_2O gave indications for a downshift of the 1,707-cm^{-1} band to 1,690 cm^{-1}. However, despite long signal averaging and the use of several samples, the signal-to-noise ratio attained in both H_2O and D_2O FTIR difference spectra did not allow us to calculate a H_2O-minus-D_2O double-difference spectrum of sufficient quality to allow an unambiguous assignment.

[5] Such an effect is observed in the photosynthetic RCs from *Rb. sphaeroides* upon formation of the Q_A^- state (Breton et al. 1997).

complicated than the simple picture just given, involving, beside the electron and proton transfer reactions, also the exchange of ubiquinone and ubiquinol between LDAO micelles. The time evolution of IR bands under and after continuous illumination (Figs. 6, 7), was simulated using a kinetics model which takes into account all the previously mentioned phenomena, leading to an overall system of 18 reactions (for details see the "Appendix"). To simulate the experimentally observed time courses of redox states it was essential to introduce a slow exchange of ubiquinone and ubiquinol molecules between pure LDAO micelles and RC-containing LDAO micelles, as already proposed by Shinkarev and Wraight (1997); see also Fig. 8. This exchange among micelles is the mechanism that can account for the early Q_A^- formation under continuous illumination, which otherwise would be expected to take place only after complete reduction of the pool of exogenous quinones. It also explains the slow (approximately 1 s) electron transfer between Q_A^- and Q_B observed after switching off the lamp. At the end of the illumination period the Q_B pocket is empty (or occupied by ubiquinol) and therefore the refilling of the Q_B pocket by an external ubiquinone molecule is rate-limiting for Q_A^- reoxidation. However, the intrinsic rate of Q_B pocket refilling, i.e., the ubiquinone exchange between the pocket and the detergent phase in the same LDAO micelle, is in the millisecond domain (Paddock et al. 1989), indicating that the Q_B pocket refilling is in turn limited by another process. This process cannot be just the reoxidation of QH_2 by external donors, as this reaction is too slow (approximately 5 s). In contrast, the observed kinetics behavior can be explained by the presence of a ubiquinone exchange equilibrium between micelles with a time constant of approximately 1 s (Shinkarev and Wraight 1997) limiting the refilling of the Q_B pocket and therefore of the electron transfer reaction between Q_A^- and Q_B.

An in-depth kinetics study of all the reactions and exchange processes would require variation of certain parameters of the system (for instance, measurements at different concentrations of exogenous ubiquinone, at different temperature, at different LDAO concentrations), and is beyond the scope of this work. Nevertheless, two conclusions can be drawn from the observations in this work. First of all, the kinetics model allows us to get an estimate of the exchange rate of ubiquinone and ubiquinol between pure LDAO micelles and RC-containing LDAO micelles. This exchange rate was found to be in the range 0.5–2 s, in good agreement with the value proposed by Shinkarev and Wraight (1997).[6] As a second point, the kinetics analysis of the spectra after illumination shows clearly that external donors reoxidize QH_2 faster than Q_B^-. This can be explained by a stabilizing effect of the Q_B pocket on the

[6]It should, however, be noted that the present measurements were carried out at 281 K, and not at 295 K as by Shinkarev and Wraight (1997).

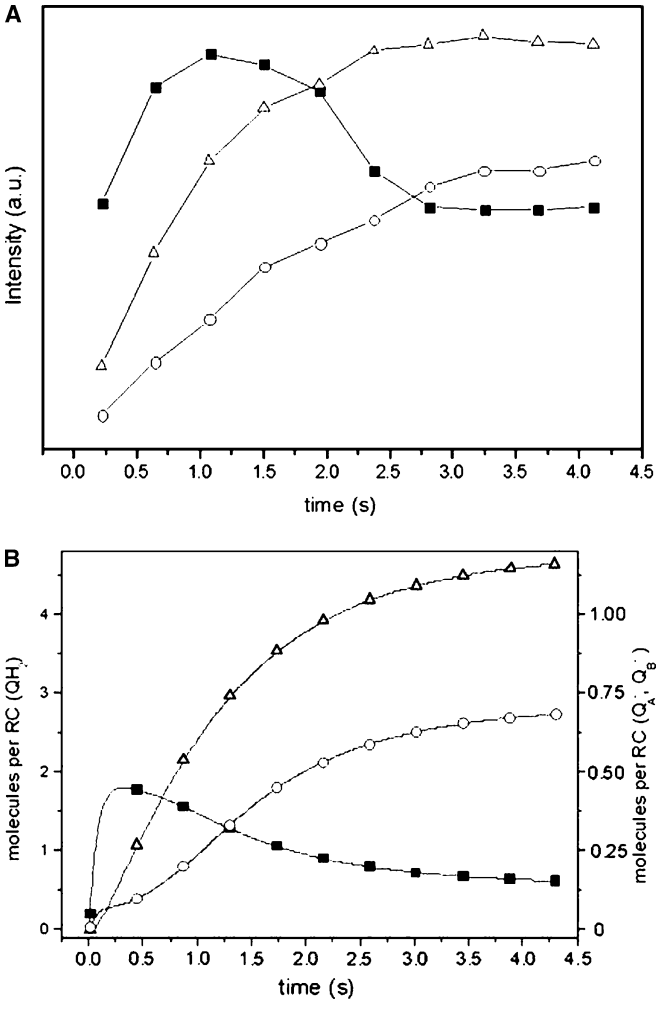

Fig. 6 **A** Kinetics evolution of IR bands under continuous illumination. *Circles* 1,735-cm^{-1} band, characteristic of Q_A^-. *Squares* 1,479-cm^{-1} band, characteristic of Q_B^-. *Triangles* 1,388-cm^{-1} band, characteristic of ubiquinol. Note that the relative amplitudes of the bands do not reflect the relative concentrations of species as the amplitude of the IR bands also depends on the oscillator strength of the particular molecular vibration (for instance, the extinction coefficient of the 1,388-cm^{-1} band for ubiquinol is much weaker than that of the 1,467-cm^{-1} band for Q_A^-). **B** Simulation, using the Gepasi 3.30 software, of the time evolution of the concentration of different species under continuous illumination. *Circles* Q_A^-; *squares* Q_B^-; *triangles* ubiquinol. The key parameters used in the simulation are as follows: Electron transfer between Q_A^- and Q_B: $k = 2{,}000\ s^{-1}$. This value is adapted from the estimated $k \approx 10^4\ s^{-1}$ (Okamura et al. 2000) at pH 7 and 298 K. The lower rate takes into account the effect of a lower temperature (281 versus 298 K). A similar temperature effect on the reaction rate has already been observed (Mezzetti et al. 2002). Electron transfer between Q_A^- and Q_B^-: $k = 200\ s^{-1}$. This value is adapted from the estimated $k \approx 10^4\ s^{-1}$ (Okamura et al. 2000) at pH 7 and 298 K. The lower rate takes into account the effect of a lower temperature (281 versus 298 K). Quinol exchange between the Q_B site and the detergent phase of the RC-containing micelle: $k = 200\ s^{-1}$. This value is in agreement with previous estimations (McPherson et al. 1989) and with the two-flash rapid-scan data of this paper. Quinone exchange between micelles: $k = 0.6\ s^{-1}$. This value is in agreement with the estimate of Shinkarev and Wraight (1997) of $\tau = 1.2$–1.5 s. Quinol exchange between micelles: $k = 0.6\ s^{-1}$. This value is in agreement with the estimate of Shinkarev and Wraight (1997) of $\tau = 1.2$–1.5 s

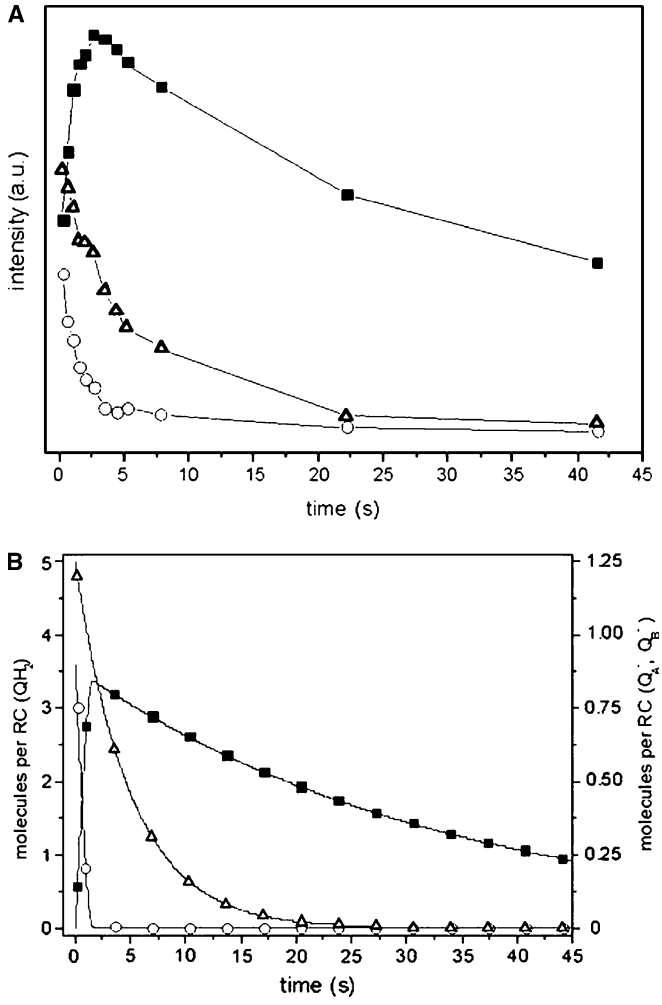

Fig. 7 **A** Kinetics evolution of IR bands after continuous illumination. *Circles* 1,735-cm^{-1} band, characteristic of Q_A^-. *Squares* 1,479-cm^{-1} band, characteristic of Q_B^-. *Triangles* 1,388-cm^{-1} band, characteristic of ubiquinol. Note that the relative amplitudes of the bands do not reflect the relative concentrations of species as the amplitude of the IR bands also depends on the oscillator strength of the particular molecular vibration considered (for instance, the 1,388-cm^{-1} band for ubiquinol is much weaker than the 1,467-cm^{-1} band for Q_A^-). **B** Simulation, using the Gepasi 3.30 software, of the time evolution of the concentration of different species after continuous illumination. *Circles* Q_A^-; *squares* Q_B^-; *triangles* ubiquinol. The key parameters used in the simulation are the same as those for Fig. 6B

Q_B^- state (which is a functionally important feature) and possibly also by some difficulty for external donors to come sufficiently close to Q_B^- for an efficient intermolecular electron transfer.

Molecular mechanism of ubiquinol formation and release

The results and the discussion presented here can be merged with the current understanding of Q_B function permitting us to draw a quite detailed scenario for the molecular mechanism of ubiquinol formation and its release from the Q_B pocket.

Okamura and coworkers (Okamura et al. 2000; Paddock et al. 2003, and references therein) have advanced a detailed molecular model for the two-step formation of ubiquinol in the RC, especially for the molecular events associated with the two light-induced electron transfer reactions between Q_A and Q_B, based on the combined use of molecular biology techniques and biophysical investigations (time-resolved UV–vis spectroscopy, X-ray diffraction, pH changes, and static FTIR difference spectroscopy). In the following, we will show how our time-resolved FTIR data strengthen the proposed model and where they can help to clarify some still unsolved issues.

According to the current model, both electron transfer reactions between Q_A and Q_B are associated with proton transfer from the cytoplasm to the Q_B pocket through well-defined pathways (Fig. 1). After the first flash, a proton is first taken up from the cytoplasm by two histidine side chains (His-H126 and HisH-128), located on the cytoplasmic protein surface. This transient protonation unfortunately cannot be confirmed by our rapid-scan FTIR data because of the limited time resolution. The proton transfer towards the interior of the protein then proceeds through intermediate protonation of the side chains of Asp-M17 and/or Asp-L210 and Asp-L213 (Fig. 1). The transient band at 1,707 cm^{-1} identified in our rapid-scan spectrum recorded immediately after (4–29 ms) the first flash could indeed reflect the transient protonation of one of these amino acid side chains, as also suggested by Remy and Gerwert (2003), who identified a similar transient band at the same wavenumber in step-scan measurements. It is worth noting that, in contrast to the cited step-scan work, in our rapid-scan FTIR spectrum the 1,707-cm^{-1} band is observed after complete reduction of P^+ (which has several intense IR bands in the 1,700–1,750-cm^{-1} spectral region). This allows us to exclude that this band arises from P^+ vibrations or that it reflects perturbations in amino acid side chains somehow related to P^+ formation (e.g., different intermolecular interaction with P and P^+); it also allows us to exclude any spectral perturbation (e.g., peak shift) arising from the presence of the intense P^+ bands.

In the model the proton is then transferred from the "intermediate" side chain to the Glu-L212 side chain. Indeed, the protonation of Glu-L212 can be observed in the two rapid-scan FTIR difference spectra recorded after the first flash through the 1,728-cm^{-1} marker band. In this case, the interpretation is not tentative but definitive, as it relies on combined steady-state FTIR difference spectroscopy/site-directed mutagenesis experiments (Nabedryk et al. 1995).

The rapid-scan FTIR spectra reveal even more information on the molecular events following the second photo-induced electron transfer reaction between Q_A and Q_B. As described in the "Introduction", this reaction is coupled to a proton transfer from the cyto-

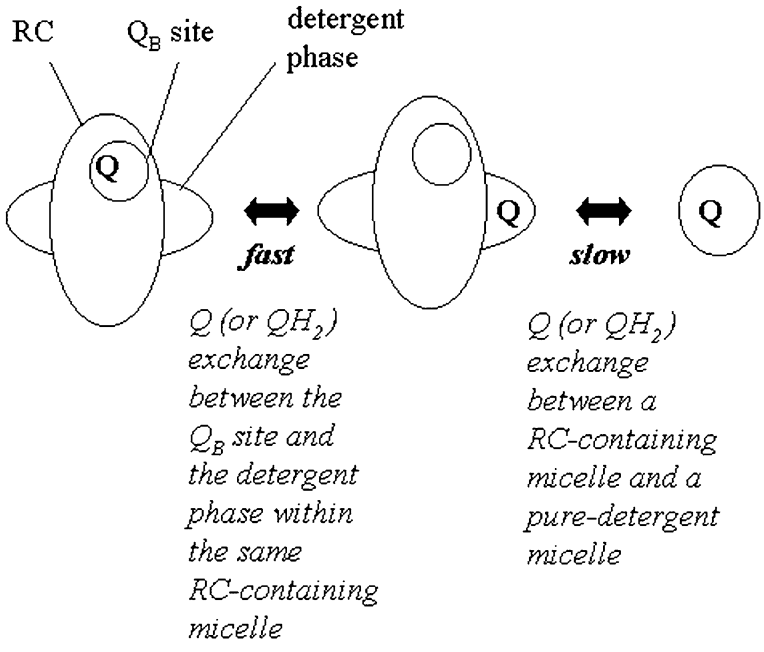

Fig. 8 Scheme of exchange of ubiquinone and ubiquinol between the Q_B site and the detergent phase within the same RC-detergent micelle and between different micelles

plasm to the Q_B pocket. The proton transfer pathway is, in its first part, the same as for the proton transfer coupled to the first electron transfer reaction: H^+ is taken up by His-H126 and His-H128 side chains, then transferred to the side chain of an amino acid located halfway between the entry point and the Q_B pocket (AspM17 and/or AspL210 and AspL213). Similarly to the situation after the first electron transfer reaction, the limited time resolution does not allow us to monitor the transient protonation of the His-H126 and His-H128 side chains. On the other hand, the presence of a 1,707-cm^{-1} positive band in the FTIR difference spectrum recorded between 4 and 29 ms after the second laser flash (spectrum corresponding to an "intermediate" state) most probably reflects the transient protonation of the amino acid located halfway between the entry point and the Q_B pocket. It is important to underline that in such an interpretation the frequency of the transient band is the same in the two spectra recorded between 4 and 29 ms after the first and the second flash, and therefore strongly suggests that this band represents two successive transient protonations (one after the first flash, the other after the second flash) of the side chain of the *same* amino acid. Rapid-scan FTIR investigations of mutants RCs could, in principle, allow us to confirm the proposed interpretation and to identify the corresponding amino acid.

According to the model, the proton is then further transferred to the Ser L223 side chain and ends up at Q_B concomitantly with the second electron to form the Q_BH^- state. Whereas the transient protonation of Ser-L223 seems to be too fast to give rise to any characteristic IR band in the spectrum of the intermediate state, the observed transient band at 1,470 cm^{-1} has been tentatively interpreted as a Q_BH^- vibration. This would mean that this spectrum shows the formation of the deprotonated ubiquinol. Furthermore, the presence in the same spectrum of a positive 1,728-cm^{-1} band, characteristic of the protonation of the Glu-L212 side chain, allows us to conclude that in the 4–29 ms time window after the second flash this amino acid side chain is still partially protonated, in agreement with the model where Glu-L212 is still protonated when Q_BH^- is formed (Glu L212 is the proton donor to Q_BH^- to yield Q_BH_2).

The spectrum recorded between 4 and 29 ms after the second flash also reflects several other molecular events, as it can be assessed by the comparison with the preceding rapid-scan FTIR spectra (Fig. 2, spectra b and c). In particular, a quite pronounced time evolution is observed in the amide I and amide II regions, probably related to some conformational rearrangement of the protein backbone upon the second photo-induced reduction step of Q_B. In the model, formation of the Q_BH_2 molecule from the deprotonated Q_BH^- occurs with a time constant of about 1 ms at 298 K (Okamura and Feher 1995), the necessary proton coming from the side chain of Glu-L212 which was protonated upon the first reduction of Q_B. Indeed, the spectrum recorded between 77 and 102 ms after the second flash shows neither the marker band from protonated Glu-L212 (positive band at 1,728 cm^{-1}) nor the putative band from Q_BH^- (positive band at 1,470 cm^{-1}). Therefore, this spectrum can be considered as representative of a fully protonated ubiquinol state. The limited time-resolution of the technique does not allow us to determine experimentally the kinetics of Q_BH^- disappearance and Glu-L212 side chain deprotonation from the temporal evolution of IR bands. An estimation of this kinetics is, however, possible by looking at the time resolution of

the technique and shows that under our experimental conditions (281 K; pH 8) this reaction takes place in the approximately 10-ms time range. If we take into account the effect of temperature on proton and electron transfer rates, such estimation is in good agreement with the current model.

The last step of the model is the release of the Q_BH_2 molecule formed from its pocket towards the membrane (in the natural RC) or the detergent phase (in the detergent-isolated RC). Our rapid-scan data do not show any further time evolution after the 77–102-ms time windows, so we can assume that any chemical or physicochemical event is already finished 77 ms after the second flash. On the one hand, this fact provides us with an estimate also of the kinetics of ubiquinol release (as for the $Q_BH^- \rightarrow Q_BH_2$ reaction, in the approximately 10-ms time range). On the other hand, it makes impossible the detailed investigation of the molecular mechanism of such a release (e.g., disappearance of specific protein bands arising from molecular interactions with Q_B/Q_BH_2 or conformational changes related to ubiquinol displacement). It is anyhow probable that the spectral evolution observed in the amide I and amide II regions (Fig. 2, cf. spectra c and d) is related to this release process rather than to the $Q_BH^- \rightarrow Q_BH_2$ reaction, as the distance between Glu-L212 and Q_BH^- is minimal and a conformational change seems unnecessary to allow this proton transfer.

Conclusions

In the present investigation rapid-scan FTIR spectroscopy allowed to follow the process of ubiquinol formation after two saturating flashes in isolated RCs. The rapid-scan spectrum immediately after the second laser flash revealed IR bands of an intermediate state prior to Q_BH_2 formation, most probably Q_BH^- with the donor of the second proton (Glu L212) still protonated. After both the first and the second laser flash indications for a transient protonation of the side chain of a glutamic or an aspartic amino acid residue on the proton pathway from the cytoplasmic surface towards the Q_B site were observed. Experiments under and after photoaccumulation conditions, on the other hand, allowed us to investigate the dynamics of ubiquinone/ubiquinol exchange at the Q_B site. The results showed that the exchange of quinone molecules between different LDAO micelles cannot be neglected and can represent the rate-limiting step for turnover of the enzyme.

The time-resolved FTIR experiments in this work did not rely on the use of internal back-reactions to reestablish the starting conditions demonstrating the possibility to follow double reduction and release of a quinone molecule from the RC protein by time-resolved rapid-scan FTIR difference spectroscopy.

A detailed investigation of the process of ubiquinol release will require improved time resolution of the technique. This might soon become possible with rapid-scan spectrometers capable of a time resolution of approximately 1 ms which are currently under development (Weinstock et al. 2004). In addition, the use of RC-containing proteoliposomes would allow problems related to Q/QH_2 exchange between LDAO micelles to be avoided. Such an approach would also allow investigation of the process in an environment closer to the photosynthetic bacterial membrane.

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Appendix: kinetics model for continuous light excitation

The overall dynamics of light-induced reactions under and after photoaccumulation conditions is the results of a series of reactions and physicochemical processes, which have been reported in the literature (Okamura et al. 2000; Shinkarev and Wraight 1997, and references therein). To describe the experimental observations it was found necessary to consider a model which consists of 12 different states connected by 18 different reactions.

As stated in the text, an essential feature of the kinetics model is the slow exchange of ubiquinone and ubiquinol molecules between different LDAO micelles (Shinkarev and Wraight 1997); see also Fig. 8. The introduction of this slow exchange in the model was necessary to account for some experimental evidence (early Q_A^- formation under continuous illumination, slow electron transfer between Q_A^- and Q_B after switching off the lamp).

The reactions taken into account are listed in the following, with reactions 12 and 13 describing ubiquinone/ubiquinol exchange between micelles. The symbols used are those defined in the text and in addition the following: QH_2 (Q), ubiquinol (ubiquinone) in the same detergent micelle as the RC; ZH_2 (Z), ubiquinol (ubiquinone) in a detergent micelle, which does not contain a RC.

1	$RCQ_A + Q = RCQ_AQ_B$	Binding equilibrium for Q in the Q_B binding site
2	$RCQ_AQ_B + light \rightarrow RCQ_A^{-}Q_B$	Light-induced reduction of Q_A in a RC containing Q_B in its oxidized state
3	$RCQ_AQ_B^{-} + light \rightarrow RCQ_A^{-}Q_B^{-}$	Light-induced reduction of Q_A in a RC already in a Q_B^{-} state
4	$RCQ_AQ_BH_2 = RCQ_A + QH_2$	Dissociation of ubiquinol from the RC
5	$RCQ_A + light \rightarrow RCQ_A^{-}$	Light-induced reduction of Q_A in a RC with an empty Q_B site
6	$QH_2 \rightarrow Q$	Reoxidation of ubiquinol in a RC-containing detergent micelle by external acceptors
7	$RCQ_A^{-} + Q = RCQ_A^{-}Q_B$	Binding equilibrium for Q in a RC with Q_A in its reduced state
8	$RQ_AQ_B^{-} \rightarrow RQ_AQ_B$	Reoxidation of Q_B^{-} by external acceptors
9	$RCQ_AQ_BH_2 + light \rightarrow RQ_A^{-}Q_BH_2$	Light-induced reduction of Q_A in a RC containing an ubiquinol bound to the Q_B site
10	$RCQ_A^{-}Q_BH_2 = QH_2 + RCQ_A^{-}$	Dissociation of ubiquinol from a RC with Q_A in its reduced state
11	$RQ_AQ_BH_2 \rightarrow RQ_AQ_B$	Reoxidation of ubiquinol bound to a RC by external acceptors
12	$Z = Q$	Ubiquinone exchange between RC-containing micelles and pure detergent micelles
13	$ZH_2 = QH_2$	Ubiquinol exchange between RC-containing micelles and pure detergent micelles
14	$ZH_2 \rightarrow Z$	Ubiquinol reoxidation in pure detergent micelles
15	$RCQ_A^{-}Q_B = RCQ_AQ_B^{-}$	Electron transfer reaction between Q_A^{-} and Q_B
16	$RCQ_A^{-}Q_B^{-} = RCQ_A^{-}Q_BH^{-}$	Electron transfer reaction between Q_A^{-} and Q_B^{-}

Line missing

The kinetics evolution of IR bands characteristic of Q_A^{-}, Q_B^{-}, and QH_2 has been simulated with good accuracy (Figs. 6, 7) using rate constants and equilibrium constants from the literature (Okamura et al. 2000; McPherson et al. 1989; Shinkarev and Wraight 1997; Sebban et al. 1995 and references therein).[7] The model was found to be not very sensitive to variations in the parameter values concerning chemical reactions and substrate binding equilibria. In contrast, the ubiquinone/ubiquinol exchange rate among pure detergent micelles and RC-containing micelles turned out to be a critical parameter in shaping the kinetics profiles of transient concentrations of the chemical species involved. Indeed, these transient concentrations are very sensitive to even relatively small variations in the kinetics constants for this exchange. This permitted the rate of this exchange to be estimated to 0.5–2 s.

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[7]The bands for each of these species do not allow us to discriminate between different states, so , for instance, the 1,467 (+)-cm^{-1} band is characteristic of Q_A^{-} regardless of its specific state, i.e., it is characteristic of the sum of the RCQ_A^{-}, $RCQ_A^{-}Q_BH_2$, $RCQ_A^{-}Q_B^{-}$, $RCQ_A^{-}Q_B$ state. Similarly, bands at 1,388 (+) and 1,433 (+)-cm^{-1} are a probe to assess the concentration of ubiquinol in any of its forms (ZH_2, QH_2, $RCQ_AQ_BH_2$ and $RCQ_A^{-}Q_BH_2$).

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