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Probing the secondary quinone (Q_B) environment in photosynthetic bacterial reaction centers by light-induced FTIR difference spectroscopy

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The photoreduction of the secondary electron acceptor, Q_B, has been characterized by light-induced Fourier transform infrared difference spectroscopy of *Rb. sphaeroides* and *Rp. viridis* reaction centers. The reaction centers were supplemented with ubiquinone (UQ_{10} or UQ_0). The Q_B-state was generated either by continuous illumination at very low intensity or by single flash in the presence of redox compounds which rapidly reduce the photooxidized primary electron donor P^+. This approach yields spectra free from P and P^+ contributions making possible the study of the microenvironment of Q_B and Q_B^-. Assignments are proposed for the C...O vibration of Q_B^- and tentatively for the C=O and C=C vibrations of Q_B.

Fourier transform infrared spectroscopy; Bacterial reaction center; Photosynthesis; Primary quinone; Secondary quinone; Ubiquinone

1. INTRODUCTION

In purple photosynthetic bacteria, the light-induced electron transfer and associated proton uptake occur in a transmembrane protein called the reaction center (RC). Following photoexcitation, the electron is transferred from the primary donor P, a dimer of bacteriochlorophyll, to the primary quinone acceptor, Q_A, and subsequently to the secondary quinone acceptor Q_B. The 2 quinones display very distinct properties, Q_A being a tightly bound one-electron acceptor, while Q_B is loosely bound, can accept 2 electrons, and serves as a mobile proton carrier [1]. While Q_A is a menaquinone in *Rp. viridis* and a ubiquinone (UQ) in *Rb. sphaeroides*, Q_B is UQ in both species. Although the recent elucidation of the three-dimensional structure of the RC of *Rp. viridis* [2] and *Rb. sphaeroides* [3] has provided essential information on the amino acid residues which make up the binding sites of Q_A and Q_B, little is known about the electronic structure of these acceptors and the structural modifications of both the protein and the quinones concomitant with the charge stabilization process.

In previous reports [4–7], it has been demonstrated that light-induced Fourier transform infrared (FTIR) difference spectroscopy has the sensitivity to monitor the molecular changes that occur at the level of individual chemical groups of the cofactor and of the protein following charge stabilization on quinones. More specifically, we have recently reported the FTIR difference spectrum of the Q_A^- state generated in RCs and chromatophore membranes by light-induced charge separation in the presence of chemical reductants and mediators which rapidly reduce P^+ [7]. In this letter we extend this approach to the study of Q_B and report on the first FTIR characterization of the photoreduction of Q_B in RCs of *Rb. sphaeroides* and *Rp. viridis*.

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Abbreviations: FTIR, Fourier transform infrared spectroscopy; Q_A (Q_B), primary (secondary) quinone electron acceptor; P, primary electron donor; RC, reaction center; UQ, ubiquinone; DAD, diaminodurene.

2. MATERIALS AND METHODS

The preparation of the RC films was essentially as described in [7] except that UQ (Sigma) solubilized in hexane (UQ_{10}) or in water (UQ_0) was added in 5-fold molar excess to the RCs. After partial drying under argon, the RCs were covered with a solution containing Na ascorbate (10 mM) and diaminodurene (DAD, 20 mM) in Tris-HCl (pH 8, 40 mM) buffer.

Cyclic light-induced FTIR measurements were performed as previously reported [7]. For flash excitation, an Nd YAG laser pulse (7 ns, 530 nm) was used.

3. RESULTS

3.1. Rb. sphaeroides *reaction centers*

Steady-state illumination of isolated RCs supplemented with an excess of UQ_{10} in the presence of DAD and Na ascorbate generates the light-minus-dark FTIR spectrum shown in Fig. 1a. This spectrum was obtained at a light intensity about 10 times lower than that allowing the saturation of the Q_A^- signal. Moreover, all the bands in this spectrum decay with the same half-time of ≈ 90 s, suggesting that only one type of reaction occurs. These observations indicate the formation of an extremely stable state as it is well established for Q_B^-

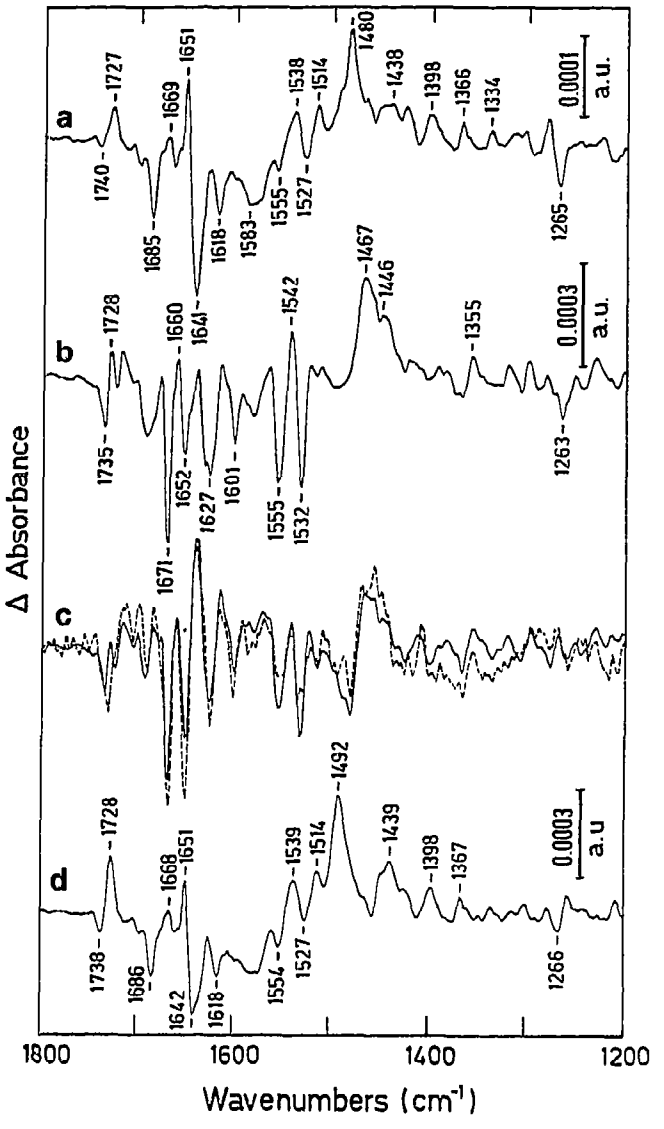

Fig. 1. Light-minus-dark FTIR Q^-/Q difference spectra at 10°C of *Rb. sphaeroides* RCs supplemented with a 5-fold molar excess of UQ_{10} in the presence of DAD and Na ascorbate; $\lambda > 715$ nm. a: Q_B^-/Q_B spectrum, without *o*-phenanthroline. b: Q_A^-/Q_A spectrum, with *o*-phenanthroline. c: Comparison of double difference $Q_A^-Q_B/Q_AQ_B^-$ spectra, (——) calculated from spectra 1a and 1b, or (– – –) measured directly by time-resolved FTIR difference spectroscopy [5,6]. d: Same as 1a except for UQ_0 instead of UQ_{10}. For all FTIR spectra, the resolution was 4 cm^{-1} and each peak frequency is given at ± 1 cm^{-1} owing to averaging on several samples.

[8]. Further evidence for Q_B^- formation is demonstrated by the following considerations. First, the spectrum in Fig. 1a is very different from that shown in Fig. 1b which was obtained on an identical sample but for the presence of 5 mM *o*-phenanthroline (an inhibitor of Q_A to Q_B electron transfer) and which has been previously assigned to Q_A^-/Q_A [7]. Second, a spectrum (not shown) almost identical to that of Fig. 1a is also obtained upon laser flash excitation. Third, a difference spectrum (Fig. 1c, continuous line) calculated from the spectra shown in Fig. 1b and 1a compares well

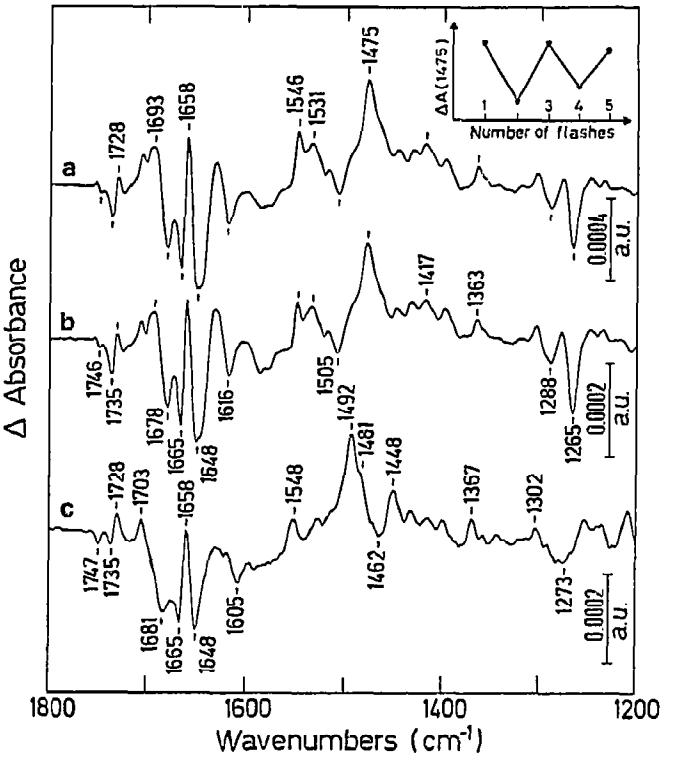

Fig. 2. Light-minus-dark FTIR Q_B^-/Q_B difference spectra of *Rp. viridis* RCs supplemented with UQ_{10} in the presence of DAD and Na ascorbate. a: excitation with a 7 ns laser flash at 530 nm. *Insert*: Amplitude of the 1475 cm^{-1} band (relative to the through at 1505 cm^{-1}) versus the number of flashes. b: excitation with steady-state light ($\lambda > 715$ nm). c: same as 2b, except for UQ_0 instead of UQ_{10}.

with that (Fig. 1c, dashed line) characterizing the transition from the state $Q_A^-Q_B$ to the state $Q_AQ_B^-$ as measured by a kinetic FTIR technique in the absence of external redox compounds [5,6]. In addition, upon decreasing the light intensity, the amplitude of all the main bands in the spectrum of Fig. 1a titrates linearly over at least a decade of intensities (the noise level is less than 10^{-5} OD). Finally, when the intensity of the continuous illumination is increased above the linear regime in which spectrum 1a was recorded, the FTIR difference spectrum becomes strongly modified, exhibiting additional contributions from both $Q_A^-Q_A$ and from a new state which we take to represent, at least in part, the state QH_2/Q_B (J.B., unpublished). On these bases, the spectrum shown in Fig. 1a can be confidently assigned to the photoreduction of Q_B and hereafter is referred to as the $Q_B^-Q_B$ spectrum.

When the RCs are supplemented with UQ_0 instead of UQ_{10}, the spectrum shown in Fig. 1d is obtained. While this spectrum is strikingly similar to that shown in Fig. 1a, notably in terms of the frequencies of most of the bands, it is also characterized by a 12 cm^{-1} upshift of the 1480 cm^{-1} positive band.

3.2. Rp. viridis *reaction centers*

When RCs of *Rp. viridis* supplemented with UQ_{10} are

excited by a saturating 7 ns flash in the presence of DAD and Na ascorbate, the spectrum depicted in Fig. 2a is obtained. All bands decay with a half time of ~60 s. The spectrum recorded under low intensity continuous illumination of the same sample is shown in Fig. 2b for comparison. The overall analogy with the Q_B^-/Q_B spectrum of *Rb. sphaeroides* suggests that these spectra can be assigned to the Q_B to Q_B^- transition. This is further demonstrated by the observation that, upon excitation with a sequence of saturating flashes separated by 1 s, the shape of this spectrum displays clear oscillations with a periodicity of 2, exhibiting very strong similarities with the spectrum shown in Fig. 2a on odd-numbered flashes and distinctly different features on even-numbered flashes. The amplitude of the 1475 cm^{-1} band, measured as a function of the number of flashes in the sequence, is depicted in the insert of Fig. 2a. Finally, the absence of contribution from changes of redox state of the bound tetraheme cytochrome has been checked by comparison with light-induced $Cyt^+ Q_A^-/CytQ_A$ spectra (E.N. and J.B., unpublished).

When the RCs are supplemented with UQ_0 instead of UQ_{10}, the spectrum shown in Fig. 2c is obtained. While for *Rb. sphaeroides* RCs, the main difference between the Q_B^-/Q_B spectra obtained with UQ_{10} (Fig. 1a) and UQ_0 (Fig. 1d) is the 12 cm^{-1} shift of the 1480 cm^{-1} anion band, many additional differences are observed between the Q_B^-/Q_B spectra of UQ_{10} (Fig. 2b) and UQ_0 (Fig. 2c) in *Rp. viridis* RCs. These differences are noticed in regions where absorption of the protein (~1540 cm^{-1}) and of the quinone (~1615 cm^{-1} and 1270 cm^{-1}, see section 4) occurs. This very different property of the two RCs suggests that the Q_B binding niche in *Rb. sphaeroides* imposes greater constraints on the quinone ring than that of *Rp. viridis*. In addition, the observation that a difference in the nature of the substituent of the quinone used to reconstitute the Q_B site brings significant changes in the spectra (Figs 1a,d,2) provides strong support to our contention that these spectra are indeed characteristic of the photoreduction of Q_B.

4. DISCUSSION

Recently, the interest in characterizing the environment and bonding pattern of the RC quinones in their neutral and anionic states has triggered investigation by several FTIR spectroscopy approaches: replacement with modified or isotopically-labelled quinones [9]; comparison of $P^+Q_A^-/PQ_A$ and $P^+Q_B^-/PQ_B$ spectra either by steady-state [9-11] or kinetic [5,6] techniques; and spectroelectrochemistry of quinones both in vitro [12] and in situ [13]. The FTIR characterization of photochemically-generated states containing pure contributions from Q_A^-/Q_A in situ has been recently reported for Photosystem II [4] and bacterial RCs [7,14]. In the present study, we report that addition of reductants and mediators, which rapidly reduce P^+, makes possible the photochemical generation of pure Q_B^-/Q_B FTIR difference spectra on isolated bacterial RCs.

The Q_B^-/Q_B spectrum obtained photochemically with *Rb. sphaeroides* RCs (Fig. 1a) shows a number of features which are distinct from those seen in the Q_A^-/Q_A spectrum (Fig. 1b) and that we assign to the specific environment and bonding interactions of the same chemical species (UQ_{10}) in the Q_A and Q_B sites. The large difference in band positions observed between these 2 spectra shows that cross-contamination between the Q_B^-/Q_B and Q_A^-/Q_A spectra can at most be very small. An identical conclusion can be drawn from a comparison of the Q_B^-/Q_B (Fig. 2) and Q_A^-/Q_A [7] spectra of *Rp. viridis*.

Taken together with the in vitro IR difference spectra of quinone anion-minus-neutral of Bauscher et al. [12], the present data allow the position of the major anion band of UQ around 1460–1500 cm^{-1} to be compared for different in vivo and in vitro conditions. This band has been assigned primarily to the C...O stretch in the semiquinone anion (see [12] and references therein). The most straightforward interpretation of the large downshift of this band for UQ_{10} acting as Q_B (1480 cm^{-1}) or Q_A (1467 cm^{-1}) in *Rb. sphaeroides* RCs, involves a tighter H-bonding of the oxygen atoms of Q_A^- than those of Q_B^-, in agreement with results from ENDOR spectroscopy [15]. The position of this band in the Q_B^-/Q_B spectrum of *Rp. viridis* (1475 cm^{-1}) is quite close to that found in *Rb. sphaeroides* (1480 cm^{-1}), an observation which suggests a similar conformation of the Q_B^- C...O bond in both species. Furthermore, the replacement of the native UQ* by UQ_0 in the Q_B site leads to an upshift of this band to 1492 cm^{-1} in both RCs.

The frequency of the C...O stretch of the UQ_0 semiquinone anion in vitro has been found to be 1500 cm^{-1} in deuterated acetonitrile and 1490 cm^{-1} in deuterated methanol [12]. The frequency of the C...O stretch of the UQ_0 semiquinone anion in vivo is thus very close to that found in methanol. This observation agrees well with the established polar character of the Q_B pocket and the H-bonding of the 2 quinonic oxygens with amino acid residues [2,3]. Although the large downshift of the frequency of the C...O stretch of the semiquinone anion in vivo when UQ_0 is replaced by UQ_{10} could suggest a tighter H-bonding of UQ_{10} in the Q_B site, it appears more likely that the presence of the isoprenoid chain influences the C...O bond strength.

*The Q_B site is occupied by UQ_{10} in *Rb. sphaeroides* and by UQ_9 in *Rp. viridis* RCs [2,3]. However the influence of the isoprenoid chain length on the IR vibrations cannot extent much beyond the first isoprenoid unit as indicated by the close identity of the frequencies of the IR bands in UQ_6 and UQ_{10} (not shown).

A more detailed analysis of the position of the 1460–1500 cm^{-1} semiquinone anion band would require spectroelectrochemistry of UQ_{10} in various solvents and a better knowledge of the other factors, such as the distortion from a planar conformation of the quinone ring and C=O groups or the influence of the polarity of the microenvironment, which contribute to the energy of this vibration.

The proximity of the frequency found for the C...O band of Q_B^- in *Rb. sphaeroides* and in *Rp. viridis* together with the comparable bonding pattern and polarity of microenvironment of Q_B demonstrated by X-ray cristallography [2,3], suggest that other vibrations of the Q_B molecule should appear at about the same position in the FTIR difference spectra of the 2 species. Three negative bands at 1265 cm^{-1}, 1616–1618 cm^{-1} and around 1645 cm^{-1} are the major common bands in these spectra (Fig. 1a and 2a). The 1265 cm^{-1} band, which corresponds to that seen at 1263 cm^{-1} in the absorption spectrum of UQ_{10} (J.B., D.T. and E.N., unpublished), is tentatively assigned on the basis of normal mode calculations to a combination of C–C stretch of the quinone ring and of C–O modes [16]. The band at 1616 cm^{-1} in *Rp. viridis* (Fig. 2a) and at 1618 cm^{-1} in *Rb. sphaeroides* (Fig. 1a) appears to be due to the C=C vibration of the quinone ring, which is located near 1610 cm^{-1} in UQ_{10} (see [12] and references therein). Furthermore, we propose that a negative band located at about 1640 cm^{-1} corresponds to the C=O vibration of the neutral Q_B in both RCs. The pronounced difference around 1650 cm^{-1} between the Q_B^-/Q_B spectra of RCs of *Rb. sphaeroides* (positive at 1651 cm^{-1}) and *Rp. viridis* (negative at 1648 cm^{-1}) can be explained by a partially overlapping amide I absorbance change. Comparable sign and position of the 1640 cm^{-1} and 1650 cm^{-1} bands are also observed for the Q_B^-/Q_B spectra of the 2 types of RCs after reconstitution with UQ_0 (Figs 1d and 2c). However, in the absence of further data on RCs reconstituted with isotopically-labelled quinones, these assignments should still be considered as tentative.

Besides the bands discussed above and assigned to vibrations of the quinone, there is only an overall analogy between the Q_B^-/Q_B spectra of *Rp. viridis* and *Rb. sphaeroides*. Although the absorption changes observed in the absorption regions of the C=O of protonated carboxylic amino acid residues (1770–1700 cm^{-1}), of the amide I protein (1690–1630 cm^{-1}), and of the amide II (around 1550 cm^{-1}) display some resemblance, they also show significant differences in the position and relative amplitude of the bands, notably in the frequency range above about 1680 cm^{-1} and around 1550 cm^{-1} where the anion-minus-neutral spectrum of UQ_0 is featureless [12]. The overall analogy can be explained by the presence of many conserved residues in the Q_B binding site [2,3] and by assuming comparable mechanisms for the stabilization of Q_B^- in the 2 species (for example protonation changes and/or pK shifts of carboxylic residues). On the other hand, we assign the differences in the IR signals of the 2 types of RC in these spectral ranges to the residues which are both functionally involved in the electron stabilization on Q_B and either not conserved or in a different environment between the 2 species. A number of non-conserved residues, such as Val L194 in *Rb. sphaeroides* (Ile in *Rp. viridis*), Asp L213 (Asn), Phe L215 (Tyr) and Thr L226 (Ala) define the Q_B pocket [2,3]. Furthermore, although for both RCs the H-bonding to one of the Q_B oxygens involves His L190, a difference has been noticed in the bonding interactions of the second oxygen with the protein in the 2 species [2,3]. In *Rb. sphaeroides* RCs, Ser L223 is the only residue involved in this H-bond while an additional H-bond between the second quinonic C=O and the peptide N–H of Gly L225 occurs in *Rp. viridis*. This difference in the bonding interactions of Q_B has already been invoked to explain the differences observed at 1650 cm^{-1} between the light-induced $P^+Q_B^-/PQ_B$ spectra of the 2 types of RCs [10]. The opposite sign of the absorbance changes at 1650 cm^{-1} seen in the spectra shown in Fig. 1a and 2a is fully consistent with the previous observations and, together with the Q_A^-/Q_A spectra for both RCs [7], support the proposed model [10].

The present study demonstrates that pure Q_B^-/Q_B FTIR difference spectra can be obtained upon reversible photogeneration of the state Q_B^- in isolated RCs of photosynthetic bacteria. The microenvironment of Q_B can thus be investigated by the analysis of the Q_B^-/Q_B spectrum of genetically-altered RCs and of RCs reconstituted with chemically-modified or isotopically-labelled quinones. Furthermore, this approach will be extended in order to study the molecular events which follow the double reduction of Q_B.

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