Volume 278, number 2, 257–260 FEBS 09328 January 1991

ADONIS 001457939100110 6

Probing the primary quinone environment in photosynthetic bacterial reaction centers by light-induced FTIR difference spectroscopy

J. Breton[1], D L. Thibodeau[1], C. Berthomieu[1], W. Mäntele[2], A. Verméglio[3] and E. Nabedryk[1]

[1]*DBCM, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France,* [2]*Institut für Biophysik und Strahlenbiologie, Universität Freiburg, Albertstr. 23, 7800 Freiburg, Germany* and [3]*DPVE, CEN Cadarache, B.P. no. 1, 13108 St. Paul lez Durance Cedex, France*

Received 15 October 1990

The photoreduction of the primary electron acceptor, Q_A, has been characterized by light-induced Fourier transform infrared difference spectroscopy for *Rb. sphaeroides* reaction centers and for *Rsp. rubrum* and *Rp. viridis* chromatophores. The samples were treated both with redox compounds, which rapidly reduce the photooxidized primary electron donor P^+, and with inhibitors of electron transfer from Q_A to the secondary quinone Q_B. This approach yields spectra free from P and P^+ contributions which makes possible the study of the microenvironment of Q_A and Q_A^-.

Fourier transform infrared spectroscopy, Bacterial reaction center, Photosynthesis, Primary quinone, Secondary quinone

1. INTRODUCTION

In the reaction center (RC) from photosynthetic purple bacteria, absorption of a photon leads to a rapid (200 ps) charge separation between the primary electron donor P, a dimer of bacteriochlorophyll, and the primary quinone acceptor Q_A. The understanding of the remarkable speed of this electron transfer step and of the even more remarkable efficiency of the subsequent charge stabilization (back-reaction in the ms time range) is still very limited. Although the recent elucidation of the three-dimensional structure of the RC has provided a wealth of information on the amino acid residues which make up the binding sites of P and Q_A, there remains a serious lack of knowledge on the electronic structure of the reactants as well as on the modifications of these structures (both geometric and electronic) concomitant with the charge separation and stabilization processes (for reviews, see [1–3]).

Vibrational infrared (IR) spectroscopy being highly sensitive to small alterations of bond energies, we have implemented light-induced Fourier transform (FT) IR difference spectroscopy techniques in order to monitor the molecular changes occurring at the level of individual chemical groups of the protein and cofactors when RCs undergo charge separation [4–10]. By comparison with electrochemically-generated cations and anions of the primary reactants [6,11], specific changes in the IR absorption of P and P^+ have been characterized and the absence of any large conformational change of the RC protein backbone has been demonstrated. The quinone bands, which are buried under the larger contributions of P and P^+, have, however, proven elusive [7,8,11]. Very recently, new strategies such as time-resolved FTIR spectroscopy [12] and in situ electrochemistry [10], have been developed in order to elicit the quinone contributions from those due to P and P^+.

In this letter we report on a different approach, namely the steady-state generation of Q_A^- in RCs and chromatophore membranes by light-induced charge separation in the presence of chemical reductants and mediators which rapidly rereduce P^+.

2. MATERIALS AND METHODS

Purified chromatophores of *Rsp. rubrum* G9 were sonicated in the presence of 150 mM NaCl, centrifuged and then washed with distilled H_2O. Purified chromatophores of *Rp. viridis* were used without further treatment. For IR samples, inhibitors and redox mediators in 40 mM Tris buffer, pH 8.0, were added to a suspension of chromatophores which was then centrifuged for 1 h at 200 000 × *g*. A fraction of the final pellet was pressed and sealed between two CaF_2 windows. The maximum absorbance at $\approx 1650\ cm^{-1}$ was kept below 1.0.

Rb. sphaeroides R-26 RCs, isolated with or without Q_B [13], were deposited on a CaF_2 window (25 mm diameter) and dried under argon. Before complete dryness, the RC film was covered with 6 μl of a 40 mM Tris buffer, pH 8.0, containing terbutryn or *o*-phenanthroline and the redox compounds, diaminodurene (DAD) and Na ascorbate, and was sealed with another CaF_2 window. This microcell was thermostated at 10°C in a cryostat.

The large IR absorption of water around $1640\ cm^{-1}$ limits the op-

Correspondence address J. Breton, DBCM, CEN Saclay, 91191 Gif-sur-Yvette Cedex, France

Abbreviations. FTIR, Fourier transform infrared spectroscopy, Q_A (Q_B), primary (secondary) quinone electron acceptor, Q_B, quinone pool, P, primary electron donor, H_A, intermediary electron acceptor, RC, reaction center, DAD, diaminodurene, PSII, photosystem II

tical path to about 10 μm. A high RC concentration (typically 1–2 mM for isolated RCs and 0.3 mM for chromatophores) is thus required and the concentration of reductants and/or mediators (see above) as well as the intensity of the actinic beam had first to be optimized. The criteria used for this optimization were that the steady-state light-induced FTIR difference spectra (i) should contain negligible contribution from the primary donor photooxidation, monitored by the absence of the bands previously assigned to the 9-keto and 10a-ester C=O of bacteriochlorophyll in the P^+Q^-/PQ spectra [4,6,8] and (ii) should exhibit good reversibility for signal averaging.

Light-induced IR and near-IR measurements were performed under steady-state illumination conditions with a Nicolet 60SX FTIR spectrometer equipped with both MCT-A and Si diode detectors. Near-IR and IR measurements were performed alternately on the same sample under identical geometry. For measurements in the near-IR, the sample as well as the Si detector were protected by red cut-off ($\lambda > 665$ nm) filters and the sample was illuminated with blue actinic light ($\lambda < 550$ nm). For IR measurements, the sample was usually illuminated with red ($\lambda > 715$ nm) or blue ($\lambda > 550$ nm) actinic light, leading to identical FTIR difference spectra. Interferograms were recorded before and during continuous illumination (25–50 s) and each light-minus-dark cycle was repeated several hundred times separated by a dark time (several minutes) sufficient to ensure full return to the ground state.

3. RESULTS

3.1. Rb. sphaeroides *reaction centers*

Cyclic steady-state illumination of isolated RCs in the presence of 100 μM terbutryn, 5 mM DAD and 1 mM Na ascorbate in 40 mM Tris, pH 8.0, buffer, generates the light-minus-dark FTIR spectrum shown in Fig. 1a. All bands in this spectrum decay with the same half-time of ≈20 s suggesting that only one type of reaction occurs. Evidence for Q_A^- formation is demonstrated by the light-induced difference spectrum in the near-IR (inset Fig. 1) obtained from the same sample. This difference spectrum has been previously assigned to electrochromic shifts, mostly of the intermediary bacteriopheophytin electron acceptor H_A, in response to the negative charge on Q_A [14]. This spectrum also demonstrates the absence of contributions from P^+/P and H_A^-/H_A, which both have characteristic spectra in this wavelength range. Any significant contribution from the state Q_A^{2-} in Fig. 1a. can be excluded on the basis of the extremely slow decay of this state [15]. In principle, signals can be expected from a change of redox state of DAD and ascorbate. However, it has been verified that the IR absorbance spectrum of a 200 mM solution of DAD and Na-ascorbate, with and without 500 mM potassium ferricyanide, in a 10 μm cell displays only very broad bands (data not shown) which thus could not contribute to the sharp signals shown in Fig. 1a. The other species which could still possibly affect this spectrum are Q_B, Q_B^-, Q_BH_2 and terbutryn. The absence of significant contribution from these species has been checked by using either RCs with *o*-phenanthroline instead of terbutryn as inhibitor of Q_A^- to Q_B electron transfer, or RCs depleted in Q_B, conditions which both lead to spectra almost indistinguishable from the one shown in Fig. 1a. This spectrum can thus be confidently assigned to the photoreduction of Q_A and hereafter is named Q_A^-/Q_A spectrum.

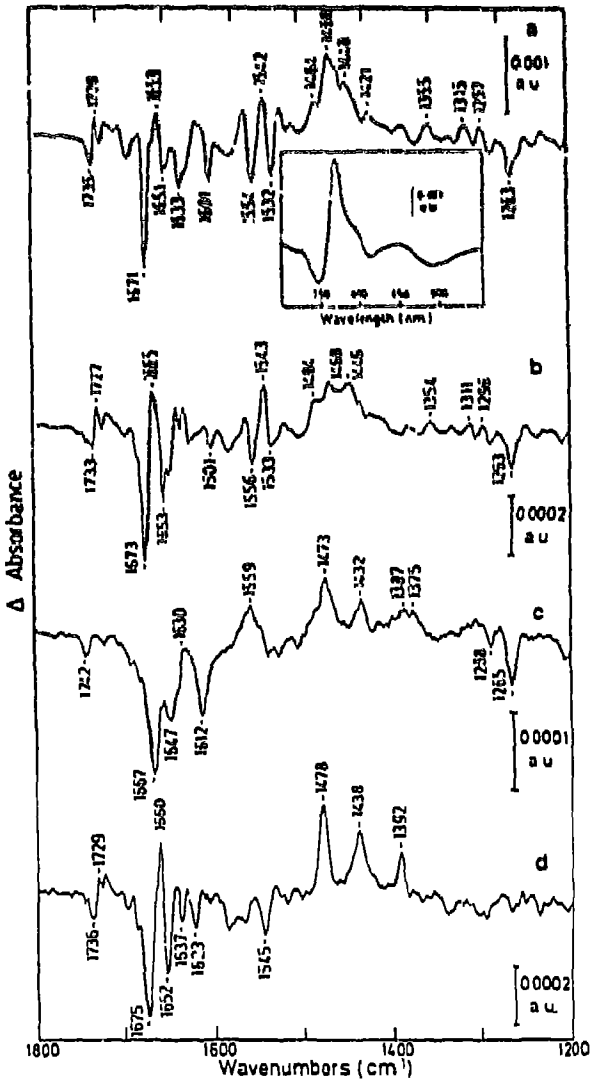

Fig. 1. Light-induced FTIR difference spectra at 10°C of (a) *Rb sphaeroides* RCs in the presence of 100 μM terbutryn, 5 mM DAD and 1 mM Na ascorbate, $\lambda > 715$ nm, 46080 interferograms co-added. Inset, light-induced spectrum in the near-IR from the same sample (see Materials and Methods), $\lambda < 550$ nm, 6400 interferograms co-added. (b) *Rsp rubrum* chromatophores in the presence of 10 mM *o*-phenanthroline, 10 mM DAD and 20 mM Na ascorbate; $\lambda < 550$ nm, 25 600 interferograms co-added. (c) *Rsp rubrum* chromatophores in the presence of 8 mM DAD and 20 mM Na ascorbate, $\lambda > 715$ nm, 25 600 interferograms co-added. (d) *Rp. viridis* chromatophores in the presence of 10 mM *o*-phenanthroline and 10 mM DAD, $\lambda > 715$ nm; 17 920 interferograms co-added. 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.

3.2. Rsp. rubrum *chromatophores*

This system was selected on the basis that it contains the same Q_A species as *Rb. sphaeroides*, but with the advantage that the cytochrome c_2 (the physiological electron donor to P^+), which could contribute to the dif-

ference spectrum, is easier to eliminate by salt washing than in *Rb. sphaeroides*. Cyclic steady-state illumination of *Rsp. rubrum* chromatophores in the presence of 10 mM *o*-phenanthroline, 10 mM DAD and 20 mM Na ascorbate in 40 mM Tris, pH 8.0, buffer yields the light-minus-dark FTIR difference spectrum shown in Fig. 1b. All the bands decay with a half-time of 40–50 s and the close analogy with the spectrum in Fig 1a as well as near-IR controls (data not shown) lead us to assign this signal to the Q_A^-/Q_A spectrum of *Rsp. rubrum*. Without *o*-phenanthroline, a very different spectrum (Fig. 1c) is obtained which we consider to contain contributions from several redox and protonation states of Q_B and/or from the quinone pool (Q_P). This signal decays with a half-time of 5–10 min.

3.3. Rp. viridis *chromatophores*

Fig. 1d shows the light-minus-dark FTIR difference spectrum of *Rp. viridis* chromatophores in the presence of 10 mM *o*-phenanthroline and 10 mM DAD. Again, all the bands decay in unison and addition of Na ascorbate (up to 20 mM) only affects the recovery time of the signal. The same spectrum is obtained when terbutryn is substituted for *o*-phenanthroline. The dominant contribution from the Q_A^-/Q_A state which is responsible for the spectrum in Fig. 1d has been monitored in the 500–600 nm spectral range with a Joliot-type flash spectrophotometer [16]; the details of these controls will be published elsewhere. Briefly, we have determined that under our experimental conditions for which both high potential cytochromes c_{559} and c_{556} are reduced in the dark, a saturating flash generates the state cytochrome c_{556}^+ Q_A^-. The reduction of cytochrome c_{556}^+ by DAD being much faster than the reoxidation of Q_A^-, the intensity of a steady-state actinic beam can be adjusted to accumulate Q_A^- while keeping all the high potential cytochromes reduced. Under these conditions, the same saturating flash delivered on top of the actinic beam is not able to generate a measurable cytochrome c_{556}^+ Q_A^- charge separation, demonstrating the accumulation of the full complement of Q_A^- in the sample.

4. DISCUSSION

Recently, the interest in characterizing the environment and bonding pattern of the RC quinones in their neutral and anionic forms has triggered investigations by several FTIR spectroscopy approaches: replacement with modified or isotopically-labelled quinones [7], comparison of $P^+Q_A^-/PQ_A$ and $P^+Q_B^-/PQ_B$ spectra either by steady-state [7,8,17,18] or kinetic [12] techniques, spectroelectrochemistry of quinones both in vitro [11] and in situ [10]. The first FTIR characterization of a photochemically-generated state containing pure contributions from Q_A^-/Q_A in situ has been recently reported [19] for the RC of photosystem II (PS II). In the present study, we report that addition of reductants and mediators, which rapidly rereduce P^+, makes possible the photochemical generation of pure Q_A^-/Q_A FTIR spectra on both isolated RCs and chromatophores from purple bacteria. Owing to the excellent reversibility of the photochemistry (the light-minus-dark spectra can be repeatedly cycled over more than 24 h without modification), FTIR difference spectra can be obtained with signal-to-noise at least equivalent to those previously reported for P^+Q^-/PQ states [8].

The Q_A^-/Q_A spectrum obtained photochemically with *Rb. sphaeroides* RCs (Fig. 1a) compares very well with that obtained electrochemically on the same RC [10]. This observation further confirms the absence of contributions from the redox mediators and the inhibitors in the photochemically-induced spectrum (Fig. 1a). Moreover, this spectrum compares strikingly well with the Q_A^-/Q_A spectrum of *Rsp. rubrum* chromatophores, which also contain ubiquinone-10 at the Q_A site. This similarity shows that the Q_A binding in both species must be highly comparable, as expected from the identity of the amino acid sequence stretches surrounding Q_A [20]. The small but reproducible differences observed between the two Q_A^-/Q_A spectra, and notably in the 1500–1400 cm^{-1} region where the semiquinone anion absorbs in vitro [11], can be assigned either to a more native environment of Q_A in chromatophores than in isolated RCs or to genuine differences in the binding of Q_A between the two species.

The spectra shown in Fig. 1b and Fig. 1c display large differences that we consider to represent the specific environments and bonding interactions of the same chemical species (ubiquinone) in the Q_A and Q_B or Q_P sites. Although the very different band positions observed between these two spectra indicate that the spectrum 1c can only be contaminated by a minute amount of Q_A^-/Q_A, we cannot so far discriminate the features in Fig. 1c which correspond to Q_B^-, Q_BH_2 or to the reduction of the quinone pool (possibly including its indirect effect on the cytochrome bc_1). A further discussion of Q_B^-/Q_B and Q_BH_2/Q_B spectra calls for additional experiments performed with sequences of saturating flashes.

The largest differences in the Q_A^-/Q_A spectra of *Rsp. rubrum* (Fig. 1b) and *Rp. viridis* (Fig. 1d) chromatophores can be attributed, at least in part, to the different chemical nature of Q_A in the two species (menaquinone in *Rp. viridis*). While this is certainly the case for the three bands observed in *Rp. viridis* at 1478 cm^{-1}, 1438 cm^{-1} and 1392 cm^{-1} in the absorption region of the semiquinone anion – these three quinone anion bands have also been recently observed in the $H_A^-Q_A^-/H_AQ_A$ spectrum of isolated *Rp. viridis* RCs [18] – the change in the 1550–1500 cm^{-1} (amide II) region more likely reflects different contributions from the protein moiety. On the other hand, it should be

noticed that the signals in the 1740 cm^{-1} to 1640 cm^{-1} carbonyl region are very similar in these two spectra (Fig. 1b and 1d). This last observation suggests that, in this spectral domain, contributions from amino acid C = O predominate over that of C = O from the neutral quinone. Although the carbonyls of the neutral ubiquinone absorb at = 1660 cm^{-1} in organic solvents ([11] and references therein), the absence of clear isotopic shifts in this region for the $P^+Q_A^-/PQ_A$ spectra of *Rb. sphaeroides* RCs reconstituted with ^{13}C- or ^{18}O-labelled ubiquinone-10 has been taken to indicate that the bands around 1650 cm^{-1} [7] and 1670 cm^{-1} [9] arise from peptide C = O groups rather than from quinone carbonyls. Possible assignments for these bands in terms of contribution from the amino acids in the vicinity of Q_A – notably a conformational change of the peptide C = O of Ala M258 – have been already discussed [7–9]. Furthermore, the Q_A^-/Q_A spectra shown in Fig. 1a,b, and 1d bear large similarities with the Q_A^-/Q_A spectrum of PSII particles [19], such as the positive anion band around 1480 cm^{-1}. Other obvious spectral analogies between bacteria and PSII are also observed in the 1740 cm^{-1} to 1610 cm^{-1} carbonyl frequency region, especially the band at = 1670 cm^{-1}. This adds support to the evidence that several of the amino acid interactions with Q_A are conserved in PSII and bacteria.

In conclusion, this study demonstrates that the Q_A^- state can be reversibly photoaccumulated so that Q_A^-/Q_A FTIR difference spectra free from contributions of P and P^+ are obtained. By this straightforward approach, the microenvironment of Q_A can be investigated by the analysis of the Q_A^-/Q_A spectrum of RCs reconstituted with chemically-modified or isotopically-labelled quinones as well as by the use of RCs genetically-altered on the residues that form the binding niche of Q_A.

Acknowledgements: The Saclay group is indebted to P. Noel (Nicolet France) for his help during the adjustment of the 60SX instrument for the sequential IR and near-IR FT-measurements. The authors acknowledge discussions with M. Bauscher and A.W. Rutherford. A NATO/NSERC postdoctoral fellowship is gratefully acknowledged by D.L.T. Part of this work was supported by EEC (SC1*0335-C) and ANRT Procope (90137) grants to J.B.

REFERENCES

[1] Parson, W.W. (1987) in: Photosynthesis (Amesz, J. ed.) pp. 43–61, Elsevier, Amsterdam.
[2] Breton, J. (1988) ISI Atlas of Science: Biochemistry 1, 323–328.
[3] The Photosynthetic Bacterial Reaction Center: Structure and Dynamics (1988) (Breton J. and Vermeglio A. eds), NATO ASI Series, Series A: Life Sci. Vol. 149, Plenum, New York.
[4] Mantele, W.G., Nabedryk, E., Tavitian, B.A., Kreutz, W. and Breton, J. (1985) FEBS Lett. 187, 227–232.
[5] Nabedryk, E., Mantele, W., Tavitian, B.A. and Breton, J. (1986) Photochem. Photobiol. 43, 461–465.
[6] Mantele, W., Wollenweber, A., Nabedryk, E. and Breton, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8468–8472.
[7] Bagley, K.A., Abresch, E., Okamura, M.Y., Feher, G., Bauscher, M., Mantele, W., Nabedryk, E. and Breton, J. (1990) in: Current Research in Photosynthesis, vol. I (Baltscheffsky, M. ed.) pp. 77–80, Kluwer, Dordrecht.
[8] Nabedryk, E., Bagley, K.A., Thibodeau, D.L., Bauscher, M., Mantele, W. and Breton, J. (1990) FEBS Lett. 266, 59–62.
[9] Thibodeau, D.L., Breton, J., Berthomieu, C., Bagley, K.A., Mantele, W. and Nabedryk, E. (1990) Proceedings of the Feldafing II Workshop, Feldafing, 24–26 March 1990, Springer-Verlag, (in press).
[10] Mantele, W., Leonhard, M., Bauscher, M., Nabedryk, E., Breton, J. and Moss, D.A. (1990) Proceedings of the Feldafing II Workshop, Feldafing, 24–26 March 1990, Springer-Verlag, (in press).
[11] Bauscher, M., Nabedryk, E., Bagley, K., Breton, J. and Mantele, W. (1990) FEBS Lett. 261, 191–195.
[12] Thibodeau, D.L., Nabedryk, E., Hienerwadel, R., Lenz, F., Mantele, W. and Breton, J. (1990) Biochim. Biophys. Acta 1020, 253–259.
[13] Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. USA 72, 3491–3495.
[14] Vermeglio, A. and Clayton, R.K. (1977) Biochim. Biophys. Acta 461, 159–165.
[15] Okamura, M., Isaacson, R.A. and Feher, G. (1979) Biochim. Biophys. Acta 546, 394–417.
[16] Joliot, P. and Joliot, A. (1984) Biochim. Biophys. Acta 765, 210–218.
[17] Buchanan, S., Michel, H. and Gerwert, K. (1990) in: Current Research in Photosynthesis, vol. I (Baltscheffsky, M. ed.) pp. 69–72, Kluwer, Dordrecht.
[18] Buchanan, S., Michel, H. and Gerwert, K. (1990) Proceedings of the Feldafing II Workshop, Feldafing, 24–26 March 1990, Springer-Verlag, (in press).
[19] Berthomieu, C., Nabedryk, E., Mantele, W. and Breton, J. (1990) FEBS Lett. 269, 363–367.
[20] Komiya, H., Yeates, T.O., Rees, D.C., Allen, J.P. and Feher, G. (1988) Proc. Natl. Acad. Sci. USA 85, 9012–9016.