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Perfusion- and light-induced ATR-FTIR redox studies of *Rb. sphaeroides* photosynthetic reaction centers

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Abstract. A new method to measure chemically induced FTIR difference spectra is applied to the study of redox transitions of primary electron donors and acceptors in photosynthetic reaction centers (RCs). Redox transitions in the film of RCs, which is deposited on an attenuated total reflection (ATR) prism, can be induced either by perfusion of buffers poised at different redox potentials or by illumination. The ATR-FTIR difference spectra are very similar to the equivalent FTIR difference spectra previously recorded upon photochemical or electrochemical excitation of the RCs in the conventional transmission mode. Possible extensions of the perfusion-induced ATR-FTIR spectroscopy to other redox active proteins and to the more general problem of the binding of substrates and ligands are discussed.

Introduction

Fourier transform infrared (FTIR) difference spectroscopy has been used to investigate the molecular changes involved in bioenergetic reactions. In the case of photoactive proteins, light-induced FTIR difference spectroscopy has been applied to investigate changes of the cofactor-protein interactions. In the more general case of non-photoactive systems, FTIR difference spectroscopy has been applied in combination with photoactivable compounds or electrochemistry.

Another approach for FTIR difference spectroscopy, using perfusion of a reagent in an attenuated total reflection (ATR) cell, has been developed for the investigation of the ligand-induced IR changes (Baenziger et al., 1993). In the ATR measurement, the IR beam penetrates the internal total reflection prism on which the sample film is attached (Fig. 1). The evanescent wave propagates through the film while it is exposed to a flow of different liquids. Therefore the ATR-FTIR technique, in principle, allows measuring the difference spectra between two states of the protein that can be manipulated by changing the composition of the external medium.

In the present study we demonstrate for the first time that chemically induced ATR-FTIR difference spectra could be obtained with a signal-to-noise ratio equivalent to that of the light-induced FTIR difference spectra. We have used the electron transfer reactions involving the primary donor P, a dimer of bacteriochlorophyll molecules, and the ubiquinone electron acceptor Q_A in the reaction center (RC) of the purple bacterium *Rhodobacter* (*Rb.*) *sphaeroides*. An extension of the measurements to the RCs of *Rhodopseudomonas* (*Rp.*) *viridis* and of photosystem I (PS I) from the cyanobacterium *Synechocystis* 6803 is also presented.

Materials and Methods

After an aliquot of the detergent-free RCs (from *Rb. sphaeroides*, *Rp. viridis* or *Synechocystis* PS I) was partially dried on the ATR prism, the RC film was hydrated with buffer (30 mM HEPES, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$). The redox states in the RCs were manipulated by changing the chemical composition of the flowing buffer (1 mL/min). The IR spectra were recorded under a continuous flow with a FTIR spectrophotometer (Magna 860, Nicolet) equipped with DTGS or MCT-A detector and with the ATR accessory (SensIR Technologies). Illumination of the RCs film was also performed to record the light-induced FTIR difference spectra. All measurements were done at room temperature.

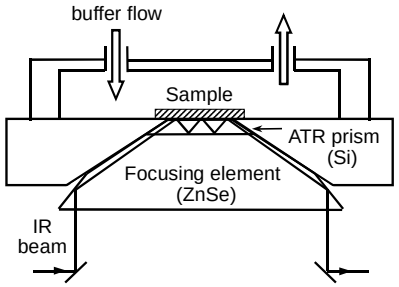

Fig. 1. ATR set-up

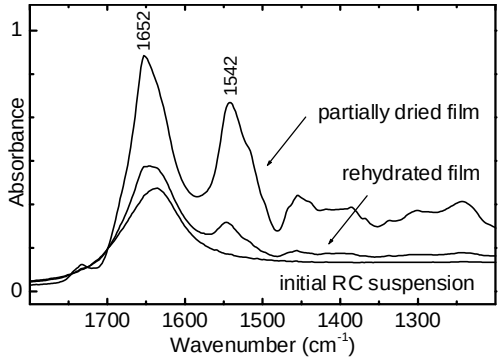

Fig. 2. ATR absorption spectra of RCs of *Rb. sphaeroides*

Results and Discussion

(1) Absorption of RC films

Figure 2 shows the ATR-FTIR absorption spectra of *Rb. sphaeroides* RCs. The spectrum of the initial suspension of RCs is dominated by water. Upon drying, the film exhibits two main protein bands of amide I (1652 cm^{-1}) and II (1542 cm^{-1}). The amplitude of the bands in the hydrated film decreased rapidly but was stable during the continuous flow for over 15 hrs. In the 1700-1500 cm^{-1} range the absorption of the hydrated film is comparable to that of transmission FTIR samples although ten times less amount of RCs is required for the ATR.

(2) Perfusion-induced P^+/P transition in Rb. sphaeroides RCs

The perfusion-induced ATR-FTIR difference spectra of P^+/P induced by chemical oxidation and the reverse (P/P^+) were measured (Fig. 3). The bands, which correspond to the CN of ferricyanide (2115 cm^{-1}) and ferrocyanide (2037 cm^{-1}), demonstrates the exchange of the buffer inside the protein layers (Fig. 3B). The excellent reversibility of the reaction is evident from the symmetry of the spectra and by the absence of distinctive features in the sum spectra (Fig. 3, middle traces). The averaged P^+/P difference spectrum (Fig 4a) is in good agreement with those measured by light- and electrochemistry-induced transmission FTIR spectroscopy (Mäntele et al. 1985, Nabedryk et al. 1990, Breton et al. 1992, Leonhard & Mäntele 1993).

(3) Perfusion-induced Q_A^-/Q_Atransition in Rb. sphaeroides RCs

The perfusion-induced ATR-FTIR difference spectrum of Q_A^-/Q_A (Fig. 4c) also agrees with those measured by light- and electrochemistry-induced transmission FTIR spectroscopy (Breton et al. 1991, Bauscher et al. 1993).

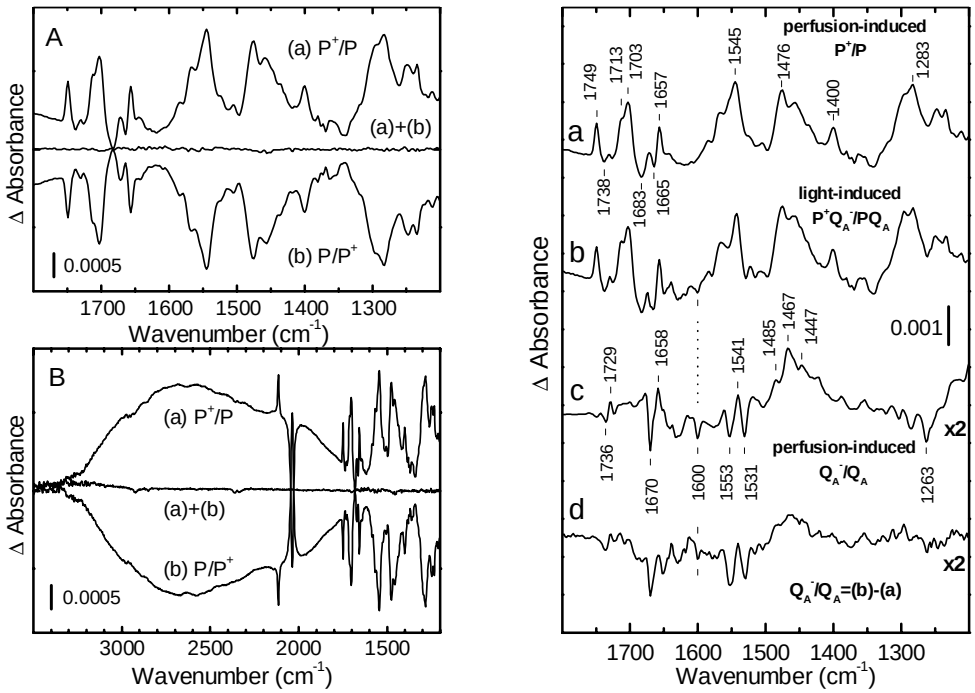

Fig. 3 (left). Perfusion-induced P^+/P and P/P^+ ATR-FTIR difference spectra in *Rb. sphaeroides* RCs. Redox control was done by switching buffers containing 4 mM ferricyanide for oxidation and 4 mM ferrocyanide for reduction.

Fig. 4 (right). Comparison of perfusion-induced and light-induced ATR-FTIR difference spectra in *Rb. sphaeroides* RCs. **(a)** Perfusion-induced P^+/P spectrum taken from Fig. 3. **(b)** Light-induced $P^+Q_A^-/PQ_A$ spectrum measured by continuous illumination under a buffer flow. **(c)** Perfusion-induced Q_A^-/Q_A spectrum measured by switching buffers containing 4 mM sodium hydrosulfite for reduction and 4 mM ferrocyanide (and 1 mM TMPD as a mediator) for oxidation. **(d)** Calculated Q_A^-/Q_A as the double difference: (b) *-minus-* (a).

(4) Comparison of perfusion-induced and light-induced ATR-FTIR

The Q_A^-/Q_A difference spectrum calculated as the double difference between the perfusion-induced P^+/P and the light-induced $P^+Q_A^-/PQ_A$ (Fig 4, d=b-a), which were measured with the same RC film, agrees with the perfusion-induced Q_A^-/Q_A spectrum (Fig 4c). Both the perfusion- and light-induced ATR-FTIR difference spectra exhibit equivalent S/N ratios to those measured by transmission FTIR.

(5) P^+/P transition in Rp. viridis RCs

The perfusion-induced ATR-FTIR difference spectrum of P^+/P in *Rp. viridis* RCs (Fig 5a) agrees with the results obtained by light-induced and electrochemistry-induced transmission FTIR (Mäntele et al. 1985, Nabedryk et al. 1990, Leonhard & Mäntele 1993). By extending these measurements to a lower potential range we have also separated the redox difference spectra of (i) the high potential cyt c_{559} and cyt c_{556} without P^+/P contribution and (ii) the low potential cyt c_{552} and cyt c_{554} together with Q_A^-/Q_A (data not shown).

(6) $P700^+/P700$ transition in Synechocystis PS I RCs

The perfusion-induced (Fig 5b) and light-induced (Fig 5c) ATR-FTIR difference spectra of $P700^+/P700$ in the *Synechocystis* PS I are essentially identical. They also agree with the results obtained by light- and electrochemistry-induced transmission FTIR (Hamacher et al. 1996, Nabedryk et al. 1996).

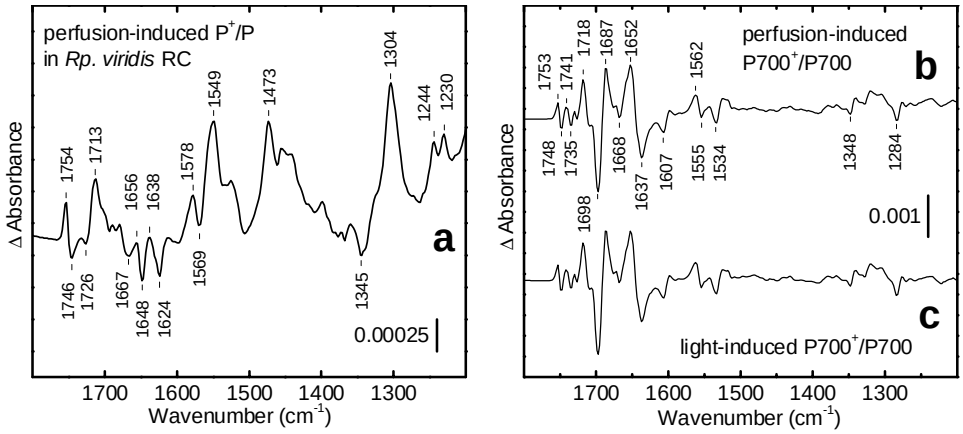

Fig. 5. ATR-FTIR difference spectra in *Rp. viridis* RCs **(a)** and *synechocystis* PS I RCs **(b and c)**. **(a)** Perfusion-induced ATR-FTIR difference spectrum of P^+/P were measured by switching buffers containing a mixture of 2 mM ferricyanide and 2 mM ferrocyanide for oxidation and 4 mM ferrocyanide for reduction. **(b)** Perfusion-induced ATR-FTIR difference spectrum of $P700^+/P700$ measured by switching buffers containing 3 mM ferricyanide and 3 mM ferrocyanide. **(c)** ATR-FTIR difference spectrum of $P700^+/P700$ induced by continuous illumination in a buffer flow containing 2.5 mM ferrocyanide and 0.5 mM ferricyanide.

Conclusions

The present work demonstrates that the perfusion-induced ATR-FTIR difference spectroscopy can be useful to investigate the individual redox transitions in the RCs. The perfusion-induced difference spectra show that the signal to noise ratios are equivalent in the light-induced difference spectra. The ATR-FTIR difference spectra in the present study are in good agreement with those previously measured by conventional transmission FTIR upon photochemical or electrochemical excitation.

Since the ATR-FTIR difference spectroscopy offers the advantage of a precise control of the aqueous environment of the sample, the perfusion-induced ATR-FTIR technique should allow the investigation of the non-photoactive or even the non-redox reactions, e.g., salt or pH changes, ligand binding, drug-receptor interactions. Notably, cytochrome *c* oxidase, Complex I, cytochrome bc_1, cytochrome b_6f, and cytochrome P450 are presently under investigation by this technique in our laboratories.

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