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Efficient conditions for the photoaccumulation of H_A^- in the photosynthetic reaction centre of *Rhodobacter sphaeroides* R26 with uniformly labelled bacteriopheophytin monitored by Fourier transform infrared difference spectroscopy

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Abstract

The native bacteriopheophytins (BPheo) *a* at sites H_A and H_B in the reaction centre (RC) of *Rhodobacter sphaeroides* R26 have been exchanged with uniformly ^{13}C , ^{15}N labelled BPheo *a*. Exchange at the H_A site was > 50% as monitored with light-induced FTIR-difference spectroscopy indicated by the shift of the band at 1590 cm^{-1} . The photoreduction of H_A has been monitored by light-induced FTIR-difference spectroscopy at 22°C in the presence of reductant and mediator: either sodium dithionite and cytochrome *c*, or sodium dithionite and dyes (potassium indigotetrasulfonate, neutral red). New experimental conditions are described for H_A^- photoaccumulation, and light-induced FTIR-difference spectra characteristic of H_A^- are reported. The H_A^-/H_A FTIR-difference spectra of *Rb. sphaeroides* R26 RCs in which uniformly ^{13}C , ^{15}N labelled BPheo *a* replaces the photoactive BPheo will allow the BPheo modes to be assigned and to be discriminated from those of the RC protein. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bacteriopheophytin; Bacterial reaction centres; Electron transfer; Isotope labelling; Fourier Transform Infra-Red; Magic Angle Spinning NMR; Photosynthesis

Abbreviations: B, bacteriochlorophyll monomer; BPheo *a*, bacteriopheophytin *a*; CP, Cross-Polarization; DEAE, diethylaminoethyl; EDTA, ethylenedinitrilotetraacetic acid; FTIR, Fourier Transform Infra Red; LDAO, *N,N*-dimethyldodecyl-amine-*N*-oxide; ITS, potassium indigotetrasulfonate; MAS NMR, Magic Angle Spinning Nuclear Magnetic Resonance; NR, neutral red; P, bacteriochlorophyll dimer; PAGE, polyacrylamide gel electrophoresis; H, bacteriopheophytin monomer; Q, quinone; *Rb.*, *Rhodobacter*; RC, Reaction Centre; RP HPLC, Reverse Phase High Performance Liquid Chromatography; Tris, Tris-hydroxymethylaminomethane; [U - ^{13}C , ^{15}N] uniformly carbon, nitrogen enriched

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1. Introduction

The reaction centre of the photosynthetic bacterium *Rhodobacter sphaeroides* is a transmembrane protein complex that consists of three polypeptide chains (L, M, H) supporting the cofactors. The two bacteriopheophytins (BPheo) in the *Rb. sphaeroides* RC are chemically identical, but only one, H_A, is known to function as an intermediary electron acceptor during the electron transfer proceeding from the donor P to the primary electron acceptor quinone Q_A, while the other bacteriopheophytin, H_B, appears not to be actively involved in electron transfer [1,2]. Although the crystal structure of the complex has been determined in great detail with up to 2.65 Å resolution, [3–5] several fundamental questions regarding the charge separation mechanism and electron pathway are still unresolved. For example, the precise role of the protein–cofactor interactions in assisting electron transport and prevention of wasteful back reactions is still poorly understood.

Selective isotopic labelling of the cofactors provides a tool for probing the RC structure and the cofactor–protein binding sites using complementary spectroscopic techniques like MAS NMR and FTIR [6–10]. However, selective isotope labelling of cofactors is difficult and often requires laborious synthesis. We recently introduced a novel concept of multispin labelling and assignment of ¹³C CP/MAS NMR resonances of ¹³C clusters of 1 kDa in a large protein complex [11]. Bacterial photosynthetic RCs of *Rb. sphaeroides* R26 were reconstituted with [*U*-¹³C] plant Pheo *a* in the two pheophytin binding sites. The present investigation aims at providing a next step into the study of the protein–BPheo interactions in *Rb. sphaeroides* R26 RCs through the isotope labelling of BPheo (Fig. 1) followed by 1-D ¹³C CP/MAS NMR of [*U*-¹³C, ¹⁵N] BPheo in *Rb. sphaeroides* R26 RCs and FTIR difference spectroscopy.

Photoaccumulation of H_A[−] under reversible conditions required for FTIR studies was reported for the first time by Nabadryk et al. [12], however, the procedure for the photoaccumulation of H_A[−] appeared to be difficult to handle. Therefore a new method for efficient generating H_A[−] in a film of *Rb. sphaeroides* R26 RCs is reported here. In the present work we describe the new experimental conditions

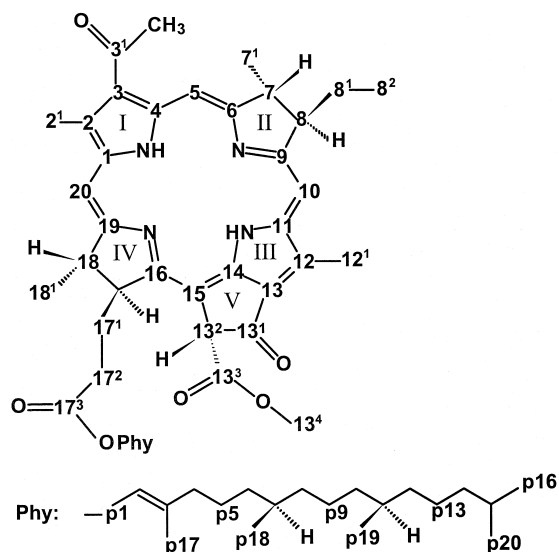


Fig. 1. Chemical structure of bacteriopheophytin *a* with IUPAC numbering scheme.

for the photoaccumulation of H_A[−] in a film of *Rb. sphaeroides* R26 RCs in the presence of reductant and mediators which rapidly reduce P⁺. Photoaccumulation of H_A[−] is achieved in FTIR film of *Rb. sphaeroides* RCs in presence of either sodium dithionite and cytochrome *c*, or sodium dithionite in combination with two types of dyes (potassium indigotetrasulfonate and neutral red). H_A[−]/H_A FTIR difference spectra of *Rb. sphaeroides* R26 RCs with unlabelled BPheo *a* and of *Rb. sphaeroides* R26 RCs with [*U*-¹³C, ¹⁵N] labelled RCs were obtained.

2. Materials and methods

To obtain uniformly enriched pigments, *Rhodospseudomonas palustris* 17001 was grown on a medium containing: [*U*-¹³C, ¹⁵N] algae hydrolysate (Heidelberg, EMBL)—2.5 g l^{−1}, KH₂PO₄ (Darmstadt, Germany)—1 g l^{−1}, MgSO₄ × 7H₂O (Darmstadt)—0.4 g l^{−1}, NaCl (Darmstadt)—0.4 g l^{−1}, CaCl₂ × 2H₂O (Darmstadt)—0.05 g l^{−1}, trace metals—10 ml l^{−1}. A quantity of 1 N NaOH was used for the adjustment to pH 7. The trace elements solution was prepared as described for M 550 in Ref. [13], but Na₂MoO₄ × 2H₂O was used instead of

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$ and EDTA was excluded. Light intensity was 2 klux from incandescent lamps.

After 20 days of growing, the cells were harvested by centrifugation (15 min at $10\,000 \times g$) and the pigments were extracted incubating under mild sonication (Standardgraph, Ultrasonic cleaner) at 4°C for 2 min in a 20-fold volume of acetone and centrifuged at $5400 \times g$ for 10 min. The supernatant consisted mostly of carotenoid pigments. The precipitate was extracted with MeOH and centrifuged at $5400 \times g$ for 20 min, releasing the BChls in the supernatant. The acetone and methanol extracts were filtered over a $0.45 \mu\text{m}$ teflon (TOSOH H-25-5) membrane filter separately, dried at reduced pressure with a rotary evaporator and subsequently dissolved in a mixture of *n*-hexane, 2-propanol and methanol (100/3/3 v/v/v) used as an eluent for HPLC. The pigments were separated on a normal phase silica HPLC column (Senshupak, 1251-N, $250 \times 4.6 \text{ mm}$ i.d.), cooled to 4°C using a mixture of *n*-hexane, 2-propanol and methanol (100/3/3 v/v/v) as an eluent. For at least 99% of the BChl in *Rb. sphaeroides* R26, phytol was found to be the esterifying alcohol. The [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a* fraction was collected and an additional amount of [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a* was obtained by pheophytinization of [$U\text{-}^{13}\text{C}$, ^{15}N] BChl *a* by bubbling a stream of nitrogen gas containing gaseous HCl into the ether solution of [$U\text{-}^{13}\text{C}$, ^{15}N] BChl *a* as described in Ref. [14]. About 30 mg of labelled BPheo *a* was used for exchange with BPheo *a* in *Rb. sphaeroides* R26 RCs. [$U\text{-}^{13}\text{C}$, ^{15}N] BChl *a* and [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a* were characterized by optical, NMR and FTIR spectroscopy.

2.1. Exchange of BPheo *a* in *Rb. sphaeroides* R26 RCs with [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a*

The RCs from *Rb. sphaeroides* R26 were isolated by treatment of chromatophores with LDAO, followed by purification with DEAE Sephacel chromatography [15]. Replacement of the BPheo *a* in *Rb. sphaeroides* R26 by [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a* was done using a slightly modified method described earlier [2,11,16]. The final yield of *Rb. sphaeroides* R26 RCs with [$U\text{-}^{13}\text{C}$, ^{15}N] BPheo *a* was about 15%.

Since reducing agents are known to react with LDAO, the detergent was replaced by Triton X-100

for photoaccumulation of H_A^- experiments by washing reaction centres bound to a DEAE cellulose column with 0.1% Triton (10 mM Tris, pH 8). The concentration of reaction centres was determined optically by using the molar extinction coefficient $\epsilon = 2.88 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 802 nm.

2.2. Photoaccumulation of H_A^- in solution

Optical samples were typically prepared in gas-tight sample cells. For the photoaccumulation of H_A^- in solution, *Rb. sphaeroides* R26 RCs (about $3 \mu\text{M}$) in 10 mM Tris-HCl (pH 8.0)/0.1% Triton X-100 buffer, containing 100 μM ITS (potassium indigotetrasulfonate) and 100 μM NR (neutral red) or cytochrome *c* (14 μM) were placed in the gas-tight cell of 1 cm path length and purged with nitrogen gas. Then a freshly prepared 0.2–0.4 M solution of $\text{Na}_2\text{S}_2\text{O}_4$ in 1 M Tris-HCl, pH 8.0 was added until a final concentration of 2–4 mM after which the sample was purged with nitrogen gas once more. Optical measurements were performed before and after actinic illumination: $I \sim 0.2 \text{ W cm}^{-2}$, 3 min, $\lambda > 645 \text{ nm}$. The times required for scanning the spectra (1–2 min) were short compared to the decay of H_A^- ($t_{1/2} \sim 15\text{--}20 \text{ min}$) [17].

2.3. Photoaccumulation of H_A^- in a film of *Rb. sphaeroides* R26 RCs in the presence of cytochrome *c* and sodium dithionite

For the FTIR measurements, 35 μl of 0.2 mM RCs solubilized in 10 mM Tris-HCl (pH 8.0)/0.1% Triton X-100 and 7 μl of 8 mM cytochrome *c* solution in water were deposited on a CaF_2 window. After partial drying of the RC under a stream of nitrogen gas, 5 μl of 0.2 M $\text{Na}_2\text{S}_2\text{O}_4$ solution in 1 M Tris buffer pH 8.0 was added and dried under a stream of nitrogen gas to 2 μl . Then 1.5 μl of 0.2 M $\text{Na}_2\text{S}_2\text{O}_4$ was added and, after partial drying, the sample was covered with another CaF_2 window.

2.4. Photoaccumulation of H_A^- in a film of *Rb. sphaeroides* R26 RCs in the presence of dyes and sodium dithionite

For the FTIR measurements, 35 μl of 0.2 mM RCs solubilized in 10 mM Tris-HCl (pH 8.0)/0.1%

Triton X-100 and 6 μl of 2 mM ITS solution in water and 6 μl of 4 mM NR solution in water were deposited on a CaF_2 window and partially dried under a stream of nitrogen gas. Then 6 μl of 0.2 M $\text{Na}_2\text{S}_2\text{O}_4$ solution in 1 M Tris buffer pH 8 was added and after partial drying under nitrogen gas, the sample was covered with another CaF_2 window.

Infra-red spectra of the RCs and of the unlabelled and $[U\text{-}^{13}\text{C},^{15}\text{N}]$ BPheo *a* were recorded on a Bruker IFS 66 v instrument, equipped with an MCT detector. A total of 256 scans were averaged for each spectrum. The difference was taken between spectra recorded during steady state illumination with light > 700 nm and in darkness. The transition H_A to H_A^- was obtained under steady-state continuous illumination at 22°C and cycles of illumination were repeated.

1-D and 2-D ^{13}C MAS NMR spectra of $[U\text{-}^{13}\text{C},^{15}\text{N}]$ BPheo were collected with a Bruker 600 MHz DMX spectrometer. 1-D ^{13}C MAS NMR spectra of the *Rb. sphaeroides* R26 RCs with $[U\text{-}^{13}\text{C},^{15}\text{N}]$ BPheo *a* were collected with a Bruker MSL400 spectrometer.

3. Results and discussion

In the present work we describe new and efficient experimental conditions for the photoaccumulation of H_A^- in presence of reductant and mediators that rapidly reduce P^+ . Photoaccumulation of H_A^- is achieved in FTIR film of the *Rb. sphaeroides* RCs in the presence of either sodium dithionite and cytochrome *c*, or sodium dithionite and dyes (potassium indigoterasulfonate and neutral red). Light-induced FTIR difference spectra characteristic of the formation of H_A^- are reported.

In order to find out optimum conditions for the photoaccumulation of H_A^- , RCs from *Rb. sphaeroides* R26 were illuminated at 22°C in the presence of reductant (dithionite) and mediator (ITS and NR) and optical spectral changes characteristic for trapped H_A^- were observed (Fig. 2). The same changes were observed with sodium dithionite and cytochrome *c* (spectra not shown). The optical spectra were found to be similar to those reported for the *Rb. sphaeroides* R26 RCs with dithionite and cytochrome *c* in solu-

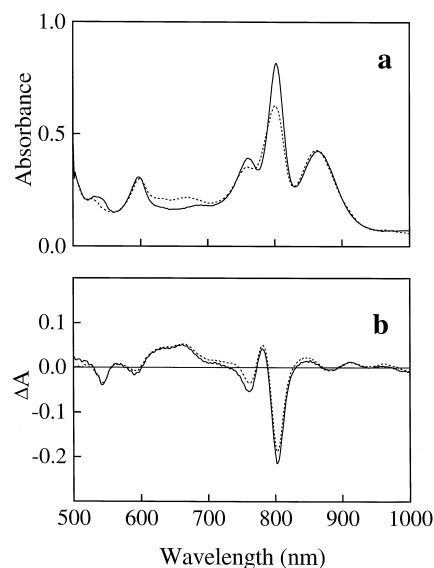


Fig. 2. Changes in the optical absorption spectrum of *Rb. sphaeroides* R26 RCs in 10 mM Tris-HCl (pH 8)/0.1% Triton X-100 buffer with actinic illumination ($I \sim 0.2 \text{ W cm}^{-2}$, 3 min, $\lambda > 645$ nm) in the presence of 100 μM potassium indigoterasulfonate and 100 μM neutral red and 2 mM sodium dithionite ($T = 22^\circ\text{C}$). $[\text{RC}] = 2.75 \mu\text{M}$. (A) Absorption spectra taken before (solid line) and after (dashed line) actinic illumination. (B) Light minus dark absorption difference spectrum for photoaccumulation of H_A^- (solid line). The dashed line represents the difference spectrum for the 20 min dark relaxation of H_A^- .

tion [17] and with dithionite and dyes systems [18]. H_A^- formation is demonstrated by the observation of characteristic optical spectral changes: the selective bleaching of the Q_y band at 760 nm and Q_x band at 542 nm, belonging to BPheo *a*, and bleaching at 590 nm and bleaching and/or a blue shift at 802 nm due to the accessory BChl(s). In addition, a broad absorption increase in the 600–700 nm region attributed to the BPheo radical anion band around 660 nm as well as small absorption increases at 846, 914 and 962 nm are observed.

For the photoaccumulation of H_A^- in a film of *Rb. sphaeroides* R26 RCs on the CaF_2 window in presence of dyes and dithionite (a) or cytochrome *c* and dithionite (b), the optical spectral changes characteristic of trapped H_A^- are observed (Fig. 3).

The light minus dark FTIR spectra (1800–1100 cm^{-1} range) of native *Rb. sphaeroides* R26 and *Rb. sphaeroides* with $[U\text{-}^{13}\text{C},^{15}\text{N}]$ labelled BPheo are

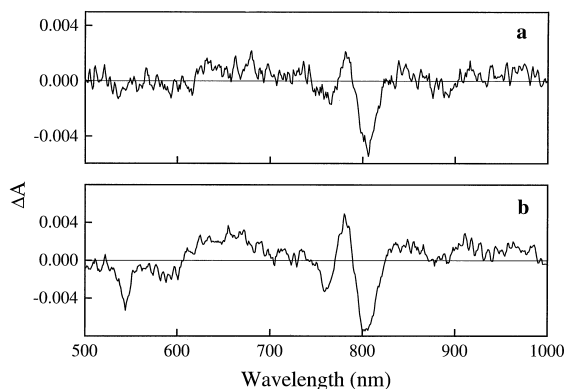


Fig. 3. Single scan spectra of the dark relaxation of H_A^- of *Rb. sphaeroides* R26 RC film on a CaF_2 window at $T = 22^\circ C$. Photoaccumulation of H_A^- was performed in the presence of (a) cytochrome *c* and sodium dithionite; (b) potassium indigotetrasulfonate and neutral red and sodium dithionite. The relaxation time was 20 min.

shown in Fig. 4. The main features are negative bands at 1745, 1732, 1674 cm^{-1} (with a shoulder at 1684 cm^{-1}), 1656 and 1623 cm^{-1} , and positive

bands at 1703 and 1590 cm^{-1} . Small features are reproducibly observed as a negative band 1395 cm^{-1} and positive bands at 1466, 1373, 1347 and 1333 cm^{-1} (Fig. 4a). The negative bands in these spectra correspond to the disappearing neutral H_A state, the positive ones to the appearing anion H_A^- state. Both states include changes in the BPheo electron acceptor and its binding site within the RC protein as well as possible changes of the accessory BChls and at more distant sites of the protein. The spectra obtained are in a good agreement with the spectra published by Nabadryk et al. [12] and assigned to the H_A^-/H_A state.

The most indicative band for the H_A^- state is the band at 1590 cm^{-1} [12,19], as it was also found in spectra of other bacterial RCs [20]. This band is reduced in intensity by more than 50% in the H_A^-/H_A spectrum of *Rb. sphaeroides* R26 RCs with [$U-^{13}C,^{15}N$] BPheo *a* (Fig. 4). Also the intensity of the band at 1466 cm^{-1} decreases by more than 50% in the H_A^-/H_A spectrum of *Rb. sphaeroides* R26 RCs with [$U-^{13}C,^{15}N$] BPheo *a*. This indicates that at least 50% of the photoactive BPheo *a* in *Rb.*

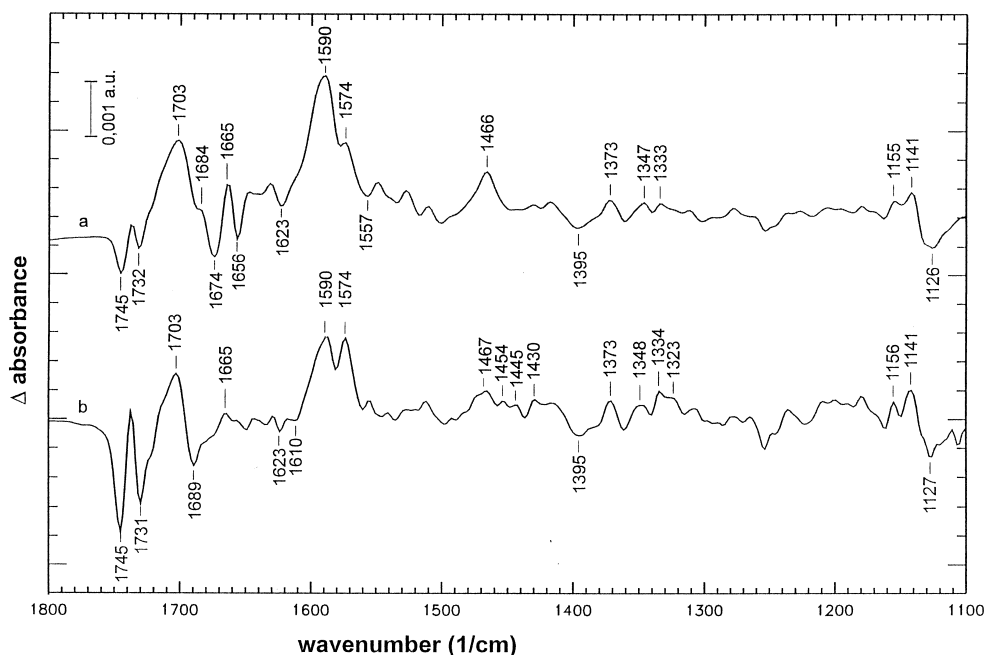


Fig. 4. Light-induced H_A^-/H_A FTIR difference spectra of *Rb. sphaeroides* R26 RCs in 1 M Tris-HCl (pH 8)/0.1% Triton X-100 buffer at $22^\circ C$ after addition of 0.2 M sodium dithionite, 2 mM potassium indigotetrasulfonate and 4 mM neutral red. (a) Unlabelled *Rb. sphaeroides* R26 RCs, (b) *Rb. sphaeroides* R26 RCs with [$U-^{13}C,^{15}N$] BPheo *a* at H_A site. 5000 interferograms co-added.

sphaeroides R26 RCs is substituted by the [U - ^{13}C , ^{15}N] BPheo *a*. The spectrum in Fig. 4b indicates a successful incorporation of [U - ^{13}C , ^{15}N] BPheo *a* in the *Rb. sphaeroides* R26 RCs.

The $\text{H}_\text{A}^-/\text{H}_\text{A}$ FTIR-difference spectra of *Rb. sphaeroides* R26 RCs in which [U - ^{13}C , ^{15}N] labelled BPheo *a* replaces the photoactive BPheo will allow the BPheo modes to be assigned and to be discriminated from those of the RC protein. A normal mode calculation analysis is in progress now and the results will be published in a future publication.

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