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Electrochemical redox titration of cofactors in the reaction center from *Rhodobacter sphaeroides*

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The electrochemical redox poising of the primary electron donor P and of the quinone electron acceptor(s) Q in isolated reaction centers from *Rhodobacter sphaeroides* in an ultra-thin-layer electrochemical cell, monitored by chronoamperometry and by spectroscopy in the visible/near-infrared region, is reported. Electrical application of a redox potential of +0.4 V (vs. Ag/AgCl/3 M KCl) leads to quantitative formation of the π-cation radical of P within a few minutes. The oxidized product can be re-reduced to the neutral species by application of 0 V, and full reversibility is maintained over many cycles. By poising at a series of intermediate potentials, a titration curve for the 865 nm P band was obtained, which could be fitted to a Nernst function with $E_m = 0.485$ vs. SHE and $n = 0.96$. By application of negative potentials (−0.2 V and −0.45 V vs. Ag/AgCl/3 M KCl), the quinone electron acceptors were reversibly reduced as demonstrated by the shift of bacteriopheophytin absorption and drastically changed kinetics of charge recombination. The use of this thin-layer electrochemical technique for the determination of midpoint potentials, for the investigation of redox-poised electron transfer reactions as well as for spectroscopy in the mid-infrared region is discussed.

Photosynthesis; Reaction center; Electrochemistry; Redox potential; Electron transfer

1. INTRODUCTION

Bacterial photosynthetic reaction centers (RC) store about 40% of the energy of the absorbed photon in a state of charge separation between the primary electron donor P (a bacteriochlorophyll dimer) and the quinone electron acceptor (Q_A or Q_B). The free energy stored in this state corresponds to the sum of the energy needed to shift the redox poise of P/P^+ and of Q/Q^- from the dark-adapted state of the RC (P and Q neutral) to the accumulation of the charged radicals.

Redox titrations of spectrophotometric signals play an important role in the study of the molecular mechanisms in photosynthetic RC, either for the determination of redox midpoint potentials or for the analysis of electron transport reactions at a given redox poise. Classically, chemical titrants in connection with redox mediators are used to obtain the desired redox potential. Electrochemical titrations, in which the potential is applied electrically, offer a number of advantages over this more traditional redox chemistry. These include fast equilibration with the applied potential, access to a wider potential range, repeated reduction and oxidation of the sample without dilution problems, direct measurement of the oxidative/reductive current and the amount of charge transferred, and the use of thin-layer cell designs in which spectrophotometric measurements in the mid-infrared region are feasible.

Techniques for electrochemical titration of redox components in thin-layer cells with spectroscopic monitoring in the visible spectral range have been reviewed [1]. Although electrochemical redox titrations of photosystem I and photosystem II subchloroplasts were reported some years ago [2,3], there has been surprisingly little further development of these techniques in the field of photosynthesis.

We have recently developed a new type of ultra-thin-layer electrochemical cell for optical and infrared investigation of redox proteins [4]. The path-length of this cell can be as small as 8–10 μm and thus allows accurate transmission measurements even in infrared spectral regions where water absorbs strongly. With this path-length, the total cell volume is within the diffusion layer. Thus, equilibration with the applied potential proceeds with a half-time in the order of tens of seconds, ideally dependent only on the diffusion constant of the protein complex or of the mediator. Our previous report was on the use of this IOTTLE (Infrared and Optically Transparent Thin-Layer Electrochemical) cell for spectroelectrochemistry of a small, water-soluble protein [4]. We now report that the technique is also applicable to a large, detergent-solubilized membrane protein complex, the bacterial

Abbreviations: RC, reaction center; *Rb.*, *Rhodobacter*; (FT)IR, (Fourier transform) infrared; P, primary electron donor; BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; Q_A and Q_B, primary and secondary quinone electron acceptor

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photosynthetic RC from *Rhodobacter (Rb.) sphaeroides*.

2. MATERIALS AND METHODS

Rhodobacter (Rb.) sphaeroides R26 reaction centers were solubilized in phosphate buffer (12.5 mM, pH 7.4) with 0.1% octylglucoside as detergent, 62.5 mM KCl as electrolyte and 0.25 mM ferrocyanide or 20 mM 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ-0) as mediators. The RC were concentrated to a final concentration of approx. 0.5 mM. 5 μl of this suspension were sufficient to fill the electrochemical cell. Filling procedure and electrical connections to the working, counter and Ag/AgCl reference electrode were as reported [4]. Spectra in the visible/near-infrared region were recorded at 8°C on an instrument built in our laboratory. Time-resolved absorbance changes at 865 nm were recorded using a Xenon flash (30 μs half-time) and a detection unit built to our design. Electrochemical measurements (chronoamperometry, coulometry) were controlled from a potentiostat and from software developed in our laboratory. All potentials quoted are vs. Ag/AgCl in 3 M KCl if not stated otherwise; add 0.208 V for potentials vs. SHE.

3. RESULTS AND DISCUSSION

Fig. 1a (solid line) shows the absorbance spectrum in the visible/near-infrared range of the RC in the IOTTLE cell equilibrated at an electrode potential of 0 V. The oxidation of P at an applied potential of +0.4 V was monitored by chronoamperometry (Fig. 1b). A current was observed which decayed over several minutes effectively to zero: the positive sign indicates that the direction of electron transfer was from the reaction center to the electrode. After 4 min, the spectrum of the electrogenerated species was recorded (dashed line in Fig. 1a). The decrease of the P absorbance band at 865 nm, the blue shift of the 800 nm absorbance, as well as the absorption increase at wavelengths above ca. 930 nm clearly indicate the transformation of the $BChl_2$ into its π-cation radical form. Difference spectra (data not shown) exhibit stable isosbestic points for the oxidative and reductive titration as well as complete reversibility for many cycles. This clearly indicates equilibrium between the two redox states and the electrode potential.

Fig. 2a shows absorbance spectra of the RC as a function of the applied potential. The amplitude of the 865 nm band, plotted vs. the applied potential, is shown in Fig. 2b. It can be approximated with a Nernst curve; a least-squares fit yields a midpoint potential of +0.277 V vs. Ag/AgCl/3 M KCl (corresponding to E_m = 0.485 vs. SHE) and 0.96 transferred electrons. In contrast to usual fitting of Nernst curves, where the number of electrons is held fixed to integral values of 1,2,..., we have left n as a variable fit parameter to confirm the validity of the data.

In seeking to compare this value of E_m with the currently accepted midpoint potential for the primary electron donor P of isolated *Rb. sphaeroides* RC, we have found that considerable confusion exists in the literature. A value of +0.45 V vs. SHE is often quoted, as

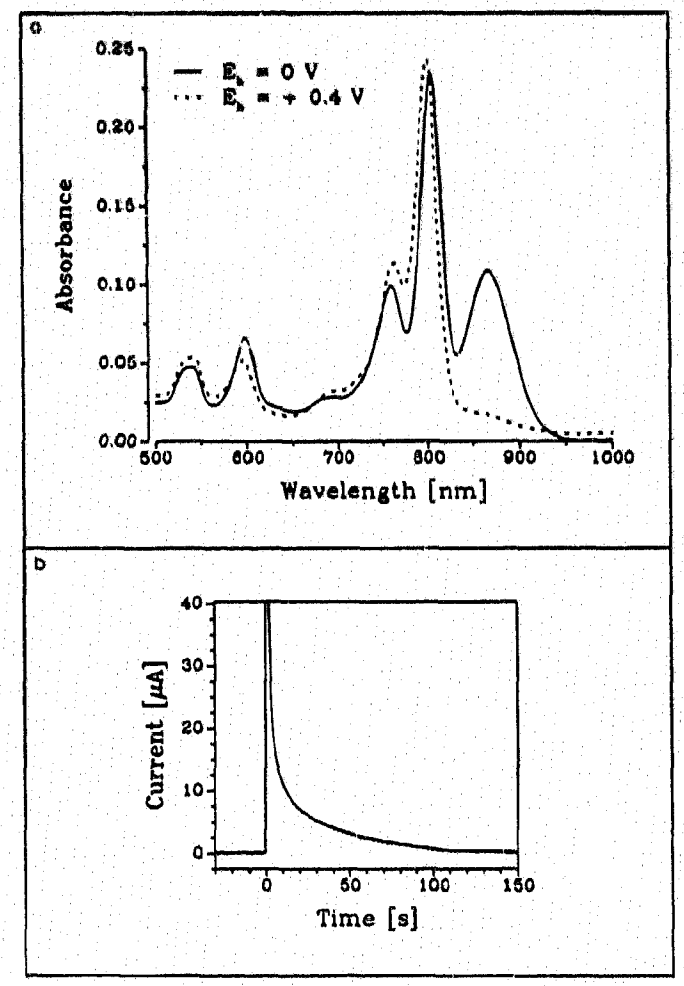

Fig. 1. (a) Absorbance spectrum of *Rb. sphaeroides* R26 RC in the spectroelectrochemical cell equilibrated at 0 V (full line) and at +0.4 V (dashed line). (b) Current as a function of time following a potential step (at t = 0) from 0 V to +0.4 V.

well as values up to +0.49 V. However, the papers cited in this context do not in fact contain original titration data, but rather cite other papers. We have attempted to trace this chain of citations back to original work, but have been forced to conclude that a precise determination of the midpoint potential by chemical titration actually does not exist. The most realistic assessment thus appears to be that of Clayton [5], who states simply that the midpoint potential lies between +0.45 V and +0.5 V vs. SHE.

When considering whether our measured value of +0.277 V (0.485 V vs. SHE) is the correct midpoint potential, it should be noted that: (i) our data points show a very much better fit to the Nernst curve than is frequently the case with chemical titrations; (ii) electrical methods permit the application of a wide range of potentials, thus ensuring that the titration is taken to its end points in both directions; (iii) monitoring of the current provides an objective measure that equilibrium

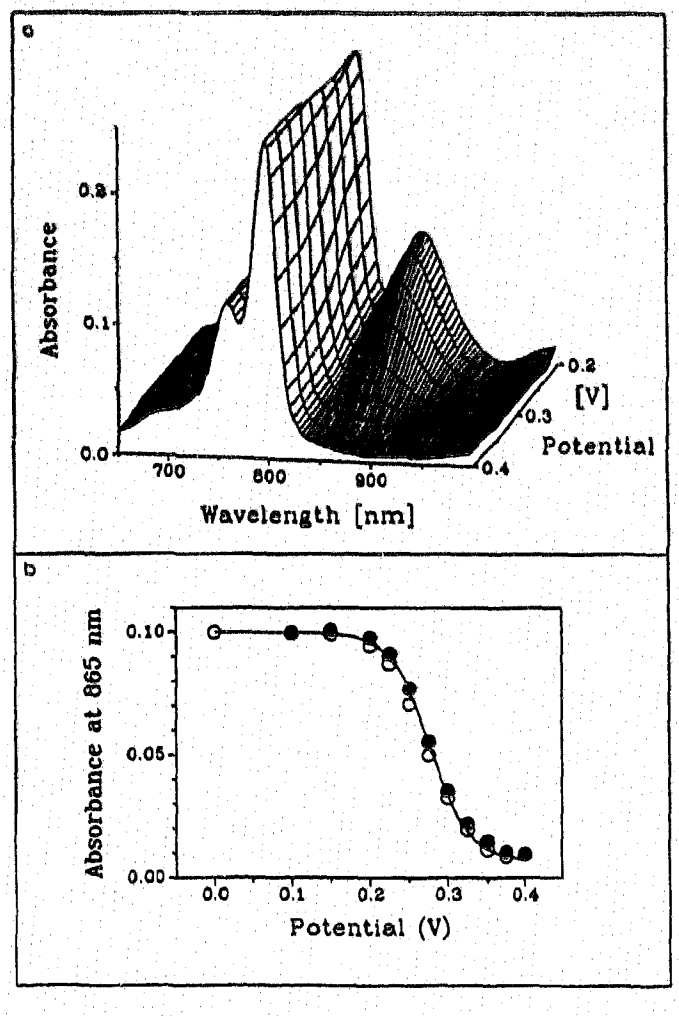

Fig. 2. (a) Absorbance spectra of *Rb. sphaeroides* R26 RC in the spectroelectrochemical cell as a function of the applied potential. (b) Reductive (○) and oxidative (●) titration of the absorbance difference at 865 nm. Solid line represents least squares fit to a Nernst function (E_m = 0.485 V vs. SHE; 0.277 V vs. Ag/AgCl/3 M KCl, n = 0.96).

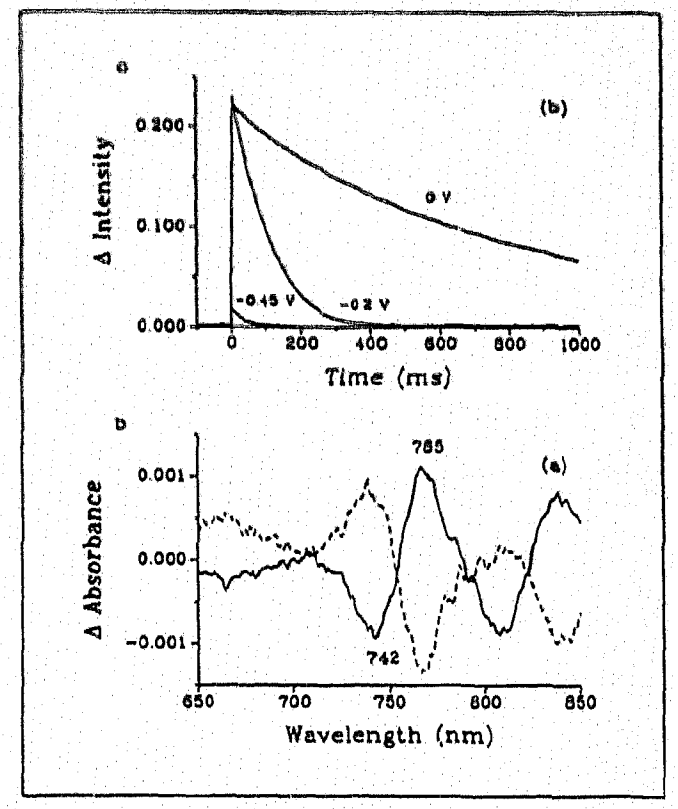

Fig. 3. (a) Time-resolved intensity changes at 865 nm (absorption of the primary electron donor P) upon equilibration of the RC with applied potentials of 0 V, −0.2 V and −0.45 V. (b) Difference spectra of *Rb. sphaeroides* R26 RC ('bacteriopheophytin shift') upon Q_A reduction (full line) and reoxidation (dashed line). For details, see text.

has been reached; (iv) one sample can be repeatedly titrated without errors arising from dilution.

Using UQ-0 as a mediator, the quinone electron acceptors of the RC can be reduced electrochemically. At equilibrium with an applied potential of 0 V, both Q_A and Q_B (if present) are in their neutral form. Lowering of the potential to approx. −0.2 V leads to reduction of UQ-0, which reduces Q_B; a potential of −0.4 V finally leads to reduction of Q_A.

Fig. 3a shows time-resolved absorbance changes at 865 nm (P absorbance) due to the primary electron donor oxidation and rereduction by charge recombination. At an electrode potential of 0 V, charge recombination $P^+Q_B^- \longrightarrow PQ_B$ proceeds with a half-time of 1.4 s, with a rapid phase (half time of 0.48 s) due to RC possessing UQ-0 as an 'artificial' Q_B (see [6] for charge recombination rates of UQ-0 at the Q_B site). When the potential is set to −0.2 V, the slow phase disappears and $P^+Q_A^- \longrightarrow PQ_A$ charge recombination is observed (half-time of 0.07 s), indicating that Q_B (the ubiquinone-10 molecules originally occupying the site as well as the UQ-0 molecules occupying 'empty' Q_B sites) had been reduced electrochemically. At a potential of −0.45 V, Q_A becomes reduced and the signal amplitude decreases drastically due to the very rapid charge recombination from the intermediary electron acceptor bacteriopheophytin. In addition, the process of electron donation from reduced UQ-0 in solution to the photooxidized primary electron donor P^+ is indicated by a rapid phase of the re-reduction (half-time of 0.015 s–0.02 s) in Fig. 3a.

The electrochromic shift of the bacteriopheophytin absorption band presents additional evidence for the reversible reduction of Q_A and/or Q_B. Fig. 3b (full line) shows the difference spectrum (−0.45 V minus 0 V; both potentials quoted vs. Ag/AgCl/3 M KCl) obtained upon Q reduction. Starting from the reduced quinone, the difference spectrum of reoxidation (dashed line) indicates full reversibility.

The redox equilibria between Q_A, Q_B and UQ-0 as well as the charge recombination mechanisms observed are consistent with the midpoint potentials of −0.26 V for Q_A [7], −0.17 V for Q_B [8] and −0.14 V for UQ-0 (M. Bauscher, unpublished results). From our data it is clear that UQ-0 in its reduced state can shuttle electrons to the native Q_B. It cannot be decided, however, if reduced Q_B is replaced by reduced UQ-0. According to McPherson et al. [6], replacement of Q_B by UQ-0 does not occur if both are in the neutral state.

For the investigation of cytochrome *c* [4,9], no mediators were necessary for rapid electrochemistry in the thin-layer cell. Although this approach may be possible with RC, the diffusion of a small mediator molecule such as ferricyanide is clearly very much faster. Ferricyanide has the advantages of a midpoint potential close to that of P_{865}, and no absorption in the spectral region of interest. In general, the selection of mediators for redox titrations of electron transport components represents a compromise between maximization of the rate of equilibration, and minimization of interfering absorbances. In chemical redox titrations, the mediators must be chosen so that redox buffering exists throughout the potential range, in order that the potential can accurately be measured with a redox electrode. For electrochemical titrations this need not be a consideration, since each potential is accurately applied and held constant by the potentiostat.

Since the IOTTLE cell used here was designed to work from the UV to the mid-IR (10 μm), vibrational spectra of enzymes in different redox states can be recorded, with a precision corresponding to single-bond absorbance. We emphasize that this would be extremely difficult, if not impossible, with chemical oxidation or reduction. Vibrational spectroscopy of redox enzymes allows the molecular basis of the stabilization of a redox state to be studied on the level of the geometry and strength of individual bonds of the cofactor or protein. We have used this approach to compare the vibrational spectra of the electrochemically-generated $BChl_2$ cation and of the Q_A anion radicals in the RC with that of the photochemically charge-separated $BChl_2$ $^*Q_A^-$ state [10].

Apart from precise determinations of redox midpoint potentials and UV-VIS as well as mid-infrared spectroscopic investigations of redox enzymes, the electrochemical titrations in a spectroelectrochemical cell permits precise and reversible poising of redox cofactors at potentials between approx. +0.60 V and −0.8 V (vs. Ag/AgCl) in aqueous media. In preliminary experiments, we have been able to reduce individually the hemes of the *Rhodopseudomonas viridis* RC. We consider that this approach is of general use for the study of biological redox reactions.

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