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Electrochemical titration of the cytochrome hemes in the *Rhodopseudomonas viridis* reaction center

Cyclic equilibrium titrations yield midpoint potentials without evidence for heme cooperativity

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The redox and spectral characteristics of the 4-heme cytochrome *c* unit of the photochemical reaction center from *Rhodopseudomonas viridis* were studied by a combination of protein electrochemistry and spectroscopy using an ultra thin-layer spectroelectrochemical cell. Quantitative and reversible reduction of the high-potential and the low-potential hemes was performed in cyclic titrations to record the optical difference spectra in the α-band region. The titration of the absorbance from the high-potential hemes can be approximated with a sum of 2 Nernst functions with E_m = 0.113 V and E_m = 0.175 V. The corresponding titration of the absorbance from the low-potential hemes yielded E_m = −0.257 V and E_m = −0.175 V (all potentials quoted vs. Ag/AgCl/3 M KCl; add 0.208 V for potentials vs. standard hydrogen electrode). The high-potential hemes equilibrate rapidly and titrate identically for oxidative and reductive titrations. Under identical conditions, the low-potential hemes exhibit a hysteresis, thus indicating much slower equilibration with the applied potential. Cyclic titrations with increasing equilibration periods, however, indicate the disappearance of the hysteresis for equilibration periods approximately twice as long as for the high-potential hemes. We take this as evidence for a slower internal equilibration, but against a cooperativity of the low-potential hemes as observed for other multi-heme cytochromes.

Rhodopseudomonas viridis; Reaction center; Cytochrome *c*; Heme interaction; Electrochemistry

1. INTRODUCTION

Cytochrome molecules play an important role as electron carriers in mitochondrial and photosynthetic electron transport. In the primary bacterial photosynthetic reactions, cytochromes act as secondary electron donors to the photosynthetic reaction center (RC). Several ways for this secondary electron donation have been characterized. In *Rhodopseudomonas*, like *Rb. sphaeroides*, *Rb. capsulatus*, *R. rubrum* or *Rps. palustris*, a water-soluble cytochrome c_2 serves as immediate electron donor to $BChl_2$, while in others, like *Rps. viridis* or *Chromatium vinosum*, a tightly-associated cytochrome subunit of the RC with 4 hemes functions as immediate electron donor, being itself re-reduced by a soluble *c*-type cytochrome ([1], and references cited therein).

The structure and function of the tightly associated cytochrome *c* subunit of *Rps. viridis* have been well-characterized. The spatial arrangement of the hemes has been deduced from X-ray structure analysis of crystallized *Rps. viridis* RC [2,3], and redox midpoint potentials of the hemes have been determined by dark titrations of optical or EPR signals [4–8]. Evidence was collected from flash-induced absorbance changes [4,5,7] that the heme with its α-band at 559 nm (E_m = 370–380 mV) is the immediate donor to $BChl_2$. However, the assignment of 4 hemes characterized by their α-band absorption and midpoint potential (559 nm, +380 mV; 556 nm, +310 mV; 552 nm, +20 mV; 553 nm, −60 mV, [5]) to the positions defined in the X-ray structure has been ambiguous for a long time. Recently, spectroscopic investigations on crystallized *Rps. viridis* RC [6] and RC oriented in Langmuir-Blodgett monolayer films [9], as well as EPR investigations of oriented chromatophores [8], have led to an assignment of the hemes in the order P_{960} c_{559} (380 mV) c_{552} (10 mV) c_{556} (310 mV) c_{553} (−60 mV). This arrangement (absorbance maxima and E_m taken from [5]) poses the question on the function of one of the low-potential hemes (c_{552}, +10 mV) between the two high-potential hemes (c_{559}, 380 mV and c_{556}, +310 mV), which cannot be conclusively answered at present.

The proximity of the hemes suggests heme–heme in-

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Abbreviations: OTTLE cell, optically transparent thin-layer electrochemical cell; SHE, standard hydrogen electrode.

teractions and possibly heme cooperativity, as has been observed for the tetraheme cytochrome c_3 [10]. Nitschke and Rutherford have investigated heme–heme interaction by monitoring the relaxation rates of the 4 hemes [8], and have proposed either fully magnetically isolated hemes or a magnetic interaction of the c_{556} (310 mV) heme with one or both of the low-potential hemes. Heme cooperativity should be detectable by a difference in the midpoint potentials in oxidative and reductive titrations at finite equilibration conditions. The difference in the measured E_m values, however, could be very small and almost undetectable in chemical titrations, especially with the heme α-bands absorbing very close to each other.

We have recently developed electrochemical techniques for the reduction and oxidation of redox proteins in electrochemical cells with spectrophotometric control [11]. These cells were developed for simultaneous optical and infrared studies and thus designed with optical path-lengths as small as 8–15 μm. With this path-length, the total cell volume is within the diffusion layer and equilibrates rapidly with the applied potential. Originally developed to study small water-soluble proteins using direct electrochemistry at modified electrodes [11], the same technique was applied to large detergent-solubilized proteins, like the bacterial RC, using modified electrodes and mediators [12,13]. Compared to 'classical' chemical titrations, electrical application of a potential in a suitable electrochemical cell offers the advantage of fast equilibration, access to a wider potential range, titration in many cycles without dilution of the sample, and the advantage of monitoring the equilibration process by the current flowing through the electrode and the charge transferred to the redox enzyme as a measure of equilibration. Redox midpoint potentials determined by electrochemical titrations with spectrophotometric control can be as precise as a few millivolts.

In the work presented here, we have used these electrochemical techniques for the determination of the heme redox midpoint potentials in the cytochrome subunit of the *Rps. viridis* RC and the analysis of possible heme cooperativity and interaction.

2. MATERIALS AND METHODS

Rps. viridis reaction centers (RC) were prepared according to the method of Welte et al. [14]. For electrochemical measurements, LDAO was exchanged against 1% octylglucoside as a detergent, and the RC were concentrated to a final concentration of approx. 0.5 mM in phosphate buffer, pH 7.4, containing 250 mM KCl as electrolyte. The following mediators were used at a final concentration of 50 μM (E_m vs. Ag/AgCl/3 M KCl in parentheses): ferricyanide (+216 mV); 1,1-dimethylferrocene (+133 mV); tetrachlorobenzoquinone (+74 mV); ruthenlumhexaminechloride (−8 mV); 1,2-naphthoquinone (−63 mV); trimethylhydroquinone (−108 mV); menadione (−220 mV); 2-OH-1,4-naphthoquinone (−333 mV); anthraquinone-2-sulphonate (−433 mV); benzylviologen (−560 mV); methylviologen (−628 mV).

The electrochemical cell used for the redox titrations was described previously [11], and used with small modifications [15]. The surface of the gold grid working electrode was chemically modified according to the procedure described in [15,16]. The filling procedure and the electrical connections to the working, counter and Ag/AgCl/3 M KCl reference electrode were as described [11]. Spectroscopic measurements were performed at 6°C on an instrument built to our design. Electrochemical measurements were performed as described [13]. All potentials quoted are vs. Ag/AgCl in 3 M KCl if not stated otherwise (add 0.208 V for potentials vs. standard hydrogen electrode, SHE) and are within ca. 5 mV precision.

3. RESULTS AND DISCUSSION

Electrochemical redox titrations of the high- and low-potential hemes were performed by first equilibrating the sample in the electrochemical cell at 0 V for 15 min, followed by recording a single-beam spectrum between 500 and 600 nm. At 0 V, the high-potential hemes should be fully reduced, thus providing a reference spectrum for the titration. The potential was then increased in 10 mV intervals to 0.26 V, either by intermediately returning to the starting potential (0 V) or by stepwise increase of the applied voltage. Each potential-step resulted in a current which decayed effectively to zero over several minutes; on the basis of these chro-

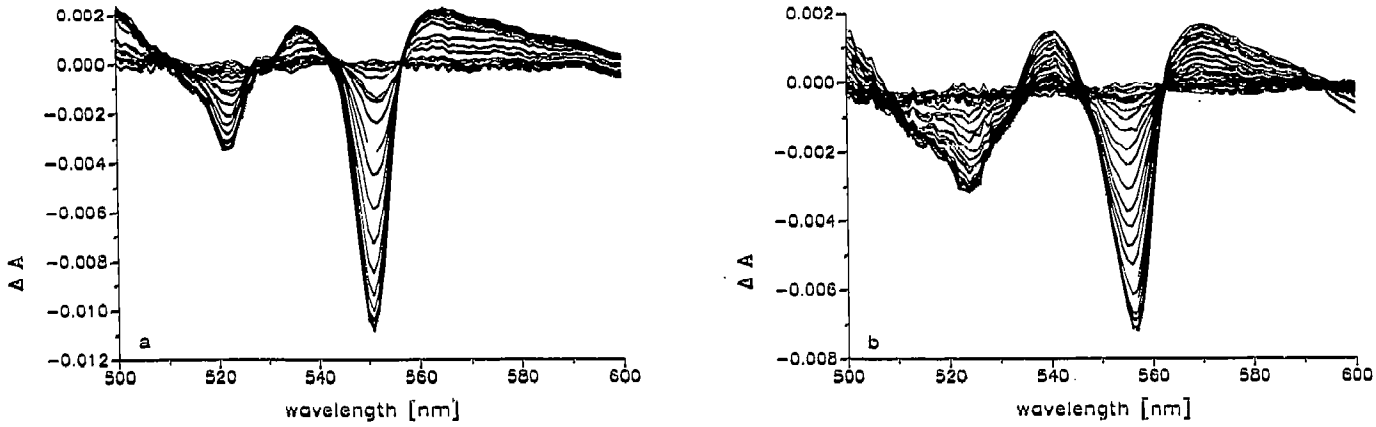

Fig. 1. Oxidative electrochemical titration of the low-potential hemes (a) and the high-potential hemes (b) of the cytochrome *c* subunit from *Rps. viridis*. Difference spectra were taken in 10 mV intervals from 0–0.26 V (vs. Ag/AgCl/3 M KCl) with 0 mV as a reference for the high-potential hemes, and in 20 mV intervals between −0.38 and 0 V with −0.38 V as a reference for the low-potential hemes.

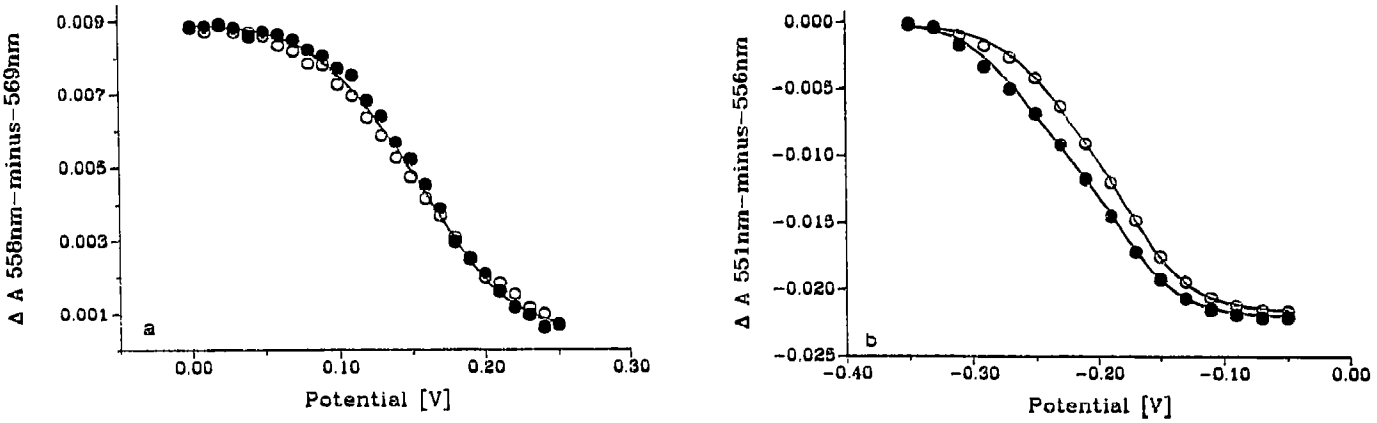

Fig. 2. Absorbance difference as a function of the applied potential in reductive (●) and oxidative (○) titrations. (a) High-potential hemes: 558-minus-569 nm. The solid line represents a least-square fit to the sum of 2 Nernst functions with $n = 1$ and $E_m = 0.113$ V and $E_m = 0.175$ V. (b) Low-potential hemes: 551-minus-556 nm. The solid lines represent least-square fits to the sum of 2 Nernst functions with $n = 1$. For the reductive titration, $E_m = -0.263$ V and -0.180 V were obtained. The oxidative titration yielded $E_m = -0.240$ V and -0.170 V.

noamperograms (data not shown) the time of equilibration was chosen to at least 10–15 min. After equilibration, another single-beam spectrum was recorded and the difference spectrum calculated with the spectrum recorded at 0 V as a reference. The low-potential hemes were titrated using the same procedure, except that the reference spectrum was recorded at −0.38 V and the potential was increased in intervals of 20 mV to 0 V.

Fig. 1 shows the redox-induced difference spectra of the low-potential hemes (Fig. 1a; −0.38 V minus x V, with x ranging from −0.38 to 0 V) and of the high-potential hemes (Fig. 1b; 0 V minus x V, with x ranging from 0.01–0.26 V). Both sets of difference spectra in this oxidative titration, within experimental error, show isosbestic points due to the close absorbance maxima of the heme couples. Nevertheless, the shifts of the α-band maxima with progressive titration are clearly seen in each set of difference spectra. Identical sets of difference spectra for the high-potential hemes were obtained for titrations performed at stepwise increasing potentials and for titrations with the intermediately applied reference potential (0 V).

The sets of redox-induced difference spectra were analyzed by calculating titration curves for all wavelength elements in the spectra, with a reference wavelength to be chosen to isosbestic points or regions outside the α-band absorption in order to correct for baseline shifts. Fig. 2 shows the amplitude differences for 558-minus-569 nm (approx. equal amplitude of the high-potential hemes, Fig. 2a) and 551-minus-566 nm (approx. equal amplitude of the low-potential hemes, Fig. 2b) as a function of the applied potential. The full lines represent least-square fits with a sum of 2 Nernst equations with $n = 1$, assuming equal contributions from the α-band of both hemes. In the case of the high-potential hemes, both the reductive (closed circles) and the oxidative (open circles) titration can be approximated with a single fit of a sum of 2 Nernst functions, yielding midpoint potentials of $E_m = 0.113$ V and $E_m = 0.175$ V).

In the case of the low-potential hemes, a hysteresis is obtained between the reductive and the oxidative titration. In a first attempt, both curves were fitted individually with sums of Nernst curves. For the oxidative titration, this fit yielded $E_{m,ox} = -0.240$ V and $E_{m,ox} = -0.170$ V. For the reductive titration, the midpoint potentials obtained were $E_{m,red} = -0.263$ and $E_{m,red} = -0.180$ V. This difference in midpoint potentials of ca. 20–30 mV was reproducibly obtained for equilibration times which yielded identical potentials for the oxidative and the reductive titrations of the high-potential hemes. A re-inspection of the chemical titrations in [5] indicates a similar effect: the reductive titration monitored at 552 nm (Fig. 1b in [5]) involves ca. 20 mV more negative potential than the oxidative titration.

In full thermodynamic equilibrium, a redox component interacting with another should exhibit identical

Table I

Heme midpoint potentials in mV (vs. standard hydrogen electrode)

	Heme[a]			
	1	2	3	4
α-band (nm)[b]	553	556	559	552
Electrochemical titration	**−49**	**+321**	**+383**	**+33**
Fritzsch et al. [6]	−60	+300	+370	+10
Nitschke & Rutherford [8]	−80	+320	+400	+20
Dracheva et al. [5]	−60	+310	+380	+20
Allegria & Dutton [7][c]	−50	+259	+370	+50
Shinkarev et al. [17][d]	−50	+312	+360	+20

[a] Numbered according to the appearance along the amino acid chain.
[b] Absorbance maxima taken from spectra in this work.
[c] Values taken were determined for Langmuir-Blodgett films of RC in lipids.
[d] Values were obtained using a decomposition method for the overlapping α-bands.

redox midpoint potentials for reductive and oxidative titrations. Finite equilibration times among interacting redox components, however, may lead to different values. Electrochemical redox titrations at nonequilibrium conditions thus may provide evidence for heme interaction and interheme equilibration kinetics.

In contrast to chemical titrations, electrical application of a redox potential offers the advantage of titrating one sample in repeated cycles without any dilution problem. We have used this advantage for the comparison of reductive and oxidative titrations of the low-potential hemes at increasing equilibration times. For these experiments, the time-course of the reductive currents at each potential step was used as a measure for complete equilibration as demonstrated in [15]. The width of the hysteresis decreased with increasing equilibration time, and almost perfect matching of the reductive and oxidative titrations was obtained for prolonged equilibration periods approximately twice as long as necessary for the high-potential hemes. Thus, within the accuracy of the electrochemical titration of a few millivolts, and in the time domain of 20–30 min, no cooperative behaviour of the low-potential hemes or of the high-potential hemes can be observed. The midpoint potentials determined for these extended equilibrations were $E_m = -175$ mV and $E_m = -257$ mV (vs. Ag/AgCl/3 M KCl).

In Table I, the electrochemically-determined potentials (for the purpose of comparison calculated vs. SHE) are summarized together with published redox midpoint potentials. Within the errors of typical chemical titrations, the potentials determined electrochemically are in good agreement with those previously determined by chemical titrations of EPR or optical signals.

For the evaluation of our redox-induced difference spectra, we have assumed that at given wavelengths equal contributions from the α-bands of the high-potential hemes or the low-potential hemes can be obtained. Shinkarev et al. [17] have analyzed the α-band region with overlapping heme spectra using a deconvolution method with a linear least-square procedure to obtain the fraction of every heme contributing to the absorbance at a specific wavelength. They pointed out that differences in the extinction coefficients of the individual hemes may lead to ambiguities in the titration curves. Nevertheless, their quantitative analysis yielded midpoint potentials in good agreement with previous determinations and our analysis, thus justifying our approach using single wavelength pairs for the high-and the low-potential hemes.

In conclusion, electrochemical titration of the tetraheme subunit of the *Rps. viridis* RC shows quantitative and reversible titration of all 4 hemes and thus allows precise determination of the redox midpoint potentials as well as individual and reversible redox poising. The electrochemically-determined potentials may serve as a basis for adjusting the estimation of the midpoint potentials from electrostatic calculations [18]. In a further paper, the use of this electrochemical poising of redox potentials to obtain redox-induced infrared difference spectra of the individual hemes as previously obtained for soluble *c* [11] and c_2 [19] cytochromes will be shown. Apart from information on the conformational changes associated with heme reduction, the infrared difference spectra provide marker bands specific for each heme; their titration should yield individual redox midpoint potentials without the need for band deconvolution.

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