Spectral, redox and kinetic characteristics of high-potential cytochrome *c* hemes in *Rhodopseudomonas viridis* reaction center

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Redox, optical and kinetic characteristics of the four-heme cytochrome *c* tightly bound to the reaction center complexes of *Rhodopseudomonas viridis* have been studied. The two high-potential hemes, previously thought to be identical, are shown to differ in midpoint potentials, absorption spectra and kinetics of photooxidation. One heme is characterized by $E_m = 380 \pm 10$ mV, and a split α-band (a peak at 559 nm and a shoulder at 553 nm) whereas the other has an $E_m = 310 \pm 10$ mV and a symmetrical α-band at 556 nm. Kinetics of laser flash oxidation of the *c*-559/553 heme by the photogenerated P-960$^+$ ($\tau \sim 0.3$ μs) matches closely that of the bacteriochlorophyll reduction and precedes oxidation of the *c*-556 heme, the latter occurring with $\tau \sim 2.5$ μs concurrently with heme *c*-559/553 re-reduction. The data point to heme *c*-559/553 being an immediate electron donor to P-960$^+$. Accordingly, this heme is tentatively identified with the iron-porphyrin group proximal to the bacteriochlorophyll special pair in the three-dimensional model of *Rps. viridis* reaction centers complexes [(1985) Nature 318, 618–624]. Thus, the following reaction sequence is assumed:

c-556 → *c*-559 → P-960$^+$.

(Rhodopseudomonas viridis) *Reaction center* *Multiheme cytochrome* c *Redox potential* *Rapid kinetics*

1. INTRODUCTION

In addition to subunits L, M and H, common for RC from most of the photosynthetic bacteria, RC from *Rps. viridis* contains a tightly bound four-heme cytochrome *c* subunit [1]. Whereas photochemical reactions on the acceptor side of the bacteriochlorophyll special pair (P-960) have been studied sufficiently well in *Rps. viridis* RC [2–7], there are only a few studies on the characteristics and function of this cytochrome.

Abbreviations: P-960 $(BChl)_2$, bacteriochlorophyll special pair; RC, reaction center complex; LDAO, lauryldimethylamine oxide; Mops, morpholinopropane sulfonic acid

According to the conventional point of view, the cytochrome contains a pair of identical high-potential hemes with $E_m = 330$–340 mV and an α-band at 557–558 nm, and two equivalent low-potential hemes with $E_m = -20/-70$ mV and an α-maximum at 552–553 nm [8–11]. Both the high-potential and low-potential hemes are considered capable of rapid (<1 μs) electron donation to P-960$^+$. The same arrangement is suggested for the tightly bound cytochrome *c* in RCs from other bacteria, such as *chromatium vinosum* [11–13], *Chromatium minutissimum* [14] and *Ectothiorhodospira shaposhnikovii* [15,16].

However, recent crystallographic data on *Rps. viridis* RCs have shown each of the 4 iron-porphyrin groups to be in different surroundings in the cytochrome *c* subunit [17]. This points to the

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physico-chemical and functional non-equivalence of all the 4 hemes.

Here we show that the two high-potential cytochrome *c* hemes in RCs from *Rps. viridis* differ in redox potentials, absorption spectra and photooxidation kinetics.

2. MATERIALS AND METHODS

Most of the chemicals were commercial products from Sigma and Fluka.

The cells of *Rps. viridis* were grown anaerobically in a medium employed by Ormerod et al. [18]. RCs were isolated according to [19] with the use of LDAO for chromatophore solubilization; subsequently, LDAO was replaced by Triton X-100 during the purification of the RCs on a DEAE-cellulose column. RC concentration was determined spectrophotometrically with ϵ = 300 $mM^{-1} \cdot cm^{-1}$ at 830 nm [9].

Redox titrations of the high-potential cytochrome *c* hemes were carried out aerobically in 1 cm rectangular optical cells in an Aminco DW-2a spectrophotometer linked to a DL-1080 transient recorder (Data Laboratories). The spectra recorded at various E_h were transferred subsequently from DL-1080 memory to a NOVA-3D computer (Data General) for data processing. The redox potential was measured with a Pt, Ag/AgCl pair of electrodes fed into a V7-27/A1 voltmeter.

P-960 was photoexcited with light pulses from either a LOMO OGM-40 ruby laser (λ = 694 nm; pulse-width, 20 ns) or a Quantel neodymium laser (λ = 1064 nm; pulse-width, 15 ns).

The rapid kinetics of cytochrome *c* and P-960 oxidation-reduction was measured in two single-beam spectrophotometers designed and built in this laboratory by L.A. Drachev. The spectrophotometers were connected to the NOVA-3D minicomputer via a DL-1080 transient recorder.

3. RESULTS

The results of a typical reversible redox titration of *Rps. viridis* RC cytochrome *c* in the 180–450 mV range are given in fig.1. At each E_h, 2–3 min were allowed for equilibration, and a difference spectrum versus the oxidized reference (E_h = 470 mV) was scanned (e.g. see fig.2A). The titration curves were constructed taking the heights of the spectra at 559 nm relative to the baseline connecting the nearly isosbestic points of the spectra at 540 and 567 nm.

The experimental points in fig.1 are nicely fitted by a Nernst curve for two one-electron components (solid line), whereas a single-component approximation is not satisfactory (dashed line). Computer analysis of this particular titration curve yields the E_{m7} values 390 and 320 mV, the high-potential component contributing 60% to ΔA_{559}. The average E_m values (± maximal deviation) for several preparations of RCs are 380 ± 10 and 310 ± 10 mV, and these latter values will be referred to below.

Fig.2A shows that in the course of reductive titration, a maximum of the α-absorption band of the high-potential cytochrome *c* hemes shifts from 559 nm to shorter wavelengths concurrently with the peak broadening and disappearance of the well-resolved shoulder at 553 nm. The individual difference spectra of the higher-potential and lower-potential components are given in fig.2B.

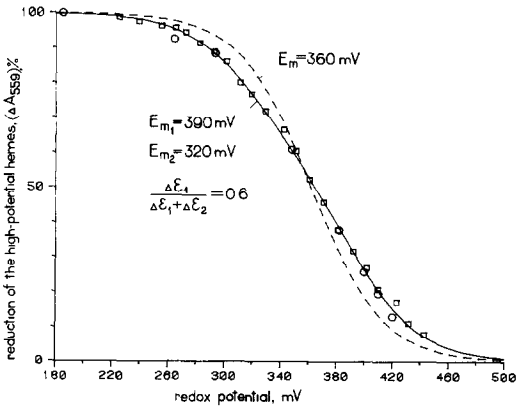


Fig.1. Redox titration of the high-potential cytochrome *c* hemes in reaction center complexes from *Rps. viridis*. The sample and reference cells contained 1 μM RCs in 0.1 M KCl, 0.1% Triton X-100, 25 mM Mops, pH 7.0, at 25°C. Redox mediators: 200 μM DAD, 50 μM 1,4-benzoquinone and up to 2 mM ferricyanide. The reference was poised at E_h = 470 mV and the sample was titrated first with ascorbate down to 150–180 mV (□) and then back with ferricyanide (○). At each point a difference spectrum vs the reference was recorded in the α-band of the cytochrome *c* hemes. The data were computer-analyzed according to the Nernst equation for 1 and 2 one-electron components (the dashed and solid lines, respectively).

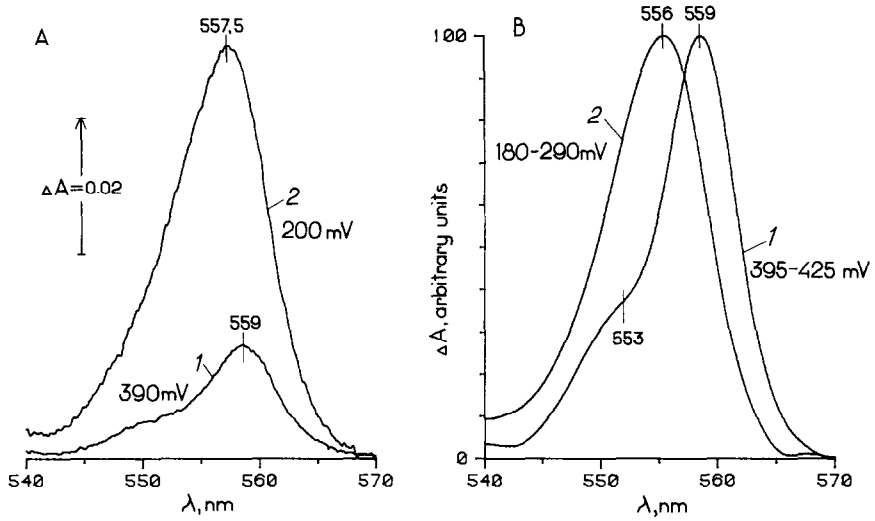


Fig.2. Difference absorption spectra of the high-potential cytochrome *c* hemes. (A) Spectra of the sample at the initial (1) and final (2) stages of the reductive titration vs the reference poised at 470 mV. For conditions, see fig.1. (B) Individual difference spectra of the two high-potential hemes; the curves obtained by subtraction of the spectra recorded at the indicated E_h values have been smoothed and normalized with respect to the amplitude.

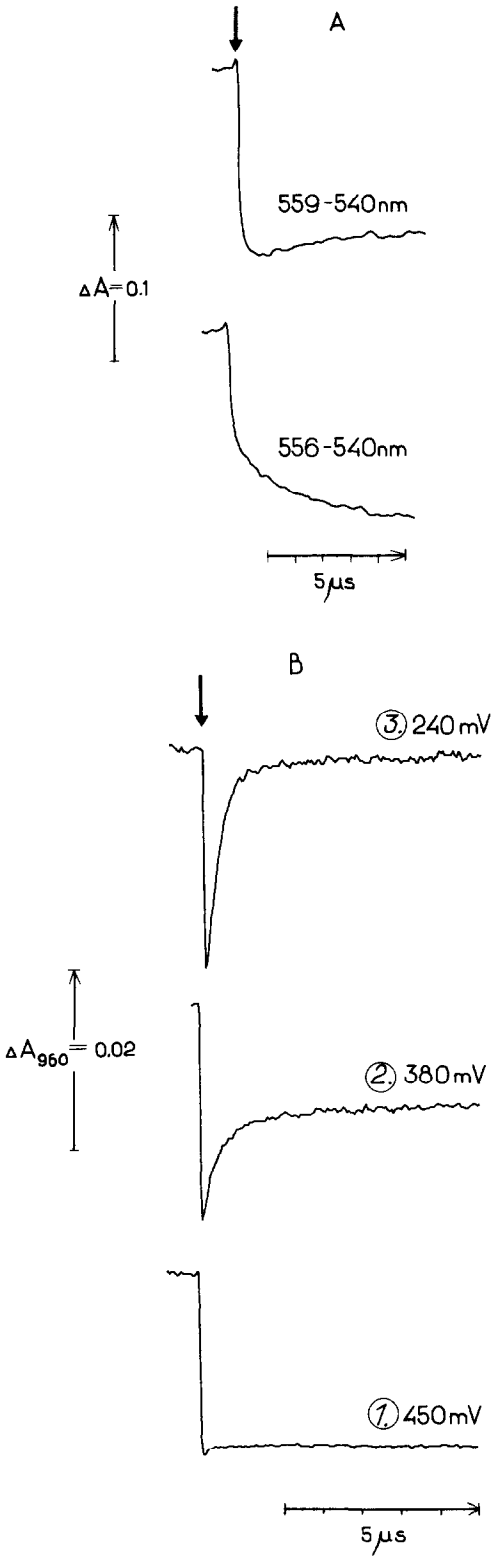
The heme with E_m = 380 mV is characterized by a narrow peak at 559 nm (half-width, 8 nm) and a shoulder at 553 nm, whereas the second heme with E_m = 310 mV reveals a symmetrical α-band centered at 556 nm which has a half-width of 10 nm.

Notably, the spectrum of heme *c*-559/553 can be viewed as a split Q_∞ band centered at 556 nm with the Q_x and Q_y transitions 6 nm apart. Magnetic circular dichroism spectra studied in collaboration with the laboratory of Dr A.M. Arutjunjan (results to be published elsewhere) show that this is indeed the case. Thus, the relationships between the two high-potential RC cytochrome *c* hemes may resemble the situation analyzed earlier for the mitochondrial hemes *b*-562 and *b*-566/558 [20], where 2 identical iron-porphyrin groups acquire different spectral characteristics because of the asymmetric protein environment.

Subsequently, we studied the kinetics of cytochrome *c* oxidation-reduction following photooxidation of P-960 by a laser flash. The experiments were carried out at various wavelengths (540–570 nm) at E_h of 240 mV, so that both *c*-559/553 and *c*-556 hemes were completely reduced before the light pulse.

As can be seen from the typical traces given in fig.3A, the kinetics of the absorption changes associated with redox transition of cytochrome *c* is biphasic and depends on the wavelength choice. At 559 nm, that is at λ_{max} of the heme with E_m = 380 mV, the rapid loss of absorbance ($\tau \approx 0.3$ μs) corresponding to heme oxidation is followed by a small increment of extinction with $\tau \simeq 2.5$ μs. At 556 nm, the amplitude of the 0.3 μs absorbance decrease is much smaller than at 559 nm and a second slower phase of oxidation with $\tau \simeq 2.5$ μs is observed. Under the same conditions, the kinetics of bacteriochlorophyll re-reduction measured at 960 nm shows a single exponent with $\tau \approx 0.3$ μs (fig.3B, trace 3). These data indicate that the photooxidized P-960 may be reduced in 0.3 μs by the heme *c*-559/553, the latter being re-reduced subsequently by *c*-556 which has a lower E_m.

This suggestion is confirmed by cytochrome *c* photooxidation spectra measured in 1 and 30 μs after the flash. Absorption changes observed in the rapid phase of the reaction are characterized by a minimum at 559 nm with a shoulder at ~553 nm typical of heme *c*-559/553 oxidation (fig.3C, crosses; cf. fig.2B, spectrum 1). On the other hand, the final effect is oxidation of the heme with the symmetrical α-band at 556 nm (fig.3C, triangles) corresponding to the heme *c*-556 with E_m = 310 mV (cf. fig.2B, spectrum 2). Notably, the amplitude of the cytochrome *c* photooxidation



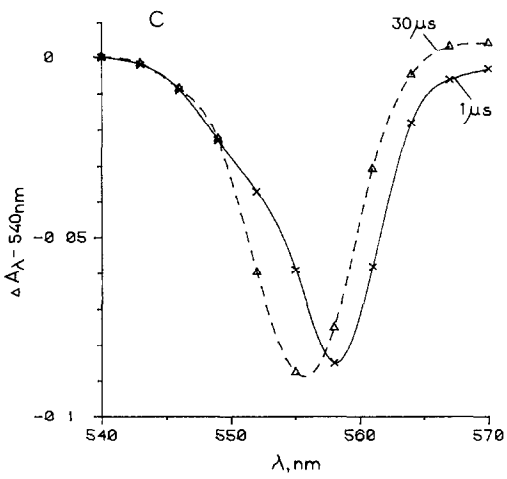


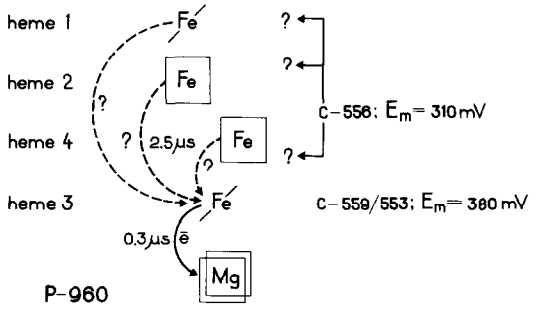
Fig.3. Laser flash-induced oxidation-reduction of cytochrome *c* and bacteriochlorophyll special pair in *Rps. viridis* reaction centers. (A) Typical kinetics of cytochrome *c* photoredox response measured in the maxima of the 2 high-potential hemes. A 1 cm spectrophotometer cell contained 5.2 μM RC in 0.1 M KCl, 25 mM Mops, pH 7.0, 0.1% Triton X-100, 200 μM DAD and 200 μM 2-methyl-1,4-naphthoquinone poised at 240 ± 5 mV. Each curve was obtained as an average of 50 individual 25 s-spaced traces after subtraction of the absorption changes at 540 nm to eliminate P-960 contribution. $\lambda_{excitation}$ = 1064 nm. (B) Typical kinetics of P-960 photoinduced oxidation-reduction at such redox potentials that before the flash (1) cytochrome *c* is completely oxidized, (2) heme *c*-556 is oxidized, *c*-559/553 is partially reduced and (3) both high-potential hemes are reduced. Conditions, as in A but [RC] = 4.8 μM and ferricyanide was added in (2) and (1) to raise E_h. The traces are individual single turnover recordings. (C) Spectro of cytochrome *c* photooxidation 1 and 30 μs after the flash. The experiments shown in A were repeated at the indicated wavelengths. RC concentration, 3 μM; $\lambda_{excitation}$ = 694 nm.

spectrum as measured in 1 and 30 μs after the flash is much the same; hence, *c*-556 oxidation during the slow phase ($\tau \approx 2.5$ μs) of absorption changes is accompanied by a stoichiometric reduction of the initially photooxidized *c*-559/553. Indeed, the spectrum of the slow phase of the reaction ($\Delta A_{30\,\mu s}$ minus $\Delta A_{1\,\mu s}$) is in good agreement with the computer-simulated difference spectrum for simultaneous *c*-556 oxidation and *c*-559/553 reduction at equimolar concentrations (not shown).

4. DISCUSSION

In contrast to the previous reports [8–11], the present data clearly show the non-equivalence of the two high-potential *c*-cytochrome hemes in RCs from *Rps. viridis*. Actually, inspection of the earlier publication [11] reveals a marked asymmetry of the high-potential cytochrome *c* spectrum (fig.1 of [11]) pointing to a presence of more than one component. Our preliminary experiments indicate that the low-potential cytochrome *c* ($E_m \sim 0$ mV [4,8]) may also be represented by 2 spectrally and potentiometrically distinct components (to be published). Thus it is likely that all the four hemes in the RC cytochrome are different, in keeping with the structural model in [17]. It is tempting to suggest that the non-equivalence of the 4 hemes may be also inherent in cytochrome *c* in RCs from other photosynthetic purple sulfur bacteria, such as *Chromatium* and *Ectothiorhodospira*.

In intriguing question arises: which of the 4 hemes is which in the 3-dimensional model of the *Rps. viridis* RC cytochrome *c*. The kinetic effectiveness of e^- donation to p-960$^+$ may give a clue to the problem. The data obtained characterize the high-potential heme *c*-559/553 with E_m = 380 mV and the split α-band as the immediate reductant of $BChl_2^+$, whereas the second high-potential heme with E_m = 310 mV and α-peak at 556 nm is likely to reduce P-960 via *c*-559/553. Consequently, it is tempting to identify the heme *c*-559/553 with the iron-porphyrin groups nearest to P-960, i.e. with heme 3, in the 3-dimensional structure of *Rps. viridis* RC presented in [17] (see scheme 1).



Scheme 1. A simplified diagram of cytochrome *c* redox centers topography is given. The hemes are numbered on the left according to [17]. The identity of the second high-potential heme (*c*-556) is not clear at present (see the text).

Photooxidation of other hemes by $BChl_2^+$ would then possibly occur indirectly via *c*-559/553. Whether *c*-556 is identical to the iron-porphyrin group next to *c*-559/553 (heme 4 in [17]) or to a more distant heme is not clear at present.

Interestingly, photooxidation of the low-potential cytochromes *c* was reported in [11] to be more rapid than that of heme *c*-559/553 as measured at higher potentials here and earlier [6]. This observation must appear incompatible with the hypothesis on the indirect oxidation of the low-potential hemes by P-960$^+$ via *c*-559/553. However, reduction of the former redox centers at low ambient redox potentials could accelerate *c*-559/553 reaction with P-960$^+$ (and, possibly, with *c*-556 as well) due to cooperative, e.g. electrostatic, interactions between the hemes within the cytochrome or bring about a conformational change of the RC complex. Given that the reduction of *c*-559/553 by the low-potential hemes is more rapid than *c*-559/553 oxidation by P-960, which is a likely possibility considering the E_m differences and distances between the redox centers in the RC, the kinetics of cytochrome *c* photooxidation at low potentials fits the present model conveniently.

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