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The electron transport system of the facultative phototroph *Rhodoferax fermentans*. II. Flash-induced oxidation of membrane-bound cytochromes *c*

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Abstract

Flash-induced oxidation of the membrane-bound *c*-type haems was studied in light-grown cells of *Rhodoferax fermentans*, a new taxon of the purple nonsulfur photosynthetic bacteria. At least three *c*-type cytochromes were found to rapidly (< 20 μs) re-reduce the photo-oxidized primary donor of the reaction centre: a first haem peaking at 556 nm with $E_m = 354$ mV, a second haem peaking at 560 nm with $E_m = 294$ mV, and a third haem peaking at 551 nm with $E_m = 79$ mV. The photo-oxidized minus reduced spectrum of the primary donor was found to be very similar to those of other purple bacteria. The primary donor midpoint potential was determined (471 mV) and *o*-phenanthroline was found to inhibit electron transfer to the secondary acceptor, thus indicating the presence of a Q-type reaction centre. The midpoint potential of the primary quinone acceptor was also determined (13 mV). A model accounting for the post-flash redox equilibria within the RC-cytochromes *c* chain is presented. A tetrahaem cytochrome *c* is proposed to operate in *Rhodoferax fermentans* and its spectral and thermodynamic features are discussed in relation with other species of purple photosynthetic bacteria.

Keywords: Bacterial photosynthesis; Photosynthesis ; Electron transfer; Cytochrome, *c*-type; (*Rf. fermentans*)

1. Introduction

The anoxygenic facultative phototrophs represent a quite heterogeneous group of bacteria able to obtain energy under aerobic/dark and anaerobic/light conditions [1]. When light is present, but also under microaerobic conditions, these microorganisms synthesize a photosynthetic apparatus, which includes the light-harvesting complexes and the photosynthetic reaction centre (RC), an integral membrane protein catalyzing the primary photochemical processes. Reaction centres are oligomeric proteins which, in purple bacteria, contain bacteriochlorophyll, bacteriopheophytin and two molecules of quinone as cofactors. Pointing from the periplasmic to the cytoplasmic side of the bacterial membrane, the following array of functional cofactors is found: a special pair of bacteriochlorophyll molecules (P), a bacteriopheophytin molecule and two quinones, indicated as Q_A and Q_B, respectively (for a review see [2]). P acts as the primary donor and, upon absorbtion of a light quantum, delivers an electron through the bacteriopheophytin molecule to Q_A in about 200 ps. The primary charge separation is stabilized by electron transfer from Q_A^- to the secondary quinone acceptor Q_B. The re-reduction of the photo-oxidized reaction centre (P^+) is mediated by *c*-type cytochromes (for reviews see [3,4]). Several studies have been reported in which the spectral and redox features of *c*-type cytochromes involved in the photochemistry of different bacterial species were analyzed [5–14]. Essentially two different patterns for the reduction of P^+ are observed. In some cases (*Rb. sphaeroides*, *Rsp. rubrum*), the immediate electron donor to P^+ is a soluble *c*-type cytochrome, cyt c_2. In other cases (e.g., *C. vinosum*, *Rps. viridis*, *Rv. gelatinosus*, *Th. pfennigii*) P^+ can be reduced by a membrane-bound multihaem cytochrome *c*. It is worth noting that a third situation has recently been described in *Rb. capsulatus* in which a

Abbreviations: RC, reaction centre; P, primary donor; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor.

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membrane-associated *c*-type cytpochrome, c_y, can substitute for the soluble cyt c_2 [15,16].

Rhodoferax fermentans, gen. nov., sp. nov., has recently been proposed [17] as a new taxon of the purple nonsulfur photosynthetic bacteria. While some microbiological and physiological features of this bacterium are established [17–21], no information is available on its photosynthetic electron transport chain. Here we report on the spectral, thermodynamic and kinetic features of the membrane-bound *c*-type cytochromes of membranes isolated from light-grown cells of *Rhodoferax fermentans*. In addition, the thermodynamic characterization of the photochemical reaction centre has also been performed.

2. Materials and methods

2.1. Organism cultivation and membrane isolation

Cells of *Rhodoferax fermentans* (a generous gift of Dr. A. Hiraishi, Konishi Co. Ltd., Tokyo) were grown and membranes isolated as described in the accompanying paper [22].

2.2. Kinetic spectrophotometry

Absorbance changes, induced by a Xenon flash lamp (3.25 J discharge energy, 15 μs pulse duration at half-maximal intensity), were measured by a single beam spectrophotometer, equipped with a double grating monochromator (bandwidth 1.5 nm). The photomultiplier was protected by a Corning glass 4/96 filter. A triggered shutter was used to gate the measuring beam (exposure of the samples to the measuring light was no longer than 2 s prior to an excitation flash). Data were acquired by a Lecroy 9410 digital oscilloscope interfaced to an Olivetti M240 computer.

2.3. Redox potentiometry

Except for the study of the effects of *o*-phenanthroline on the kinetics of charge recombination within the reaction centre, all the experiments were carried out in a glass cuvette made anaerobic by means of a stream of nitrogen. A platinum electrode was fitted to the cuvette and the redox potential measured against an external calomel electrode (connected via a salt bridge). The pH for all the experiments was 7.0 (50 mM Mops buffer). The following redox mediators were used: 1,2-naphthoquinone, 1,4-naphthoquinone, duroquinone and *p*-benzoquinone 10 μM each; phenazine ethosulfate, phenazine methosulfate and diaminodurene 1 μM each. When Q_A was titrated the same redox mediators mixture was used, but 4 μM Methylene blue and 20 μM 2-hydroxy-1,4-naphthoquinone were added to the mixture. Redox titrations were carried out using sodium ascorbate as reductant and potassium ferricyanide as oxidant. All the experiments were performed in the presence of nigericin and valinomycin (2 μg/ml each).

2.4. Bacterioclorophyll determination

Bacteriochlorophyll was extracted with acetone/methanol (7:2, v:v) and the concentration of the pigment was estimated using an extinction coefficient of 75 mM^{-1} cm^{-1} at 775 nm [23].

3. Results

3.1. The photosynthetic reaction centre

Fig. 1 shows a spectrum of flash-induced absorbance changes measured in *Rhodoferax* membranes at an ambient potential equal to 482 mV. Under these conditions all of the photo-oxidizable *c*-type cytochromes are pre-oxidized before the exciting flash (see [22] and the data presented here in the following paragraph) and the spectrum obtained is clearly due to the contribution of the reaction centre only. The same spectrum is obtained when considering the absorbance changes as measured after the first (filled circles) or the fourth (empty circles) flash in a train of closely spaced photoexcitations: this coincidence suggests the absence of any component able to re-reduce (at 482 mV) the photo-oxidized reaction centre. The spectrum, showing a broad band between 530 and 570 nm and a bleaching centered at 605 nm, is very similar to the light-induced difference spectrum observed in purple bacteria reaction centres [14,24,25]. The data of Fig. 1 have been used to subtract the contribution of RC photo-oxidation from flash-induced spectra when cytochrome *c*

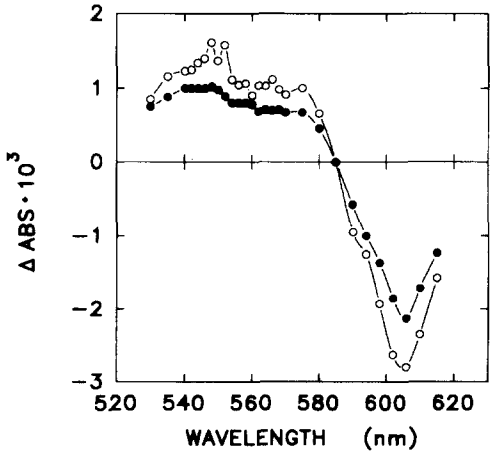

Fig. 1. Photo-oxidized minus reduced spectrum of the primary electron donor in *Rhodoferax fermentans* membranes measured after the first (filled circles) and after the fourth (empty circles) excitation in a train of flashes fired 100 ms apart. Bacteriochlorophyll concentration was 28 μM, and the redox potential was poised at 482 mV.

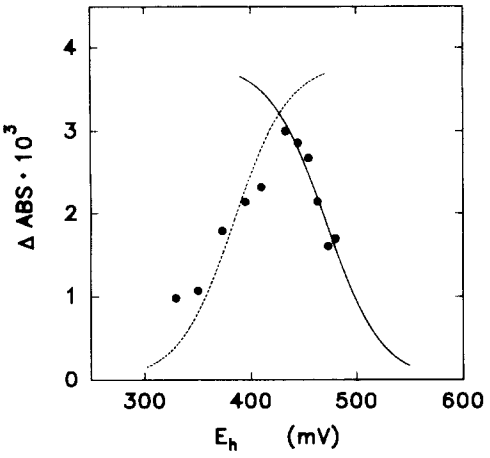

Fig. 2. Redox titration of the primary electron donor, P. Absorbance changes induced by a single flash of light were measured at 540 nm. Continuous and dotted curves represent Nernst titrations ($n = 1$) with $E_m = 471$ mV and $E_m = 384$ mV, respectively. Bacteriochlorophyll concentration was 44 μM.

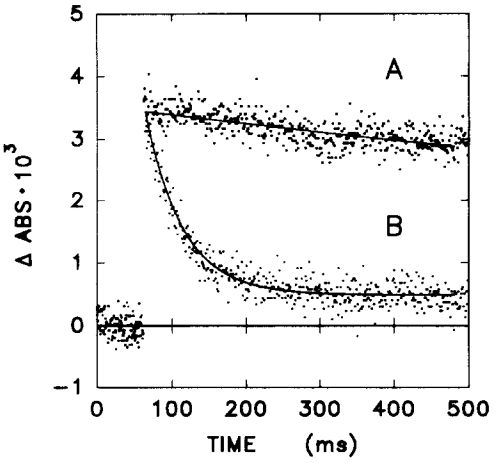

Fig. 3. Kinetics of P^+ decay by charge recombination following a single photoexcitation. Absorbance changes were measured at 540 nm, in the absence (trace A) and in the presence (trace B) of *o*-phenanthroline (1 mM). Continuous curves represent best fits of the kinetic traces to a single exponential function (A) and to an exponential decay (accounting for 86% of the total recovery, half-time 34.5 ms) plus a constant (B). Bacteriochlorophyll was 47 μM and the redox potential was poised at 430 mV with equimolar ferri- and ferrocyanide in an aerobic cuvette.

photo-oxidation was studied (as discussed in detail later).

Fig. 2 shows a redox titration of the reaction centre signal at 540 nm. Upon lowering the redox potential from 480 to 430 mV, reducing the primary RC donor before the flash, the extent of P photo-oxidation increases, with E_m = 470 mV (continuous curve). When the E_h is lowered further, the P^+ signal decreases, with an apparent E_m = 384 mV (dashed curve). As shown in the following paragraph, at E_h lower than 430 mV fast electron donation (not resolved on the timescale of our measurements) occurs to the photo-oxidized P^+ from a *c*-type cytochrome which becomes progressively reduced before the flash. The midpoint potential of the P/P^+ couple estimated from Fig. 2 is close to the midpoint potential reported for the primary donor in *Rhodopseudomonas viridis* [26,27] and *Chromatium vinosum* RC [8,28].

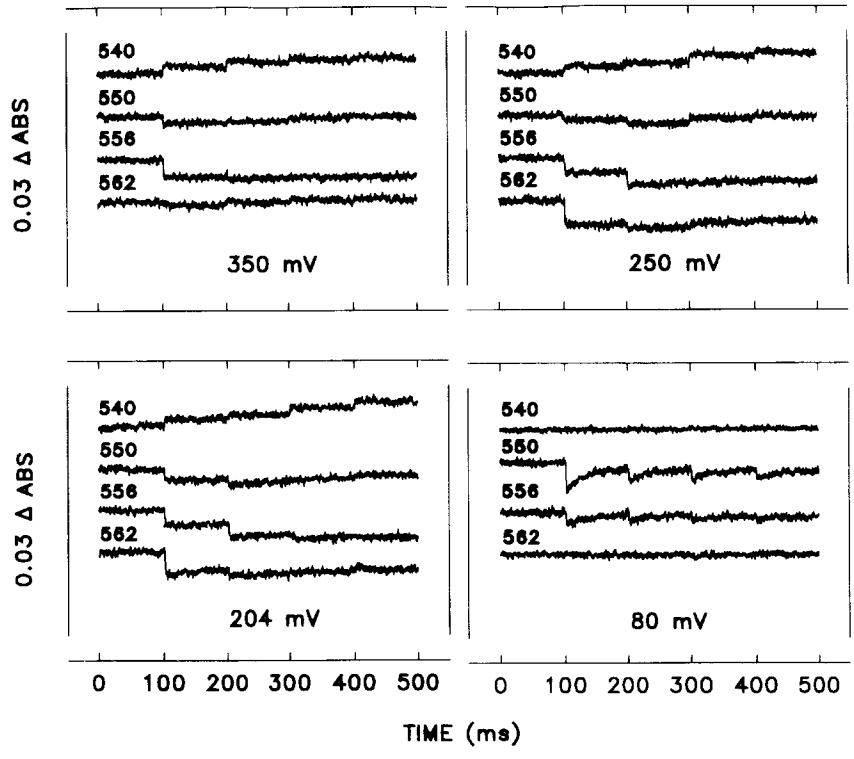

Fig. 4. Light-induced absorbance changes detected at 540, 550, 556 and 562 nm at different ambient potentials, following a train of 4 flashes, fired 100 ms apart. Bacteriochlorophyll and *o*-phenanthroline concentrations were 44 μM and 1 mM, respectively.

Fig. 3 shows the effect of *o*-phenanthroline on the dark re-reduction of flash-generated P^+ measured at 540 nm ($E_h = 430$ mV). Under these redox conditions, where no pre-reduced cytochrome *c* can donate to the photo-oxidized pigment, and in the absence of the inhibitor, P^+ decays by charge recombination of the $P^+Q_B^-$ state with a half-time larger than 1.5 s (trace A). As Fig. 3 shows, 1 mM *o*-phenanthroline was efficient in preventing $Q_A^- \rightarrow Q_B$ electron transfer, leading to the faster charge recombination of the $P^+Q_A^-$ state (trace B); this process occurs monoexponentially with a half-time of 34.5 ± 0.3 ms.

3.2. Reaction centre associated cytochromes

To study the spectral and redox properties of photo-oxidizable *c*-type cytochromes in *Rhodoferax* membranes, flash-induced absorbance changes were monitored at different redox potentials (from 400 to 70 mV) in the 540–570 nm wavelength interval. As shown in Fig. 4, over this spectral region, a train of closely spaced flashes induced a complex pattern of absorbance changes attributable to fast sub-millisecond oxidation of *c*-type cytochromes coupled to P^+ re-reduction. Measurements performed at the maximal time resolution of our instrument indicated that, at all redox poises, absorbance changes due to cytochrome(s) *c* photo-oxidation had a rise time of less than 20 μs (not shown). Under these conditions, re-opening of the photo-excited RC is presumably rate-limited by Q_A^- to Q_B electron transfer, a process which in purple bacterium RC's can occur with a half-time of tens of microseconds (e.g., 25 μs in *Rps. viridis* [29]). Given the relatively long duration of the light pulse used in our experiments (15 μs half-width, see Materials and Methods), double photo-excitations of the pigment are likely to occur following a single flash, at least in a fraction of the RC population. In order to prevent (or reduce) this possibility, cytochrome *c* photo-oxidation measurements were performed in the presence of *o*-phenanthroline (1 mM), which blocks electron transfer to Q_B. Subsequent flashes in a train were fired 100 ms apart, a dark time long enough to allow (even in the presence of *o*-phenanthroline) a substantial reoxidation of Q_A^- after each flash and therefore recovery of photoactivity in multiple flash experiments.

Oxidized minus reduced spectra of *c*-type cytochromes were assumed to have an isosbestic point at 540 nm: only reaction centre photo-oxidation was assumed responsible for the absorbance changes recorded at this wavelength. On the basis of the trace recorded at 540 nm and of the reaction centre photo-oxidation spectrum of Fig. 1, at each wavelength and redox potential the P^+ contribution to the 542–570 nm signals was calculated and subtracted. At 401 mV the first flash induces small absorbance changes (Fig. 5, closed circles), peaking at 556 nm. The same maximum was retained when the measurements were performed at 385 mV; lowering the redox potential from 401 to 385 mV led only to increased amplitudes of the signals recorded. In both cases a second flash was unable to produce a further absorbance change (empty circles in the figure), suggesting that, over this redox potential range, only one haem (peaking at 556 nm, cytochrome *c*-556) is titrating in and donates to P^+. When the potential was lowered to 328 mV a prominent shoulder appeared in the 558–562 region of

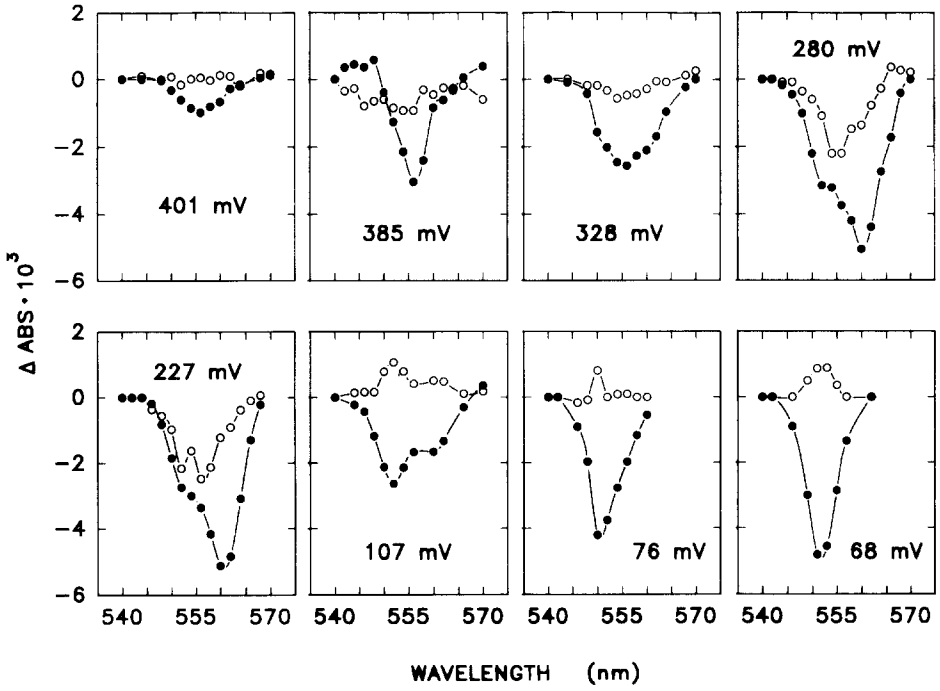

Fig. 5. Cytochrome photo-oxidation spectra as a function of redox potential determined after the first (filled circles) and after the second (empty circles) excitation flash. Bacteriochlorophyll and *o*-phenanthroline concentrations were 29 μM and 1 mM, respectively. Dark time between flashes was 100 ms.

the spectrum induced by the first flash; the second excitation was still unable to produce significant further absorbance changes. A sharper distinct spectrum was recorded when the potential was lowered down to 280 and 227 mV (see panels in Fig. 5); at both these redox poises a maximum absorbance change was detected at 560 nm, indicating the flash-induced oxidation of a different RC-associated cytochrome. The amplitudes of the photo-oxidation spectra recorded at 280 and 227 mV (cytochrome *c*-560) are greater than the amplitudes recorded for the cytochrome *c*-556. Full oxidation of the 556-haem was probably not observed due to the fast equilibration of P^+ with pre-reduced cytochrome *c*-560, possibly via 556-haem (see Discussion). Quite interestingly, at E_h equal to 280 and 227 mV the second excitation flash induced absorbance changes peaking at 556 nm, thus indicating that, when cytochrome *c*-560 has been already oxidized following the first excitation, P^+ produced by the second flash again equilibrated with cytochrome *c*-556. Upon lowering the redox potential to 107 mV, a broad photo-oxidation spectrum was observed after the first flash, exhibiting a shoulder at 560 nm and a new peak positioned at 552 nm. This peak became sharper and shifted to 551 nm when the redox potential was poised to 68 mV. The spectrum measured at this lowest potential (attributed to oxidation of a third haem, cytochrome *c*-551) was free from contributions of the other haems, suggesting a rapid re-reduction of the 556 and 560 cytochromes at the expense of cytochrome *c*-551 (or direct P^+ re-reduction by cytochrome *c*-551). The apparent absorbance increase following the second excitation flash when the redox potential was poised at 107, 76 and 68 mV does not reflect a genuine spectral component. As is shown by the lowest right panel of Fig. 4 it simply originates from a lower extent of cytochrome *c*-551 photo-oxidation induced by the second and subsequent flashes as compared to the one produced by the first photoexcitation in a train. This behaviour, observed only at low redox potentials and in the presence of *o*-phenanthroline, reflects an incomplete recovery of primary photochemistry during the dark time between flashes. Reducing conditions are likely to hamper the re-oxidation of Q_A^- produced by the first flash in the presence of *o*-phenanthroline, thus partially impairing RC photoactivity in subsequent multiple photoexcitations. On the other side, preliminary experiments (not shown) indicated that, in the absence of *o*-phenanthroline, a single 15 μs-flash induced double turnovers of the RC, leading, at intermediate and low redox poises, to broader spectra in the 542–570 nm region, which prevented spectral resolution of the different haems.

3.3. Redox features of reaction centre associated cytochromes

As shown by the results presented in the previous paragraph, *Rhodoferax* membranes contain at least three cytochromes (peaking at 551, 556 and 560 nm) involved in sub-millisecond electron donation to flash generated P^+. Fig. 6 presents full redox titrations of the absorbance changes induced by a single flash at 550, 556 and 562 nm. Each of these wavelengths, although representative for one of the three photo-oxidizable haems, includes spectral contributions from the others. Upon lowering the redox poise, the light-induced 556 nm signal was the first to reach its maximal amplitude, followed by the signal at 562 nm. The signal at 550 nm fully titrates in at E_h lower than 100 mV, a redox poise at which the amplitude of the 556 nm signal is more than halved and that of the 562 absorbance change is practically zero. These data are therefore consistent with the E_m sequence indicated by the spectra of Fig. 5: E_m(cytochrome *c*-556) > E_m(cytochrome *c*-560) > E_m(cytochrome *c*-551). In agreement with this relation and with electron distribution within the chain, the decrease in the 556 absorbance change observed below 320 mV is paralleled by an increase at 562 nm. This signal, in turn, titrates out at E_h values at which the 550 nm absorbance change fully titrates in. A simple, direct fitting to Nernst components is, however, prevented by the considerable spectral overlapping of the three haems (see Fig. 5). A numerical simulation of these titrations, which takes into account reciprocal spectral interference of the haems, as well as equilibration of the oxidizing equivalents produced by the flash in the electron transport chain, is presented in the Discussion.

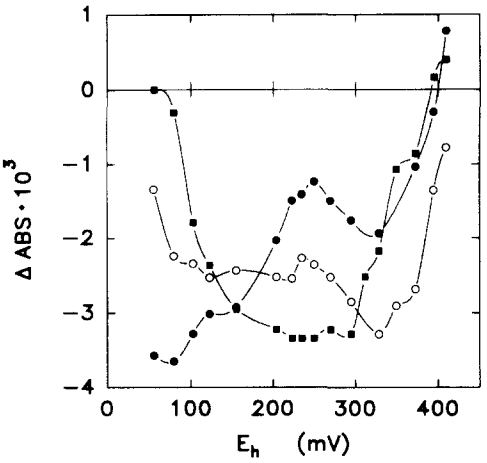

Fig. 6. Redox titrations of flash-induced absorbance changes at 550 (filled circles), 556 (empty circles), and 562 nm (filled squares). Experimental conditions as in Fig. 4.

3.4. The primary acceptor

At E_h values lower than 100 mV, the amplitude of the photo-oxidation signal at 550 nm was found to decrease monotonically upon decreasing of the redox poise, presumably reflecting the reduction of the primary quinone acceptor before the flash. In these measurements the sample was supplemented with 4 μM Methylene blue ($E_{m,7}$ = 11 mV,

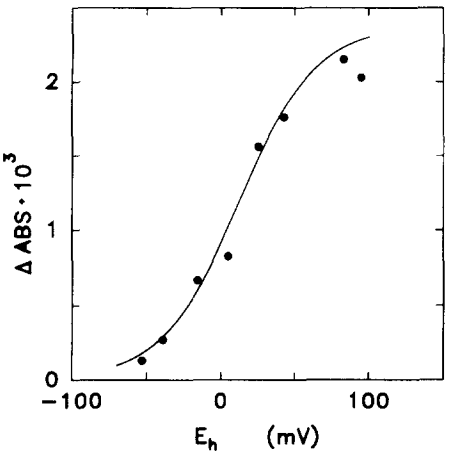

Fig. 7. Redox titration of the primary quinone acceptor, as determined by measuring light-induced absorbance changes at 550 nm. The data points are fitted to a theoretical Nernst curve ($n = 1$, $E_m = 13$ mV). Bacteriochlorophyll concentration as in Fig. 1.

[30]) and 20 μM 2-hydroxy-1,4-naphthoquinone ($E_{m,7} = -145$ mV, [31]), in order to establish a satisfactory dark equilibration of the primary acceptor during averaging of the signals. In the absence of these additional redox mediators, substantial deviations from a Nernstian behaviour were observed, even allowing for a dark adaptation between flashes longer than 5 min (not shown). The redox titration (Fig. 7) is in good agreement with a Nernstian curve ($n = 1$) with $E_m = 13$ mV.

4. Discussion

The oxidized minus reduced spectrum of the primary donor of *Rhodoferax* reaction centre resembles that of other purple bacteria [14,24,25]. The midpoint potential of the primary acceptor (13 mV) is similar to that measured in species containing ubiquinone as Q_A (e.g., *Rhodobacter sphaeroides* (−20 mV) [13,32] and *Rhodobacter capsulatus* (−25, −30 mV) [33,34]). Since a large amount of ubiquinone was found in *Rhodoferax* membranes [18,19], we suggest that also in this species ubiquinone acts as the primary (Q_A) and secondary (Q_B) acceptor. Consistently with the notion that a Q-type RC operates in *Rhodoferax*, *o*-phenanthroline dramatically accelerates the kinetics of charge recombination, blocking Q_A^- to Q_B electron transfer.

A common feature among the species possessing membrane-bound *c*-type cytochromes is the presence of a multihaem unit (tetrahaem) associated with the photosynthetic reaction centre. The spectral features of the haems present in *Rhodoferax fermentans* resemble those of *Rhodopseudomonas viridis*, since at least three different α maxima are detectable in *Rhodoferax*. A fourth haem could be present in the RC-associated multihaem unit of *Rhodoferax* membranes: as described in the accompanying paper [22], when membranes from photosynthetically-grown cells were titrated, an haem with a midpoint potential close to 0 mV was found, whereas in membranes from dark-aerobically grown cells this haem was not detected.

As previously described the *c*-type cytochromes present in *Rhodoferax* membranes have been characterized by dark equilibrium redox titrations (see [22]). In the present paper, the E_h dependence of light-induced cytochrome *c* oxidation was examined (see Figs. 5 and 6). To check whether these two different redox titrations were consistent we have assigned the midpoint potentials obtained from the dark-equilibrium titration [22] to the three haems (556, 560 and 551) detected following flash excitation of the samples: the haem at 556 nm was assumed to have a midpoint potential equal to 358 mV, the haem at 560 nm was considered half-reduced at 296 mV and the 551 nm haem at 78 mV (see [22] and Fig. 5); the primary donor (P) and acceptor (Q_A) of the reaction centre were assumed to have midpoint potentials equal to 471 and 13 mV, respectively (see Figs. 2 and 7). Using these assumptions and the spectral information of Fig. 5, we simulated redox titrations of the cytochrome *c* oxidation induced by a single turnover flash, according to the following procedure.

(i) At each value of the ambient potential, E_h, the concentration of oxidation equivalents produced by the photoexcitation was set equal to the concentration of RC's in which the primary donor P is reduced and the primary acceptor Q_A is oxidized before the flash.

For each of the three haems a stoichiometry of one per RC was assumed and the oxidation equivalent generated on the RC by the flash was considered to rapidly equilibrate within the P-cytochrome *c* redox chain according to:

$$\sum_{i=1}^{3}(o_i - o_{io}) = \{\exp[F(E_h - E_{mP})/RT] + 1\}^{-1} \times \{\exp[F(E_{mQ} - E_h/RT)] + 1\}^{-1} \quad (1)$$

$$E_{mi} + (RT/F)\ln[o_i/(1-o_i)] = E_{mP} + (RT/F)\ln[o_P/(1-o_P)] \quad i = 1,3 \quad (2)$$

where o_{io}, o_i are the normalized equilibrium concentrations of oxidized haem *i* before and after the flash, respectively, E_{mi} are the midpoint potentials of haem *i*, o_P is the concentration of oxidized P following equilibration in the chain after the flash, and E_{mP} and E_{mQ} are the midpoint potentials of P and of the primary quinone acceptor Q_A, respectively, R is the gas constant, F the Faraday constant and T the absolute temperature. All the concentrations are normalized to the total concentration of RC.

(ii) Eqs. (1) and (2) were solved numerically, yielding a redox titration of the expected flash-induced oxidation ($\Delta o_i = o_i - o_{io}$) for each of the three haems after equilibration. The result is presented in Fig. 8A.

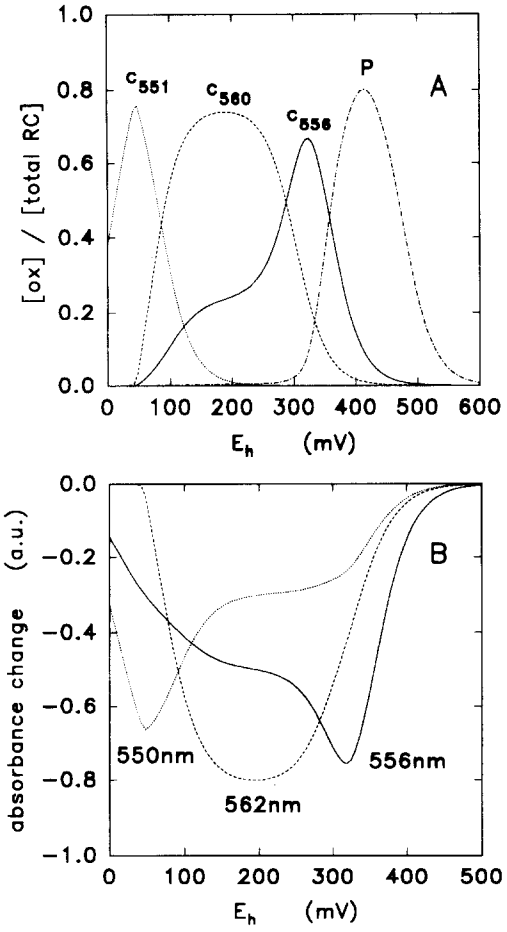

Fig. 8. Theoretical post-flash redox equilibria within the P-cytochromes *c* redox chain. (A) Calculated redox state of the primary donor (P), cyt *c*-556, cyt *c*-560 and cyt *c*-551 following a single photoexcitation of the RC, as a function of E_h. (B) Simulated redox titrations of the flash-induced absorbance changes expected at 556, 562 and 550 nm. See text for details of the model.

(iii) On this basis, in order to simulate the experimental data of Fig. 6, the absorbance changes induced by photo-oxidation of haem 1 (cyt *c*-556), haem 2 (cyt *c*-560) and haem 3 (cyt *c*-551) were calculated at the three selected wavelengths. In view of the spectral overlapping of the haems:

$$\Delta A_i = -\sum_{j=1}^{3} \epsilon_{ij} \Delta o_j \quad i = 1,3 \tag{3}$$

where ΔA_i are the absorbance changes at 556, 562 and 550 nm for $i = 1,3$, respectively. The ϵ_{ij} coefficients, normalized to the absorption coefficients of the three haems at the respective peak wavelengths, account for the relative spectral contribution of haem j at wavelengths i. The spectral contributions due to photo-oxidation of cyt *c*-556 ($\epsilon_{11} = 1$, $\epsilon_{21} = 0.24$, $\epsilon_{31} = 0.23$) and of cyt *c*-551 ($\epsilon_{13} = 0.38$, $\epsilon_{23} = 0.00$, $\epsilon_{33} = 0.86$) can be easily obtained from Fig. 5 (spectra at $E_h = 401$–385 mV and $E_h = 68$ mV, respectively). In fact, as Fig. 8A shows, at the more oxidizing and more reducing conditions of our measurements only cyt *c*-556 and cyt *c*-551 respectively are expected to be oxidized following rapid redox equilibration after the flash. The coefficients ϵ_{i2} (cyt *c*-562) have been evaluated from the spectrum at $E_h = 207$ mV of Fig. 5, corrected for the contribution of cyt *c*-556 according to the calculated redox equilibrium between cyt *c*-562 and cyt *c*-556 ($\epsilon_{12} = 0.34$, $\epsilon_{22} = 1.00$, $\epsilon_{32} = 0.32$, see Fig. 8A).

The resulting redox titrations are plotted in Fig. 8B and give a satisfactory account of the correspondent experimental data (Fig. 6). Deviations from the measured absorbance changes, more prominent at 550 nm at intermediate redox poises, are likely to reflect the uncertainty in the ϵ_{ij} values used in the model, as well as the experimental error in the determination of the E_m values of the haems from dark redox titrations. As described in the accompanying paper [22], we detected in *Rhodoferax* cell-free extracts the presence of a soluble *c*-type cytochrome (α maximum at 551 nm), the midpoint potential of which was estimated to be 287 mV. The discrepancy between the experimental redox titration (Fig. 6) and the simulation (Fig. 8B) at 550 nm could also be due to a small amount of the soluble 551 cytochrome, undergoing photo-oxidation and rapidly equilibrating with the considered redox chain. Since no parameter (spectral or thermodynamic) was allowed to be adjusted in the numerical simulation, we conclude that the midpoint potential of the haems measured in dark equilibrium titrations are reasonably consistent with the behaviour observed in flash experiments.

On the basis of the available information it is therefore tempting to propose that in *Rf. fermentans* the RC is associated with a tetrahaem unit, which includes cyt *c*-556 ($E_m = 354$ mV), cyt *c*-560 ($E_m = 294$ mV), cyt *c*-551 ($E_m = 79$ mV) and a fourth haem with unknown α maximum and midpoint potential close to 0 mV. This pattern would be quite similar to that of the tetrahaem cytochrome *c* of *Rhodopseudomonas viridis* [5,6]: cyt *c*-559 ($E_m = 380$ mV), cyt *c*-556 ($E_m = 310$ mV), cyt *c*-552 ($E_m = 20$ mV), cyt *c*-554 ($E_m = -60$ mV). If this is correct, the two high-potential haems would have very close midpoint potentials in the two species, but would exhibit reversed spectroscopic features.

We have indeed shown that in *Rhodoferax fermentans* the oxidizing equivalents generated on the RC by a single photo-excitation rapidly equilibrate within the (P)-(cyt *c*-556)-(cyt *c*-560)-(cyt *c*-551) chain according to the redox poise of the system before the flash. In *Rhodopseudomonas viridis* it has convincingly been demonstrated that, when both high-potential haems are pre-reduced, cyt *c*-556 donates its electron to P^+ via cyt *c*-559 [35]. The time resolution of our measurements does not allow us to discriminate between a similar interhaem electron transfer involving the two high-potential cytochromes of *Rhodoferax* and a direct re-reduction of P^+ by each pre-reduced haem.

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