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On the role of high-potential iron–sulfur proteins and cytochromes in the respiratory chain of two facultative phototrophs

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Abstract

The capability of high potential iron–sulfur proteins (HiPIPs) and soluble cytochromes to shuttle electrons between the bc_1 complex and the terminal oxidase in aerobically grown cells of *Rhodoferax fermentans* and *Rhodospirillum salinarum*, two facultative phototrophs, was evaluated. In *Rs. salinarum*, HiPIP and a *c*-type cytochrome (α-band at 550 nm, $E_{m,7}$ = +290 mV) are both involved in the electron transfer step from the bc_1 complex to the terminal oxidase. Kinetic studies indicate that cytochrome c_{550} is more efficient than HiPIP in oxidizing the bc_1 complex, and that HiPIP is a more efficient reductant of the terminal oxidase as compared to cytochrome c_{550}. *Rs. salinarum* cells contain an additional *c*-type cytochrome (asymmetric α-band at 556 nm, $E_{m,7}$ = +180 mV) which is able to reduce the terminal oxidase, but unable to oxidize the bc_1 complex. *c*-type cytochromes could not be isolated from *Rf. fermentans*, in which HiPIP, the most abundant soluble electron carrier, is reduced by the bc_1 complex (zero-order kinetics) and oxidized by the terminal oxidase (first-order kinetics), respectively. These data, taken together, indicate for the first time that HiPIPs play a significant role in bacterial respiratory electron transfer.

Keywords: High potential iron–sulfur protein; Cytochrome; Bacterial respiration; Facultative phototroph; Halophile

1. Introduction

The process of aerobic respiration in chemo-organotrophic prokaryotes involves the following main steps: (1) the oxidation of an organic substrate yields a reduced electron donor (i.e. NADH, succinate, formate, etc.), that in turn can be oxidized by the respiratory electron transfer chain located in the plasma membrane; (2) specific dehydrogenases oxidize these electron donors with the concurrent formation of ubiquinol; (3) ubiquinol can be oxidized by quinol oxidases, when present, and/or by the bc_1 complex; and (4) in the latter case, the reduced cytochrome c_1 of the bc_1 complex is oxidized by an electron carrier that in turn reduces the terminal oxidase (usually known as cytochrome *c* oxidase) [1]. The latter catalyzes the reduction of dioxygen concomitantly with the generation of a proton electrochemical gradient ($\Delta\mu_{H^+}$) across the plasma membrane, useful for bacterial growth. This respiratory electron transfer chain is mainly composed of membrane-bound protein complexes, such as the different dehydrogenases, quinol oxidases, bc_1 complexes, and terminal oxidases, while the only electron transfer step involving a solu-

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ble periplasmic component is the redox link between the bc_1 complex and the terminal oxidase. This step is usually effected by a soluble *c*-type cytochrome.

Facultative phototrophic prokaryotes belong to a physiologically well-defined group, comprising bacterial species able to grow under photoheterotrophic, photoautotrophic, chemo-organotrophic and, in very few cases, fermentative conditions [2]. Most of these species belong to the α-subclass of proteobacteria, a phylogenetic group of prokaryotes closely related to mitochondria [3]. The major soluble *c*-type cytochromes usually found in phototrophs belong to the c_2 class [4], are structurally similar to the mitochondrial cytochrome *c* [5], and feature reduction potentials in the range from +250 to +470 mV [6], compatible with the high potential part of both photosynthetic and respiratory electron transfer pathways.

Several species of facultative phototrophs do not express soluble *c*-type cytochromes when grown under anaerobic light (photosynthetic) conditions, while considerable amounts of the so-called high potential iron–sulfur proteins (HiPIPs) are found [7]. HiPIPs are small proteins (M_r 8.5–11 kDa) possessing a cluster $[Fe_4S_4]^{3+/2+}$ [8–10] characterized by reduction potentials (ranging from +50 to +450 mV) similar to those displayed by cytochromes c_2, and therefore compatible with their involvement in both respiratory and photosynthetic electron transfer. Indeed, direct kinetic competence of HiPIPs in bacterial photosynthesis has been demonstrated [11–14], and a role in respiration has been proposed for a membrane-associated HiPIP from *Rhodotermus marinus* [15]. However, little information is available on the expression and role of HiPIPs in facultative phototrophs grown under aerobic dark (respiratory) conditions. Although the presence of HiPIPs in the facultative phototrophs *Rhodoferax fermentans* [16] and *Rhodospirillum salinarum* [17] grown under aerobic chemo-organotrophic conditions has been demonstrated, and the competence of *Rf. fermentans* HiPIP as respiratory electron transfer has been suggested [16], no detailed kinetic studies are yet available. *Rs. salinarum* is characterized, in analogy with mitochondria, by a linear respiratory electron transfer chain constituted by a bc_1 complex, highly sensitive to the inhibitors antimycin A and myxothiazol, and by a terminal oxidase of the aa_3 type [17].

In this report, the kinetics of electron transfer between the bc_1 complexes and the terminal oxidases of *Rf. fermentans* and *Rs. salinarum*, mediated by HiPIPs, are investigated. A functional comparison with homologous *c*-type cytochromes is then carried out, in order to solve the question of whether HiPIPs should be considered functional counterparts of soluble cytochromes in bacterial respiration.

2. Materials and methods

2.1. Cells growth and membranes isolation

Rf. fermentans and *Rs. salinarum* cells were grown at 30°C under chemoheterotrophic conditions, using the MYCA [18] and SAL [19] media, respectively, in a 12-l fermentor (New Brunswick) sparged continuously with air (6 l min^{-1}). Plasma membrane fragments were isolated as described [20] and resuspended in 10 mM Tris-HCl buffer (pH 7.3).

2.2. Protein isolation and purification

Rf. fermentans HiPIP was isolated in the reduced form as previously reported [13]. The soluble fraction of *Rs. salinarum* cells was treated as follows: ammonium sulfate was added (40% saturation) and the pellet precipitated after centrifugation (15 000×*g*, 20 min) was discarded; the supernatant was then extensively dialyzed using a 3-kDa M_r cut-off membrane. The dialyzed extract was used for the purification of two HiPIP isoforms (iso-1 and iso-2) in the reduced form according to Meyer et al. [21]. Two cytochromes were further identified and separated, according to the following procedure: the dialyzed extract was loaded onto a DEAE-cellulose column (2.6×25 cm), previously equilibrated with 10 mM Tris-HCl buffer (pH 7.3, buffer A). After elution of HiPIP iso-1 with buffer A containing 80 mM NaCl, a first cytochrome (α-band at 550 nm, cytochrome c_{550}) was eluted using buffer A containing 150 mM NaCl. The column was then washed with 200 mM NaCl, and a second cytochrome (asymmetric α-band at 556 nm, cytochrome c_{556}) was eluted using buffer A containing 250 mM NaCl. Ammonium sulfate (1.3 M) was subsequently added to both cytochrome fractions, that were separately loaded onto a Phenyl Se-

pharose CL-4B column (1.6×15 cm), equilibrated with 50 mM Tris-HCl buffer, pH 7.3, containing 1.3 M ammonium sulfate (buffer B). The column was extensively washed with buffer B, and a linear decreasing gradient (1–0.6 M ammonium sulfate, 10 column volumes) was used for the elution of cytochrome c_{550} at approximately 0.8 M ammonium sulfate. Cytochrome c_{556} was found to be extremely hydrophobic, being retained by the Phenyl Sepharose CL-4B column using buffer B with no ammonium sulfate, and was eluted only using 5 mM Tris-HCl buffer, pH 7.3. Both cytochromes were then subjected to size-exclusion chromatography on a 1.6×65 cm. Superdex 75 column (flow rate 0.6 ml min^{-1}). Further purification of cytochrome c_{556} was not pursued. Fractions containing cytochrome c_{550} were subsequently loaded onto a MonoQ column (1-ml bed volume, equilibrated with buffer A) and eluted with a linear gradient from 150 to 300 mM NaCl (25 column volumes, flow rate 0.7 ml min^{-1}). Cytochrome c_{550} was found to elute in two peaks, at 240 and 260 mM NaCl, the latter containing pure protein. Cytochrome c_{550} and cytochrome c_{556} were isolated in the reduced and oxidized forms, respectively. Homogeneity was evaluated by native electrophoresis according to Davis [22], using 10% acrylamide gels. The gels were stained for heme [23], and then for protein with Coomassie blue R-250.

2.3. Redox potentiometry

Equilibrium redox titrations were performed according to Dutton [24]. The following redox mediators were used: *p*-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, 2.5 μM each for the titration of cytochrome c_{550}; *p*-benzoquinone, 1,2-

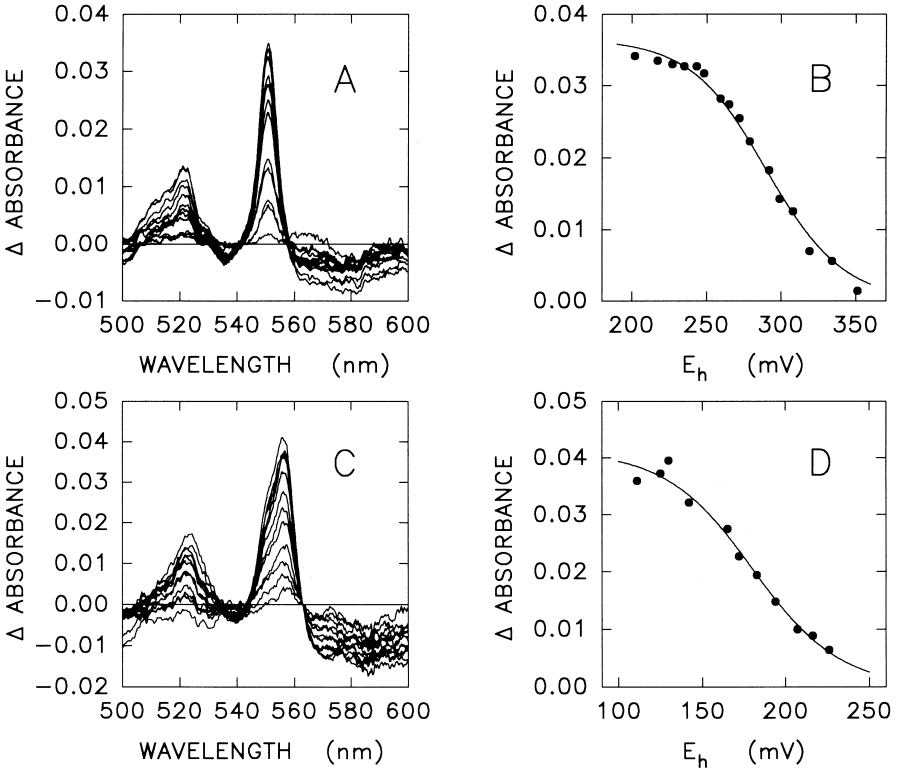

Fig. 1. (A) Reduced-minus-oxidized difference spectra of *Rs. salinarum* cytochrome c_{550} as a function of the ambient redox potential (ranging from +205 to +353 mV). A spectrum of the oxidized cytochrome was determined at E_h = +424 mV, and subtracted to all the spectra recorded at lower potentials. The corresponding absorbance changes as determined at 550 minus 540 nm are shown in B; the continuous line represents a fit of the experimental points to a nernstian curve (n = 1). (C) Reduced-minus-oxidized difference spectra of *Rs. salinarum* cytochrome c_{556} as a function of the ambient potential (ranging from +111 to +263 mV). The reference spectrum of the oxidized cytochrome was determined at E_h = +276 mV. The corresponding absorbance changes as determined at 556 minus 546 nm are shown in D; the continuous line represents a fit of the experimental points to a nernstian curve (n = 1).

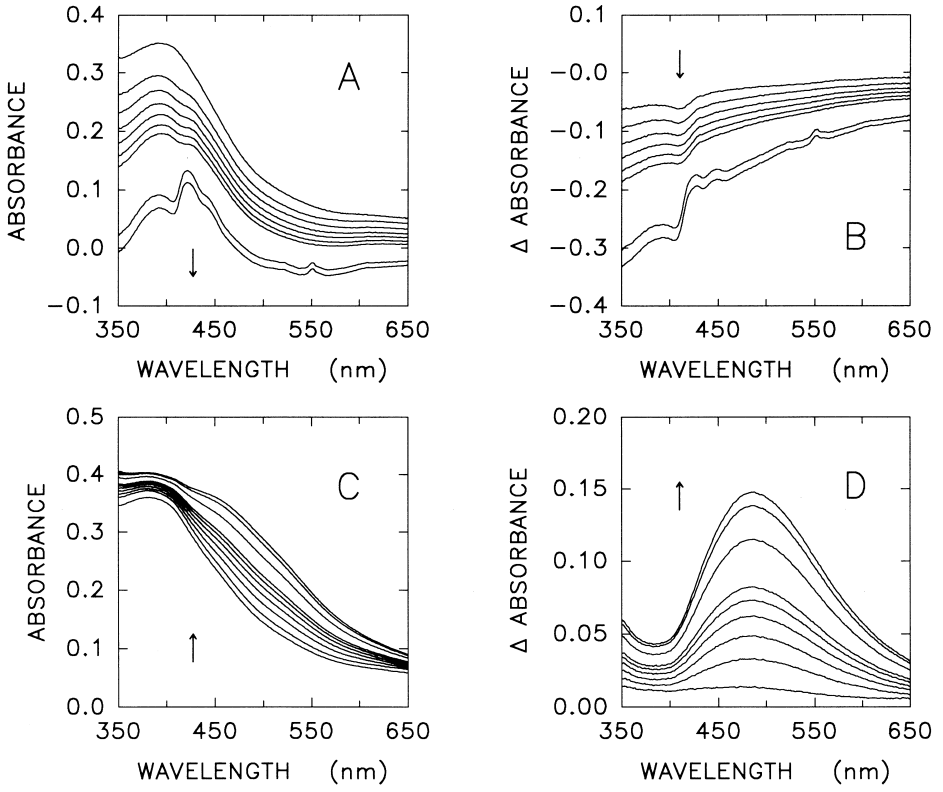

Fig. 2. (A) Time-dependent absorbance changes of HiPIP iso-2 (20 μM) in the presence of *Rs. salinarum* membranes (0.1 μM oxidase); the spectra were recorded during a 90-min time interval. (B) Time-dependent difference spectra determined by subtracting the initial spectrum recorded immediately after the addition of membranes. (C,D) Same as in A and B, but corresponding to a time interval of 80 min and in the presence of 20 μM HiPIP iso-1 and 0.5 nM oxidase.

naphthoquinone, 1,4-naphthoquinone, duroquinone, 1,4-dihydroxynaphthoquinone, 2.5 μM each for the titration of cytochrome c_{556}. Sodium ascorbate and hexacyanoferrate(III) were used as reductant and oxidant, respectively.

2.4. Spectrophotometric measurements

All the spectrophotometric measurements were carried out at pH 7.3 (10 mM Tris-HCl), 25°C, using a Jasco 7850 instrument. The molar extinction coefficients of *Rs. salinarum* HiPIPs, and cytochrome c_{550} were determined as described [13]. The concentration of bc_1 complexes and terminal oxidases was determined using 19, 21, and 23 mM^{-1} cm^{-1} as the extinction coefficients of *c*-, *b*-, and *a*-type cytochromes, respectively. The oxidized-minus-reduced difference spectrum of HiPIP iso-1 features a broad band with maximum at 490 nm ($\Delta\varepsilon = 8$ mM^{-1} cm^{-1}), while the reduced-minus-oxidized difference spectra of cytochrome c_{550} and of partially purified cytochrome c_{556} display maxima at 417 nm ($\Delta\varepsilon = 60$ mM^{-1} cm^{-1}) and at 420 nm, respectively. Therefore, spectrophotometric measurements were carried out at 490, 417, and 420 nm in order to determine the kinetic parameters of the redox changes of HiPIP iso-1, cytochrome c_{550}, and cytochrome c_{556}, respectively.

3. Results

3.1. Rhodospirillum salinarum

Four soluble electron transfer proteins (HiPIP iso-1 and iso-2, and cytochromes c_{550} and c_{556}) were isolated from the extract of *Rs. salinarum* cells grown under respiratory conditions, using classic chromatographic separation techniques. The two HiPIPs and cytochrome c_{550} were also purified, while the purification of cytochrome c_{556} was not pursued. The Hi-

PIP iso-2/HiPIP iso-1/cytochrome c_{550} molar ratio was estimated to be ca. 2:1:0.25. The redox properties of HiPIP iso-1 and iso-2 were identical to those previously reported for the same HiPIPs isoforms isolated from phototrophically grown *Rs. salinarum* [21]. The reduction potentials of cytochromes c_{550} and c_{556} were determined using spectrophotometric redox titrations. Fig. 1A shows a series of spectra of cytochrome c_{550}, measured under different redox potentials, while in Fig. 1B the corresponding values of the absorbance changes determined at 550 minus 540 nm are reported. The experimental points can be fitted to a nernstian curve ($n=1$), yielding $E_{m,7} = +290 \pm 2$ mV. When the redox titration of cytochrome c_{556} was performed, and the corresponding absorbance changes determined at 556 minus 546 nm, a reduction potential of $+180 \pm 3$ mV was obtained (Fig. 1C,D).

The competence of HiPIP iso-1 and iso-2, cytochrome c_{550}, and c_{556} in the respiratory electron transfer chain of *Rs. salinarum* was investigated. Fig. 2A shows a series of spectra recorded as a function of time in the presence of 20 μM HiPIP iso-2 and a suspension of *Rs. salinarum* membranes obtained from aerobically dark-grown cells, while the corresponding difference spectra are reported in Fig. 2B. The oxidized-minus-reduced difference spectrum of HiPIP iso-2 was not observed after 90 min: this result suggests that the oxidized form of the protein is unstable. The unspecific reduction of all membrane-bound cytochromes is instead observed, possibly a consequence of the HiPIP iso-2 $[Fe_4S_4]^{3+}$ cluster degradation, with concomitant release of sulfide [21]. Analogous results were obtained when a similar experiment was carried out in the presence of 20 μM HiPIP iso-2 as a function of membrane concentration (corresponding to a concentration of terminal oxidase ranging from 3 to 100 nM, data not shown). Oxidation of HiPIP iso-1 was detected upon addition of the reduced protein to a suspension of *Rs. salinarum* membranes. As shown in Fig. 2C,D, the oxidized-minus-reduced difference spectrum of HiPIP iso-1 is observed, suggesting that HiPIP iso-1 is oxidized by the terminal oxidase in a straightforward process. Addition of 50 μM KCN, a concentration previously shown to specifically inhibit the aa_3 oxidase of *Rs. salinarum* [17] strongly inhibits HiPIP iso-1 oxidation (Fig. 3A). Moreover, HiPIP iso-1 reduction is essentially blocked by the presence of myxothiazol, a specific inhibitor of the bc_1 complex (Fig. 3B).

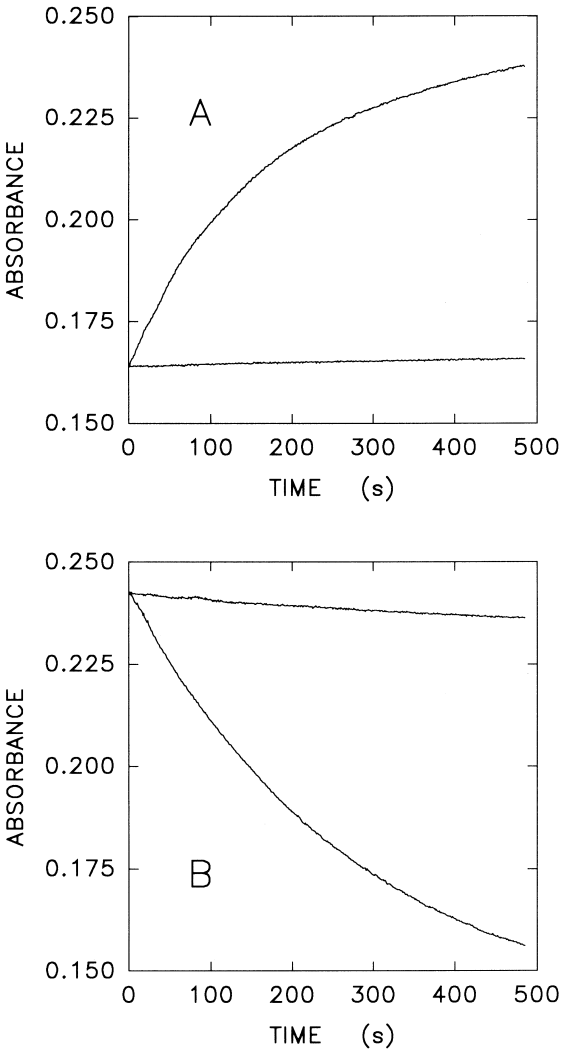

Fig. 3. (A) Time course of oxidation of 12 μM HiPIP iso-1 catalyzed by *Rs. salinarum* membranes (16 nM oxidase), in the absence (upper trace) or in the presence (lower trace) of 50 μM KCN. (B) time course of reduction of 12 μM HiPIP iso-1 catalyzed by *Rs. salinarum* membranes (63 nM bc_1) in the absence (lower trace) or in the presence (upper trace) of 1 μM myxothiazol.

The time courses of oxidation of cytochrome c_{550} and HiPIP iso-1 in the presence of *Rs. salinarum* membranes are shown in Fig. 4A and B, respectively. In both cases, the experimental points can be interpreted using a first-order kinetic model. The dependence of the oxidation rate constant on the concentration of each soluble electron donor is different

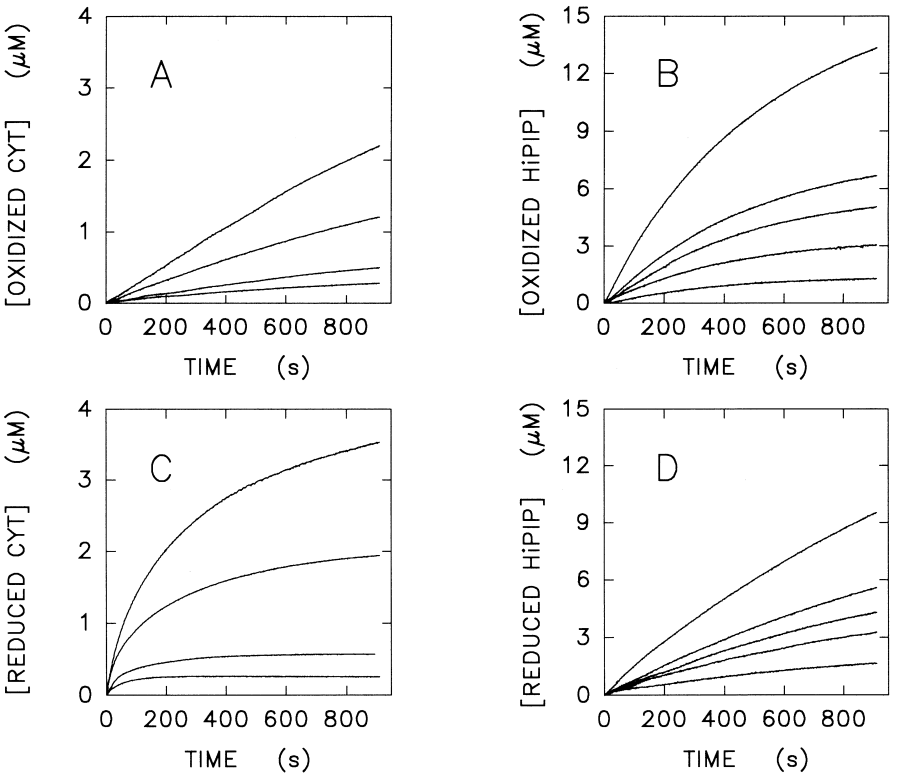

Fig. 4. Time course of oxidation (A,B, 0.2 nM oxidase) and reduction (C,D, 0.73 nM bc_1) of cytochrome c_{550} (A,C) and HiPIP iso-1 (B,D), catalyzed by *Rs. salinarum* membranes. The following concentrations of cytochrome c_{550} and HiPIP iso-1 were used: 0.4, 0.8, 3 and 6 μM (cytochrome c_{550}), and 2.5, 5, 7.5, 10, and 20 μM (HiPIP iso-1).

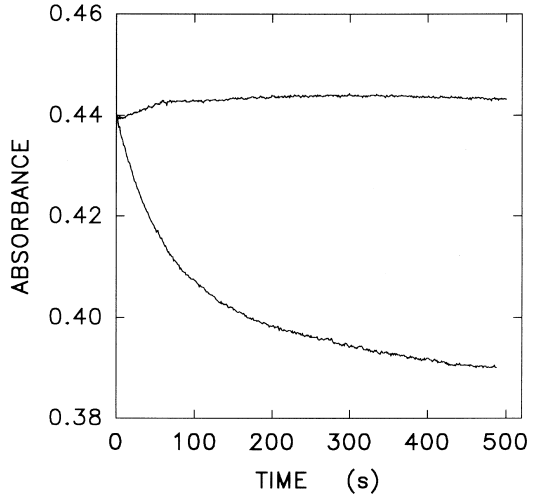

Fig. 5. Time course of oxidation (lower trace) of cytochrome c_{556} by *Rs. salinarum* membranes (0.48 nM oxidase). The time dependence of reduction of cytochrome c_{556} by *Rs. salinarum* membranes in the presence of 5 mM succinate and 50 μM KCN is also shown (upper trace, 1.8 nM bc_1).

when considering cytochrome c_{550} or HiPIP iso-1: in the first case, k_{obs} slightly decreases as the concentration of cytochrome increases in the interval 0.4–6 μM, ranging from 1×10^{-3} to 4.5×10^{-4} s^{-1}. On the contrary, the rate constant of HiPIP iso-1 oxidation appears to be independent of its concentration, varying in the range 2.5–20 μM, with a mean value of $k_{obs}=(2.4\pm0.5)\times10^{-3}$ s^{-1}.

The time courses of cytochrome c_{550} and HiPIP iso-1 reduction by the bc_1 complex are shown in Fig. 4C and D, respectively. The kinetics of cytochrome c_{550} reduction appears to be biphasic, composed of an initial fast phase, tentatively interpreted as a first-order process ($k_{obs}=0.2\times10^{-2}$ s^{-1}), followed by a much slower phase. The reduction of HiPIP iso-1 by the bc_1 complex was found to follow a first-order kinetics, independent of HiPIP iso-1 concentration, yielding a mean value of $k_{obs}=(1\pm0.16)\times10^{-3}$ s^{-1}.

The kinetics of oxidation and reduction of cytochrome c_{556} by *Rs. salinarum* membranes are shown in Fig. 5. While the oxidation of cytochrome c_{556} can

be readily detected (Fig. 5, lower trace), the reduction of cytochrome c_{556} by the bc_1 appears to be, if at all, a very slow electron transfer process (Fig. 5, upper trace).

3.2. *Rhodoferax fermentans*

In order to extend our analysis on the role of HiPIPs as respiratory electron carriers, the rate of HiPIP oxidation and reduction by *Rf. fermentans* membranes was investigated. Fig. 6A shows that the time course of HiPIP oxidation catalyzed by the terminal oxidase follows a first-order kinetics independently of the HiPIP concentration. The mean value of $k_{obs} = (3.3 \pm 0.5) \times 10^{-3}$ s^{-1} is in excellent agreement with our previous observations at a single HiPIP concentration (20 μM), which yielded

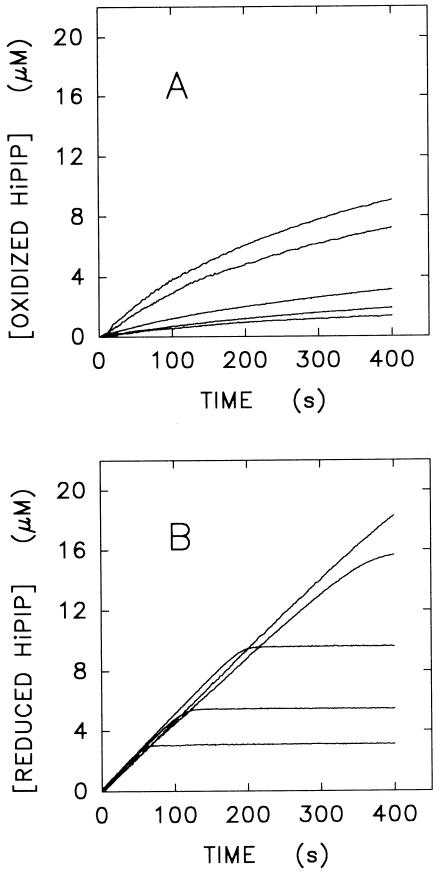

Fig. 6. Kinetics of *Rf. fermentans* HiPIP oxidation (A, 6 nM oxidase) and reduction (B, in the presence of 5.8 nM bc_1 complex, 5 mM succinate, and 50 μM KCN) catalyzed by *Rf. fermentans* membranes, and monitored by absorbance changes at 490 nm, at HiPIP concentrations of 2.9, 5, 10, 16, and 25 μM.

$k_{obs} = (2.9 \pm 0.6) \times 10^{-3}$ s^{-1} [16]. To evaluate the competence of HiPIP in *Rf. fermentans* respiration, this values should be compared with the rate constants of oxidation of alternative electron donors to the terminal oxidase. On the other hand, it is known that aerobically dark-grown cells of *Rf. fermentans* contain hardly detectable amounts of soluble high potential *c*-type cytochromes [20]. Therefore, the soluble fraction of anaerobically light-grown cells of *Rf. fermentans* was used to isolate and purify the only detectable high potential *c*-type cytochrome, known to belong to the c_8 class [25]. Under the same experimental conditions used to determine the rate of HiPIP oxidation, the rate of oxidation of cytochrome c_8 (4.8 μM) was found to follow a biphasic kinetics, composed by an initial first-order process (yielding k_{obs} of 1.2×10^{-2} s^{-1}, but accounting for only 8% of the total amplitude), followed by a very slow phase not obeying a first-order kinetics (data not shown).

Fig. 6B shows the time course of HiPIP reduction (at concentrations ranging from 2.9 to 25 μM), by the *Rf. fermentans* bc_1 complex. The observed rate of reduction clearly follows a zero-order kinetics independently of HiPIP concentration, yielding a mean value of V_{in} equal to $(4.9 \pm 0.36) \times 10^{-2}$ μM s^{-1}.

4. Discussion

The composition of the soluble extract of *Rs. salinarum* cells grown under anaerobic light (photosynthetic) conditions was investigated in detail by Meyer et al. [21]. In particular, *Rs. salinarum* was shown to contain two HiPIP isoforms (iso-1 and iso-2) characterized by molecular masses of 10 and 45 kDa, respectively, along with a high potential *c*-type cytochrome [21], exhibiting an α-band centered at 550 nm (cytochrome c_{550}). The reduction potential of HiPIP iso-1 was reported to be +265 mV, while HiPIP iso-2 was shown to be unstable in the oxidized form; its reduction potential was proposed to be as high as, or higher than, the reduction potential of HiPIP iso-1 [21]. The reduction potential of cytochrome c_{550} was not determined.

In the present study, the composition of the soluble fraction of *Rs. salinarum* cells grown under aerobic dark (respiratory) conditions has been analyzed

in detail. HiPIP iso-1, HiPIP iso-2 and cytochrome c_{550} were also found in aerobically dark-grown cells. In addition, a soluble *c*-type cytochrome, featuring an asymmetric α-band at 556 nm (cytochrome c_{556}) was isolated. This cytochrome was not detected in photosynthetically grown cells of *Rs. salinarum* [21]. Cytochromes c_{550} and c_{556}, characterized by reduction potentials of +290 and +180 mV, respectively, are the most abundant *c*-type cytochromes present in aerobically dark-grown *Rs. salinarum* cells, in agreement with previous observations of two high potential *c*-type cytochromes in the soluble fraction of crude cell-free extracts of *Rs. salinarum* cells grown under respiratory conditions [17].

Experiments carried out to determine the kinetics of HiPIP iso-1 oxidation and reduction by *Rs. salinarum* membranes obtained from cells grown under respiratory conditions, in the absence and presence of cyanide or myxothiazol, indicate that these processes are specifically catalyzed by the terminal aa_3 oxidase and the bc_1 complex, respectively. In agreement with previous observations [21], the oxidized form of HiPIP iso-2 was shown to be unstable. While cytochrome c_{550} is able to both oxidize the bc_1 complex and reduce the terminal oxidase, cytochrome c_{556} efficiently reduces the terminal oxidase, but cannot oxidize the bc_1 complex, a result that can be explained by considering that the reduction potential of cytochrome c_{556} (+180 mV) is lower than that of *Rs. salinarum* cytochrome c_1 (+244 mV) [17]. Therefore, cytochrome c_{550} and HiPIP iso-1 represent the only major electron shuttles between the bc_1 complex and the terminal aa_3 oxidase in *Rs. salinarum*.

Comparing the rate constants of oxidation and reduction of cytochrome c_{550} and HiPIP iso-1 in the presence of *Rs. salinarum* membranes, it can be concluded that HiPIP is the more efficient reductant of the terminal oxidase, displaying a reaction rate constant ca. 2–5 times larger, while cytochrome c_{550} is the more efficient oxidant of the bc_1 complex, featuring a rate constant ca. five-fold larger with respect to the HiPIP, at comparable concentrations of the soluble protein donor. The latter finding correlates well with the observation that the soluble cytochrome c_8 from *Chromatium vinosum* is reduced by the bc_1 complex more efficiently than the HiPIP [26]. The observed rate constant of oxidation of cytochrome c_{550} by *Rs. salinarum* membranes was found to decrease by a factor of two as the concentration of cytochrome was increased from 0.4 to 6 μM. This behavior is similar to the dependence of the rate constant of oxidation of horse heart cytochrome *c* by the mitochondrial terminal oxidase, for which a decrease by a factor of 2–5 for the observed rate constant, upon cytochrome *c* concentration increase from 0.3 to 5 μM, was reported [27,28]. Similar results have also been obtained for the kinetics of oxidation of horse heart and *Paracoccus denitrificans* cytochromes *c* by the *P. denitrificans* aa_3 oxidase [29,30]. These latter findings can be rationalized using the steady-state approximation and the reaction mechanism proposed by Minnaert [31]. On the other hand, the rate constant of HiPIP oxidation by *Rs. salinarum* membranes does not depend on HiPIP concentration, suggesting that no rate-limiting steps occur under the experimental conditions used.

It is known that in aerobically dark-grown cells of *Rf. fermentans* a large quantity of HiPIP is expressed [16], and that its addition to a suspension of membranes stimulates the rate of succinate-dependent respiration [16]. On the other hand, high potential *c*-type cytochromes are hardly detectable [20]. The present study indicates that the rate of oxidation of *Rf. fermentans* HiPIP by a suspension of membranes obeys a first-order kinetics. Furthermore, the oxidation rate of the high potential *Rf. fermentans* cytochrome c_8 (isolated and purified from anaerobically light-grown cells) by the terminal oxidase is very slow. It is noteworthy that the oxidation rate of c_8-type cytochromes by the mitochondrial cytochrome *c* oxidase has also been reported to be slow, and does not obey first-order kinetics [32].

The reduction of HiPIP by the *Rf. fermentans* bc_1 complex obeys zero-order kinetics independently of HiPIP concentration, suggesting the formation of a complex between HiPIP and the bc_1. The observed kinetics was analyzed by using the integrated Michaelis–Menten equation, yielding $K_m \cong 0.4$ μM, and $V_{max} = (5.6 \pm 0.82) \times 10^{-2}$ μM × s^{-1}, this latter value similar to those ($V_{max} \cong 5 \times 10^{-2}$ μM × s^{-1}) reported for the reduction of horse heart cytochrome *c* and *Rhodobacter sphaeroides* cytochrome c_2 by the mitochondrial bc_1 complex [33]. A comparison of the efficiency of different *c*-type cytochromes as oxidants of the mitochondrial bc_1 complex has revealed that only few cytochromes (from *Euglena gracilis*,

Crithidia fasciculata, *Crithidia oncopelti*, and *Rb. sphaeroides*) are able to be reduced at rates comparable with the one sustained by horse heart cytochrome *c* [32]. To the best of our knowledge, this is the first time that a soluble non-heme iron–sulfur protein is shown to oxidize the bc_1 complex at a rate comparable with that observed with *c*-type cytochromes.

In summary, we have found that HiPIPs isolated from *Rf. fermentans* and *Rs. salinarum* grown under aerobic dark conditions play a significant role in bacterial respiration, by virtue of their interaction with both the bc_1 complex and the terminal oxidase. Such function, usually featured in bacterial respiration by *c*-type cytochromes, cannot be considered any more as exclusively played by heme-containing proteins.

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References

[1] B.L. Trumpower, R.B. Gennis, Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration, Annu. Rev. Biochem. 63 (1994) 675–716.
[2] R.E. Blankenship, M.T. Madigan, C.E. Bauer (Eds.), Anoxygenic Photosynthetic Bacteria, Kluwer Academic, Dordrecht, The Netherlands, 1995.
[3] D. Yang, Y. Oyaizu, H. Oyaizu, G.J. Olsen, C.R. Woese, Mitochondrial origins, Proc. Natl. Acad. Sci. USA 82 (1985) 4443–4447.
[4] R.P. Ambler, M. Daniel, J. Hermoso, T.E. Meyer, R.G. Bartsch, M.D. Kamen, Cytochrome c_2 sequence variation among the recognised species of purple nonsulfur photosynthetic bacteria, Nature 278 (1979) 659–660.
[5] F.R. Salemme, S.T. Freer, Ng.H. Xuong, R.A. Alden, J. Kraut, The structure of oxidized cytochrome c_2 of *Rhodospirillum rubrum*, J. Biol. Chem. 248 (1973) 3910–3921.
[6] M. Caffrey, E. Davidson, M.A. Cusanovich, F. Daldal, Cytochrome c_2 mutants of *Rhodobacter capsulatus*, Arch. Biochem. Biophys. 292 (1992) 419–426.
[7] R.G. Bartsch, The distribution of soluble metallo-redox proteins in purple phototrophic bacteria, Biochim. Biophys. Acta 1058 (1991) 28–30.
[8] R.G. Bartsch, High potential iron proteins: bacterial, Methods Enzymol. 23 (1971) 644–649.
[9] T.E. Meyer, Purification and properties of high-potential iron–sulfur proteins, Methods Enzymol. 243 (1994) 435–447.
[10] I. Bertini, S. Ciurli, C. Luchinat, The electronic structure of FeS centers in proteins and models. A contribution to the understanding of their electron transfer properties, Struct. Bonding 83 (1995) 1–53.
[11] A. Hochkoeppler, S. Ciurli, G. Venturoli, D. Zannoni, The high potential iron–sulfur protein (HiPIP) from *Rhodoferax fermentans* is competent in photosynthetic electron transfer, FEBS Lett. 357 (1995) 70–74.
[12] B. Schoepp, P. Parot, M. Menin, J. Gaillard, P. Richaud, A. Vermeglio, In vivo participation of a high potential iron–sulfur protein as electron donor to the photochemical reaction center of *Rubrivivax gelatinosus*, Biochemistry 34 (1995) 11736–11742.
[13] A. Hochkoeppler, P. Kofod, G. Ferro, S. Ciurli, Isolation, characterization, and functional role of the high-potential iron–sulfur protein (HiPIP) from *Rhodoferax fermentans*, Arch. Biochem. Biophys. 322 (1995) 313–318.
[14] A. Hochkoeppler, D. Zannoni, S. Ciurli, M.A. Cusanovich, T.E. Meyer, G. Tollin, Kinetics of photo-induced electron transfer from high-potential iron–sulfur protein to the photosynthetic reaction center of the purple phototroph *Rhodoferax fermentans*, Proc. Natl. Acad. Sci. USA 93 (1996) 6998–7002.
[15] M.M. Pereira, A.M. Antunes, O.C. Nunes, M.S. da Costa, M. Teixeira, A membrane-bound HiPIP type center in the thermohalophile *Rhodotermus marinus*, FEBS Lett. 352 (1994) 327–330.
[16] A. Hochkoeppler, P. Kofod, D. Zannoni, HiPIP oxido-reductase activity in membranes from aerobically grown cells of the facultative phototroph *Rhodoferax fermentans*, FEBS Lett. 375 (1995) 197–200.
[17] G. Moschettini, A. Hochkoeppler, B. Monti, B. Benelli, D. Zannoni, The electron transport system of the halophilic purple nonsulfur bacterium *Rhodospirillum salinarum*. 1. A functional and thermodynamic analysis of the respiratory chain in aerobically and photosynthetically grown cells, Arch. Microbiol. 168 (1997) 302–309.
[18] A. Hiraishi, Y. Hoshino, T. Satoh, *Rhodoferax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the '*Rhodocyclus gelatinosus*-like' group, Arch. Microbiol. 155 (1991) 330–336.
[19] E.E. Mack, L. Mandelco, C.R. Woese, M.T. Madigan, *Rhodospirillum sodomense*, sp. nov., a dead sea *Rhodospirillum* species, Arch. Microbiol. 160 (1993) 363–371.
[20] A. Hochkoeppler, G. Moschettini, D. Zannoni, The electron transport system of the facultative phototroph *Rhodoferax fermentans*. I. A functional, thermodynamic and spectroscopic study of the respiratory chain of dark- and light-grown cells, Biochim. Biophys. Acta 1229 (1995) 73–80.
[21] T.E. Meyer, J. Fitch, R.G. Bartsch, G. Tollin, M.A. Cusanovich, Unusual high redox potential ferredoxins and soluble cytochromes from the moderately halophilic purple pho-

totrophic bacterium *Rhodospirillum salinarum*, Biochim. Biophys. Acta 1017 (1990) 118–124.
[22] B.J. Davis, Disc electrophoresis-II. Method and application to human serum proteins, Ann. New York Acad. Sci. 121 (1964) 404–427.
[23] P.E. Thomas, D. Ryan, W. Levin, An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels, Anal. Biochem. 75 (1976) 168–176.
[24] P.L. Dutton, Redox potentiometry: determination of midpoint potentials of oxidation-reduction components of biological electron transfer systems, Methods Enzymol. 54 (1978) 411–435.
[25] A. Hochkoeppler, S. Ciurli, P. Kofod, G. Venturoli, D. Zannoni, On the role of cytochrome c_8 in photosynthetic electron transfer of the purple nonsulfur bacterium *Rhodoferax fermentans*, Photos. Res. 53 (1997) 13–21.
[26] J. Tan, G.E. Corson, Y.L. Chen, M.C. Garcia, S. Güner, D.B. Knaff, The ubiquinol:cytochrome c_2/c oxidoreductase of *Chromatium vinosum*, Biochim. Biophys. Acta 1144 (1993) 69–76.
[27] L. Smith, H. Conrad, A study of the kinetics of the oxidation of cytochrome c by cytochrome c oxidase, Arch. Biochem. Biophys. 63 (1956) 403–413.
[28] B. Errede, G.P. Haight Jr., M.D. Kamen, Oxidation of ferrocytochrome c by mitochondrial cytochrome c oxidase, Proc. Natl. Acad. Sci. USA 73 (1976) 113–117.
[29] B. Bolgiano, L. Smith, H.C. Davies, Electron transport reactions in a cytochrome c-deficient mutant of *Paracoccus denitrificans*, Biochim. Biophys. Acta 973 (1989) 227–234.
[30] B. Bolgiano, L. Smith, H.C. Davies, Kinetics of the interaction of the cytochrome c oxidase of *Paracoccus denitrificans* with its own and bovine cytochrome c, Biochim. Biophys. Acta 933 (1988) 341–350.
[31] K. Minnaert, The kinetics of cytochrome c oxidase. I. The system: cytochrome c-cytochrome oxidase-oxygen, Biochim. Biophys. Acta 50 (1961) 23–34.
[32] B. Errede, M.D. Kamen, Comparative kinetic studies of cytochromes c in reactions with mitochondrial cytochrome c oxidase and reductase, Biochemistry 17 (1978) 1015–1027.
[33] J. Hall, X. Zha, L. Yu, C. Yu, F. Millett, The binding domain of horse cytochrome c and *Rhodobacter sphaeroides* cytochrome c_2 for *the Rhodobacter sphaeroides* cytochrome bc_1 complex, Biochemistry 26 (1987) 4501–4504.