HiPIP oxido-reductase activity in membranes from aerobically grown cells of the facultative phototroph *Rhodoferax fermentans*

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Received 2 October 1995

Abstract The role of the periplasmically located, water-soluble, HiPIP (high-potential iron-sulfur protein) in the respiratory chain of the facultative phototroph *Rhodoferax fermentans* has been examined. The oxidized HiPIP is reduced by succinate-dependent respiration via the bc_1 complex, this reaction being inhibited by myxothiazol and/or stigmatellin. The reduced HiPIP can be oxidized by the membrane-bound cytochrome oxidase, this reaction being inhibited by 0.1 mM cyanide. We conclude that aerobically grown *Rf. fermentans* contains a redox chain in which HiPIP mediates electron transfer between the bc_1 complex and the *cb*-type cytochrome oxidase.

Key words: HiPIP; Electron transfer; Bacterial respiration; Facultative phototrophs; *Rhodoferax fermentans*

1. Introduction

The respiratory activities of facultative phototrophic bacteria are catalyzed by a series of membrane-bound redox protein complexes such as the NADH- and succinate-dehydrogenases, the bc_1 complex, quinol oxidases, and cytochrome *c* oxidases [ ]. These redox complexes are linked via both lipophilic quinone molecules and water soluble, periplasmically located, proteins such as cyt c_2 and cyt *iso*-c_2 [1]. Exceptions to this rule are the electron transport chains of *Rhodobacter capsulatus*, in which a membrane-associated cyt *c* (cyt c_y) has been shown to be alternative to cyt c_2 [2], and species such as for example *Rhodospirillum salinarum*, *Rubrivivax gelatinosus* and *Rhodoferax fermentans* in which no significant amounts of high-potential, water soluble, *c*-type cytochromes have been found [3,4]. Conversely, these latter species, are endowed with high-potential iron-sulfur proteins (HiPIP) [3,5] and it has been suggested that HiPIPs might complement the absence of cyt c_2 [6]. The structural and spectroscopic properties of HiPIPs have recently been reviewed [7], but their functional role is still a matter for discussion [6].

HiPIP from *Rf. fermentans* has a molecular mass of 8.7 kDa, a reduction potential at pH 7.0 of +351 mV [5] and it is highly homologous to the HiPIP of *R. gelatinosus* (J. van Beeumen, personal communication). The purified HiPIP is obtained in its reduced form also under aerobic conditions, but can be oxidized by chemicals such as hexacyanoferrate(III) [5]. It has recently been demonstrated that reduced HiPIP from *Rf. fermentans* can also be oxidized upon photoexcitation of the photochemical reaction center (RC) through specific redox interaction with cyt *c*-556 [8], one of the four *c*-type heme groups associated with the RC in light-grown cells of this bacterial species [8,9]. This finding is among the very few indications for a role of HiPIPs in bacterial photosynthesis [10,11]. On the other hand, HiPIPs might also have a role in respiratory oxidative processes of facultative phototrophs as recently suggested for a membrane-bound protein containing a HiPIP-type cluster core in membranes from the thermohalophilic aerobe, *Rhodothermus marinus* [12].

In this study we report that membranes from aerobically dark-grown cells of the facultative phototroph *Rf. fermentans* show HiPIP oxido-reductase activities. Thus, similarly to the role of cyt c_2 in species such as *Rb. capsulatus*, *Rb. sphaeroides* and *Rsp. rubrum* [1], the HiPIP of *Rf. fermentans* mediates electron transfer between the bc_1 complex and the cyanide-sensitive cytochrome oxidase.

2. Materials and methods

2.1. Cell growth and membrane isolation procedure

Cells of *Rf. fermentans* were aerobically dark-grown at 30°C using a Microferm Fermentor (New Brunswick Sci. Co., Inc., Edison NJ, USA) under a continuous air flux of 6 $l \cdot min^{-1}$ in MYCA medium as previously described [4]. Cells were harvested at late log phase and membrane fragments prepared according to ref. [4].

2.2. Purification of HiPIP

HiPIP from aerobically dark-grown cells of *Rf. fermentans* was purified using a previously reported procedure for purification of HiPIP from anaerobically light-grown cells [5].

2.3. Respiratory activities and absorption spectra

All experiments were performed in 50 mM MOPS buffer containing 5 mM $MgCl_2$ (pH 7.2). The rates of oxygen consumption were determined polarographically using a Clark-type oxygen electrode at 30°C. The rates were measured over a time period of ca. 15 min with a membrane concentration of 0.7 mg protein $\cdot ml^{-1}$ (final volume 1.9 ml). Absorption spectra were measured at 25°C using a Jasco 7850 spectrophotometer. Notably, addition of 22 μg protein $\cdot ml^{-1}$ membranes contributed to variation of the base line; however, the membrane absorption spectrum did not change with time. Concentration of the reduced HiPIP was calculated using absorption coefficients of 44 $mM^{-1} \cdot cm^{-1}$ and 18 $mM^{-1} \cdot cm^{-1}$ at 278 nm and 388 nm, respectively [5].

2.4. Protein determination

The protein concentration of the membrane suspensions was determined according to Bradford [13], using bovine serum albumine (BSA) as standard.

3. Results and discussion

3.1. Isolation and purification of HiPIP

The HiPIP isolated from aerobically dark-grown cells of *Rf. fermentans* (approx. 7 mg per 100 g of wet-cell paste) shows

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SDI 0014-5793(95)01188-9

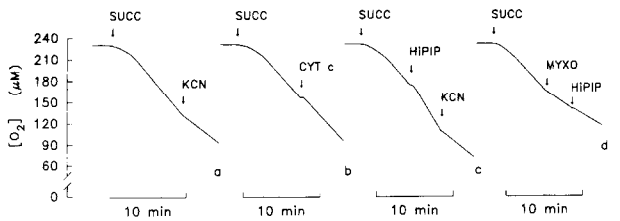

Fig. 1. Determination of the succinate-dependent oxygen consumption as catalyzed by *Rf. fermentans* membranes (0.7 mg protein·ml^{-1}). The concentration of molecular oxygen was determined polarographically as a function of time.

thermodynamic ($Em_{7.0}$ = +351 mV) and absorption spectroscopic properties (see following paragraphs) similar to those reported for the HiPIP of anaerobically light-grown cells [5]. To our knowledge, this is the first report on isolation of a HiPIP from aerobically grown cells of a facultative phototroph (see ref. [6]). Notably, the purification procedure adopted here lead to purification of a *c'*-type cytochrome from the water-soluble fraction of anaerobically light-grown cells of *Rf. fermentans* [5]. Conversely, no detectable amount of this *c'*-type cytochrome was isolated from the soluble fraction of aerobically dark-grown cells.

3.2. Respiratory activities

It is widely established that the overall respiratory activities catalyzed by membrane fragments isolated from aerobically grown cells of facultative phototrophs such as *Rb. capsulatus*, *Rb. sphaeroides* and *Rsp. rubrum* are significantly accelerated by addition of exogenously added horse-heart cytochrome *c* (50 μM) [1]. Indeed, during cell-disruption and membrane-isolation procedure, a large amount of the hydrophilic, periplasmically located cytochrome c_2 is lost; thus, the physiological rates of oxygen consumption by membrane fragments can only be recovered through addition of either cyt c_2 or homologous *c*-type cytochromes. To investigate the possible role of the water soluble, periplasmically located [6] HiPIP in the respiratory chain of *Rf. fermentans*, we have measured the overall rates of oxygen consumption in membrane suspensions isolated from aerobically dark-grown cells either in the presence or in the absence of HiPIP. The results of Fig. 1 (trace a) show that upon addition of succinate the O_2 consumption by membrane fragments was 0.21 μM·s^{-1} while the overall rate decreased to 0.14 μM·s^{-1} in the presence of 0.1 mM cyanide, a concentration previously shown to completely inhibit the cytochrome oxidase activity of this bacterial species [4]. Significantly, in the presence of 15 μM HiPIP the rate of oxygen consumption increased to 0.32 μM·s^{-1} and decreased to 0.16 μM·s^{-1} upon addition of 0.1 mM cyanide (see trace c). It is noteworthy that addition of 50 μM horse-heart cytochrome *c* did not affect the rate of succinate-dependent respiration (trace b). In Fig. 1 (trace d) it is also shown that addition of the bc_1 complex specific inhibitors, myxothiazol and/or stigmatellin (5 μM each) decreased the rates of oxygen consumption from 0.21 and 0.32 μM·s^{-1} to 0.13 and 0.11 μM·s^{-1} in the absence or in the presence of HiPIP, respectively. It is apparent that the strong inhibitory effect observed also in the presence of HiPIP indicates that the reduction of HiPIP occurs via the bc_1 complex.

In Fig. 2, the rate of succinate-dependent oxygen consumption catalyzed by membranes (0.7 mg protein·ml^{-1}) as a function of HiPIP concentration, is shown. Clearly, under these experimental conditions, the rate of succinate respiration is strongly enhanced and close to saturation at 40 μM HiPIP (% rate enhancement = 62). This pattern is similar to that observed in preparations of membranes from facultative phototrophs with cyt c_2 as electron carrier, upon addition of horse-heart cyt *c* [14]. In this latter case, the percentage of rate enhancement is an indirect measurement of the amount of inside-out vesicles because the accessibility of exogenous cyt *c* to its reacting site is limited to plasma membrane vesicles having the same cells membrane orientation (right side-out vesicles) and/or to membrane sheets [15]. By applying a formula originally proposed for the case of cytochrome *c* [16], the percentage inverted orientation of membrane vesicles can be calculated as follows:

$$\% \text{ inverted orientation} = (\text{non-enhanced rate/enhanced rate}) \times 100.$$

In this way it is estimated that 40 μM HiPIP has access to only 38% of the total *Rf. fermentans* membrane preparation; that is, based on a bc_1 content of 0.6 nmol per mg protein [4], the estimated HiPIP/bc_1 molar ratio would be in the order of 250, a value similar to the horse-heart cyt *c*/bc_1 molar ratio measured in analogous membrane preparations from aerobically dark-grown *Rb. capsulatus* [2,14]. Thus, the respiratory chain of *Rf. fermentans* seems to interact with exogenously added HiPIP as cyt *c* normally does in *Rb. capsulatus*.

3.3. HiPIP oxido-reductase activity

To test for the presence of a HiPIP oxido-reductase activity in membranes of aerobically grown *Rf. fermentans*, a series of absorption spectra recorded in the 400–600 nm wavelength range as a function of time was performed. In accordance with the observation that purified HiPIP remains reduced even in the presence of molecular oxygen [5], no time dependence of the absorption spectrum of the reduced HiPIP was observed in the absence of membranes (not shown). Conversely, when reduced HiPIP (20 μM) was added to the membrane suspension a time-dependent process resulting from HiPIP oxidation [5] was ob-

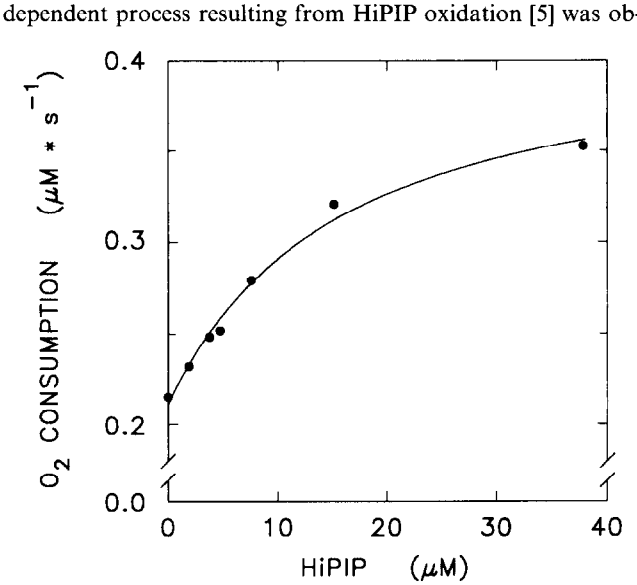

Fig. 2. Rate of succinate-dependent respiration as a function of HiPIP concentration. The solid line through the experimental points is described by a hyperbolic curve. Conditions as for Fig. 1.

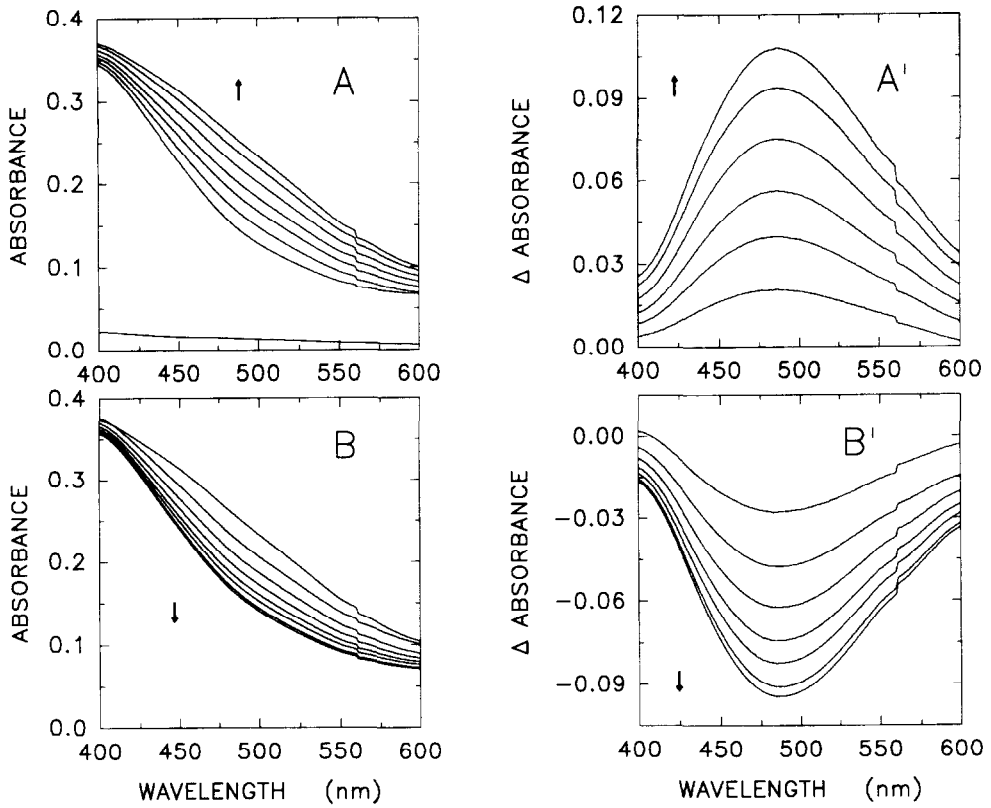

Fig. 3. Spectrophotometric detection of HiPIP oxidation and reduction as catalyzed by membranes from aerobically-grown *Rf. fermentans*. Experimental conditions: T, 25°C; scan-rate, 400 nm·min^{-1}; assay volume, 0.8 ml. Note that a filter change is seen at 560 nm. (A) Time dependence (from the bottom to the top, see arrow) of the oxidation of reduced HiPIP (20 μM) as catalyzed by an aerobic suspension of membranes (97 μg protein·ml^{-1}). The lower trace represents the absorption spectrum of membranes. Spectra were recorded over a time period of 130 min. (A′) Time-dependent difference spectra obtained by substracting the zero-time spectrum recorded immediately after addition of reduced-HiPIP. (B) Time dependence (from the top to the bottom, see arrow) of the reduction of oxidized HiPIP (sample of panel A) as catalyzed by an aerobic suspension of membranes in the presence of 2 mM succinate. (B′) Time-dependent difference spectra obtained by subtracting the 130 min time spectrum of panel A recorded immediately before addition of succinate.

served (Fig. 3, panel A), this conclusion being confirmed by the difference spectra of panel A′. Notably, upon addition of 2 mM succinate the oxidized HiPIP was subsequently reduced (Fig. 3, panel B) as also clearly established by the difference spectra of panel B′.

By monitoring the absorbance variation at 490 nm as a function of time, the kinetics of HiPIP oxidation (shown in Fig. 4, panel A) was found to follow a single-exponential curve with a pseudo first order rate constant $k_{obs} = (2.9 \pm 0.6) \times 10^{-3}$ s^{-1}; the total absorbance change observed (ΔABS = 0.152 ± 0.011) was in line with that expected (ΔABS = 0.168) using a difference (oxidized minus reduced) absorption coefficient of 8.4 mM^{-1}·cm^{-1} at 490 nm [5]. As predicted by the data reported in Fig. 1 (trace c), addition of 0.1 mM KCN to the membrane suspension prior to addition of reduced HiPIP completely inhibited the oxidation of HiPIP (Fig. 4, panel A′). This inhibition was not due to side effects of cyanide on the chemical stability of HiPIP: when 20 μM HiPIP was incubated with 0.1 mM cyanide in the absence of membranes, HiPIP was found to be spectroscopically stable over a time interval of 6 h. Moreover, when reduced HiPIP was added to the membrane suspension under anaerobic conditions the rate of HiPIP oxidation decreased by a factor of 90 (data not shown). Panel B of Fig. 4 shows that addition of 2 mM succinate to an assay mixture containing 20 μM HiPIP previously oxidized by the membrane-bound HiPIP oxidase, induced a rapid HiPIP reduction ($V_i = 0.52$ μM·s^{-1}) following an initial pseudo zeroth-order reaction which turns into a pseudo first-order reaction. Notably, the total change of absorbance perfectly matched that of the experiment of panel A. It is also apparent that under our experimental conditions, HiPIP reduction was approximately 10 times faster than HiPIP oxidation. However, as shown in panel B′, the succinate-dependent HiPIP reduction was fully inhibited by the bc_1-complex inhibitors, myxothiazol and/or stigmatellin (5 μM each), indicating that reduction of HiPIP requires this redox protein complex.

4. Conclusions

The results of this study demonstrate that the periplasmically located, water-soluble, high-potential iron-sulfur protein (HiPIP) from aerobically dark-grown cells of the facultative phototroph *Rf. fermentans* mediates electron transfer between the bc_1 complex and the cyanide-sensitive cytochrome oxidase, previously shown to contain haems of *cb*-type [4].

This is the first time that a HiPIP oxido-reductase activity has been found in membranes from facultative phototrophs. This finding raises therefore the question whether the role of the HiPIP as electron transport carrier is restricted to *Rf. fermentans* or it is more widely distributed among those species defi-

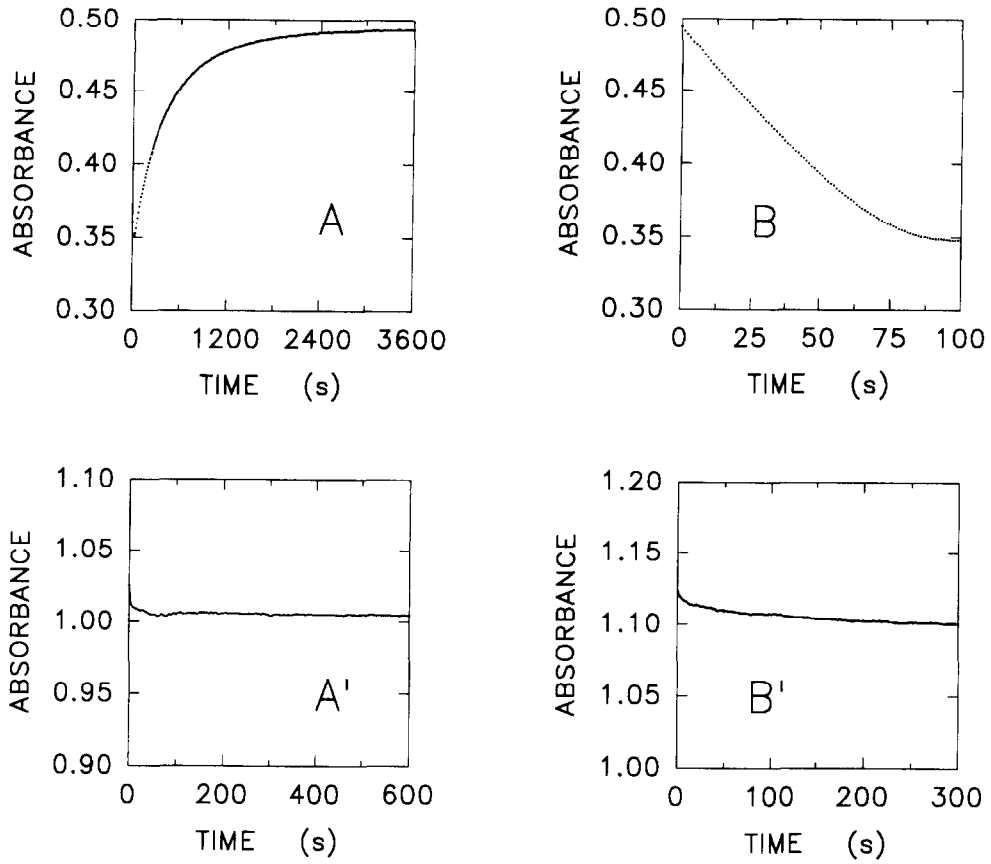

Fig. 4. Kinetics of HiPIP oxidation and reduction as monitored by absorbance changes at 490 nm. Experimental conditions as in Fig. 3. Panels A and A′: (A) HiPIP oxidation as catalyzed by an aerobic suspension of membranes and (A′) its inhibition by 0.1 mM KCN. Note that in A′, the initial absorbance was higher due to membrane turbidity as the assay membrane concentration was raised to 0.48 mg protein·ml^{-1}. Panel B and B′: (B) Succinate-dependent HiPIP reduction (following HiPIP oxidation, see panel A) and (B′) its inhibition by myxothiazol and stigmatellin (5 μM each).

cient in periplasmically located soluble *c*-type cytochromes [6]. Further investigation will be necessary to verify this important question.

Acknowledgements: A.H. and P.K. were recipients of grants from the A. Buzzati-Traverso Foundation (Italy) and the EC Human Capital and Mobility network MASIMO, respectively. Work supported by C.N.R. (BTBS program, Italy).

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