The high potential iron-sulfur protein (HiPIP) from *Rhodoferax fermentans* is competent in photosynthetic electron transfer

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Abstract The functional role of the High Potential Iron-sulfur Protein (HiPIP) from the photosynthetic bacterium *Rhodoferax fermentans* was investigated. We demonstrated that the HiPIP increased the rate of light-induced oxygen reduction mediated by the photosynthetic reaction center (RC); this stimulation reached half-saturation at [HiPIP]/[RC] ca. 15. The capability of the HiPIP in delivering electrons to the reaction center of *Rhodoferax fermentans* was demonstrated through kinetic spectrophotometry of cytochrome *c*-556 oxidation in the presence or in the absence of HiPIP. It is concluded that the HiPIP is competent in the photosynthetic electron transfer chain of *Rhodoferax fermentans*.

Key words: HiPIP; Bacterial photosynthesis; Electron transfer; Cytochrome

1. Introduction

HiPIPs (high potential iron-sulfur proteins) are a small class of proteins found in purple photosynthetic bacteria, containing a cubane $[Fe_4S_4]$ cluster [1]. HiPIPs are peculiar among iron-sulfur proteins because of the high redox potential at which the cluster exchanges electrons (from +50 to +450 mV) [2] using the $[Fe_4S_4]^{3+/2+}$ redox couple [3]. A much larger number of ferredoxins show a lower redox potential range (from −250 to −650 mV) [4] and a $[Fe_4S_4]^{2+/1+}$ redox couple [3].

Although the chemical and biophysical properties of HiPIPs are relatively well defined [5], their functional role is still uncertain [6]. In purple sulfur bacteria, HiPIPs have been shown to participate in direct [7] and indirect [8] substrate oxidation reactions and in electron donation to the photo-oxidized reaction center (RC) of *Chromatium vinosum* [9]. On the other hand, it has also been shown that *C. vinosum* HiPIP is unable to accept electrons from the homologous bc_1 complex [10], thus not participating in cyclic photosynthetic electron flow. In purple nonsulfur bacteria the electron transfer between three different HiPIPs (from *Rhodocyclus gelatinosus*, *Rhodospirillum salinarum* and *Rhodopseudomonas marina*) and the reaction center from *Rhodopseudomonas viridis* was recently analyzed [11]. Although electron donation to the photo-oxidized RC was observed, the HiPIPs were shown to be barely reactive, especially when compared to the reactivity of Rhodopseudomonas viridis cytochrome cM_2. A new HiPIP associated with the membranes of the chemoorganotrophic bacterium *Rhodotermus marinus* has recently been described, and its possible function in the respiratory electron transfer chain has been suggested [12].

Rhodoferax fermentans is a purple nonsulfur bacterium [13,14] which shows some interesting properties such as the presence of a membrane-bound *c*-type tetrahaem rapidly delivering electrons to the photo-oxidized RC [15,16], and the presence of amounts of HiPIP higher than the soluble *c*-type cytochromes (Hochkoeppler, A., Ferro, G., Kofod, P. and Ciurli, S., submitted). This latter finding would be consistent with the complementary role of these two types of proteins as potential electron carriers [6].

Here the role of *Rf. fermentans* HiPIP in photosynthetic electron transfer catalyzed by membranes from light-grown cells is analyzed. The results clearly indicate that the HiPIP donates electrons to the photo-oxidized reaction center, operating via the membrane-bound *c*-type tetrahaem.

2. Materials and methods

2.1. Cell growth and membrane isolation

Rhodoferax fermentans was grown photoheterotrophically in completely filled screw-capped bottles (25°C). The growth medium (pH 6.8) contained yeast extract (3.0 g/l), casaminoacids (2.0 g/l), malic acid (1.0 g/l) and $(NH_4)_2SO_4$ (0.5 g/l) as previously described [13,14]. The cells were washed twice and finally suspended in 50 mM Tris buffer (pH 7.6, 4 ml/g of cell paste) containing 1 mM EDTA and 10% (w/v) sucrose. Lysozyme (300 mg/l), DNAse (Fluka, 3,000 units/g of cell paste), and 1 mM PMSF were added to the suspension and, after 1 h, the cells were disrupted using a French pressure cell operating at 20,000 psi (three cycles). Cell debris and membrane fragments were removed by differential centrifugation at 20,000 × *g* (30 min) and 190,000 × *g* (2 h), respectively.

For light-induced oxygen uptake measurements, both purified HiPIP (Hochkoeppler A., Ferro G., Kofod P., and Ciurli S., submitted) and *Rf. fermentans* membranes were kept in 50 mM MOPS buffer (pH 7.2). The purity of HiPIP was checked by SDS-PAGE, using 7.5% acrylamide stacking gel and 20% acrylamide running gel (pH 6.5, Tris-acetate buffer).

2.2. Light-induced oxygen uptake measurements

Light-induced oxygen uptake was determined as previously reported [17]: oxygen consumption was measured with a Clark-type oxygen electrode at 30°C, using an infrared light source constituted by a 150 W spot lamp and a Kodak Wratten 88A filter.

2.3. Kinetic spectrophotometry

Absorbance changes, induced by a xenon flash lamp (3.25 J discharge energy, 15 μs pulse duration at half-maximal intensity) filtered through two layers of Kodak Wratten 88A filter, 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 seconds prior to an excitation flash). Data were acquired by a Lecroy 9410 digital oscilloscope interfaced to an Olivetti M240 computer.

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The concentration of photo-oxidizable reaction centers in *Rf. fermentans* membranes was determined by measuring the absorbance changes at 540 and 605 nm induced by a train of 8 flashes (fired 50 ms apart). The reaction center concentration was then calculated using extinction coefficients of 10.3 $mM^{-1} \cdot cm^{-1}$ and 19.5 $mM^{-1} \cdot cm^{-1}$ at 540 and 605 nm, respectively [18].

All the measurements were performed aerobically in the absence of exogenous reductants and redox mediators.

The absorbance changes at 556 nm due to cytochrome oxidation were obtained by subtracting the contribution of RC photo-oxidation at the same wavelength. The latter accounted for 0.8 of the absorbance change measured at 540 nm [16].

2.4. Protein assay

HiPIP concentration was determined by the BCA method [19], using bovine serum albumine as standard.

3. Results and discussion

The possible role of HiPIP in the photosynthetic electron transfer chain of *Rhodoferax fermentans* has been investigated measuring the light-induced oxygen uptake (LIOU) [17]. Previous studies have indeed demonstrated that, in membranes from facultative phototrophs, a high rate of oxygen consumption can be driven by continuous light when reducing equivalents from exogenously added donors are delivered to oxygen through the following electron transfer chain: electron donor → RC → quinone → quinol oxidase → oxygen [17]. This light-driven respiration is resistant to cyanide concentrations inhibiting the cyt *c* oxidase activity, and it can be rate-limited by two factors: (i) the rate of re-reduction of the photo-oxidized RC, and (ii) the activity of the membrane-bound quinol oxidase.

Fig. 1 compares the polarographic traces of dark- and light-induced oxygen consumption reactions catalyzed by membranes from *Rf. fermentans* in the presence or in the absence of different soluble components. Fig. 1A shows that a slow respiratory activity is observed in the dark upon addition of 5 mM ascorbate to the membranes, this activity being almost totally inhibited by 10 μM cyanide. However, a rapid oxygen consumption can be induced by illumination of the oxygraph reaction chamber with infrared continuous light. The LIOU reaction catalyzed by membranes supplemented with an amount of the 190,000 × *g* soluble fraction, such as to obtain the same in vivo membrane/supernatant ratio, is shown in Fig. 1B. Apparently, the rate of LIOU reaction is increased (44%) in the presence of the supernatant, suggesting that the soluble fraction contains component(s) accelerating this reaction. Interestingly, Fig. 1C shows that membranes supplemented with 8.5 μM of the purified HiPIP catalyze an even faster (49%) LIOU reaction as compared to that observed in Fig. 1A, with no effect on dark respiration. Conversely, addition of 50 μM horse heart cytochrome *c* accelerates significantly (230%) only the dark oxygen consumption, with a slight (19%) inhibitory effect on light-induced respiration (Fig. 1D).

A series of controls were carried out in order to ascertain whether the measured LIOU activities were related to RC photochemistry or they were affected by auto-oxidation of pigments and/or other RC-unrelated components. In the absence of membranes (and in the presence of 5 mM ascorbate) no activity was detectable, while in the presence of membranes only (in the absence of ascorbate), the measured rate of LIOU accounted for 20% of the control activity. Moreover, the LIOU activity was 30% inhibited by 20 μM of the $Q_A \rightarrow Q_B$ electron transfer inhibitor tridecylstigmatellin, and completely inhibited by 10 mM cyanide, a concentration of inhibitor capable to block the quinol oxidase [17] (data not shown).

The stimulation of LIOU reaction as a function of HiPIP

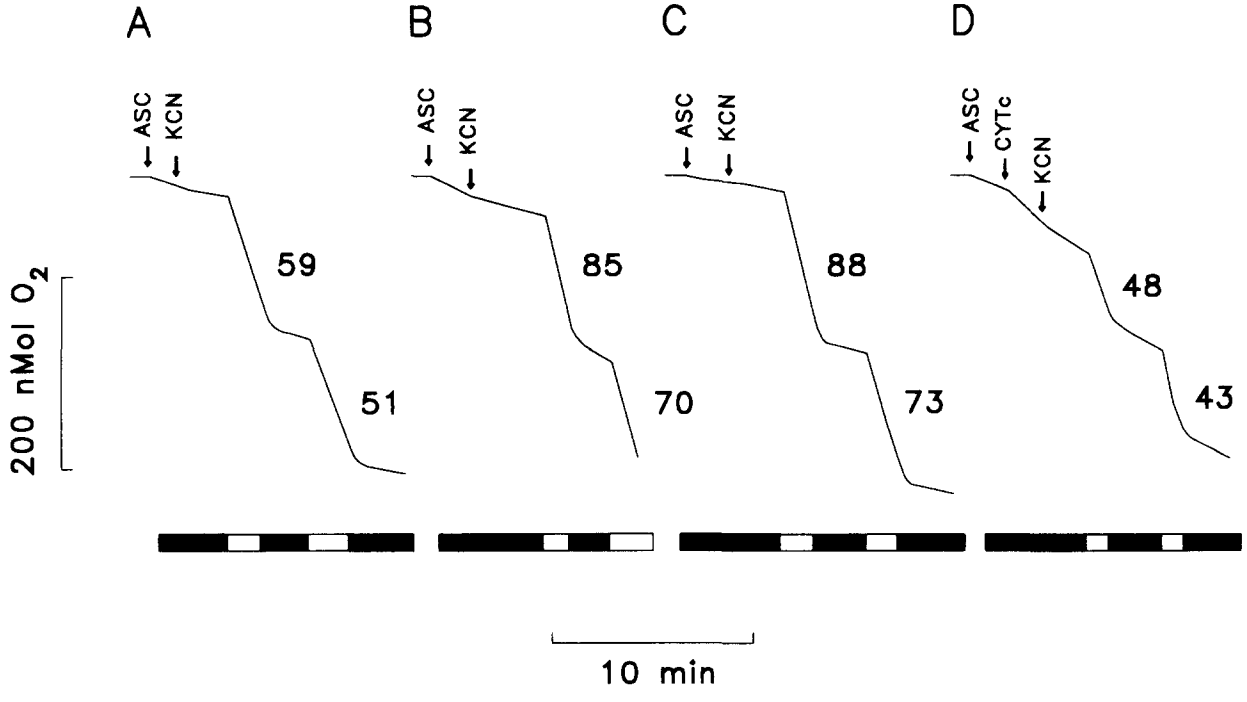

Fig. 1. Polarographic traces of oxygen consumption in the presence of 50 mM MOPS buffer (pH 7.2), 5 mM ascorbate, 10 μM KCN, 0.225 μM RC (trace A); this assay mixture was supplemented with supernatant (trace B), HiPIP (trace C), and horse heart cytochrome *c* (trace D) as described in the text. Filled and empty bars stand for dark and light periods, respectively. Numbers indicate the rates of LIOU reaction (oxygen consumption in the light minus oxygen consumption in the dark) as nmol O_2/min.

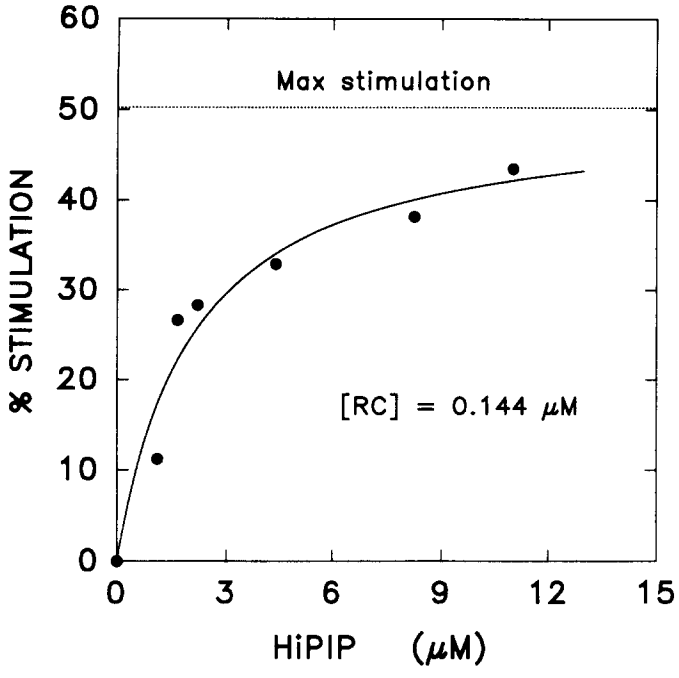

Fig. 2. Stimulation of the light-induced oxygen uptake as a function of HiPIP concentration. All the measurements were performed in the presence of 0.144 μM RC. Other experimental conditions as in Fig. 1A. The data points are fitted to a Michaelis–Menten curve (solid line).

concentration is shown in Fig. 2. At HiPIP/RC molar ratios larger than ca. 15 a plateau is approached. Additional experiments have also demonstrated that in the presence of 5 μM antimycin A and of saturating amounts of HiPIP the LIOU reaction rate can be 20% further enhanced (not shown). This indicates that the quinol oxidase, which accepts electrons from the RC-generated quinols, is not saturated under our experimental conditions. These findings suggest that electron transfer would occur between HiPIP and RC, possibly via the formation of a complex, as previously observed in analogous photosynthetic systems involving soluble *c*-type cytochromes and RCs [11,20–22]. To test this hypothesis, we analyzed, using kinetic spectrophotometry, the absorbance changes related to RC-induced cytochrome oxidation. In membranes from light-grown cells of *Rf. fermentans* a *c*-type tetrahaem was indeed shown to deliver electrons to the photo-oxidized primary donor (P^+) [15,16]. Three haems were spectroscopically and potentiometrically resolved: (i) a first *c*-type haem having an α maximum band at 556 nm (cyt *c*-556) and a mid-point potential at 354 mV, (ii) a second cytochrome with an α band centered at 560 nm and E_m = 294 mV, and (iii) a third haem with an α maximum at 551 nm with E_m = 79 mV. The mid-point potential of the fourth haem (E_m = 0 mV) was also determined [15,16]. Finally, a mid-point potential of 351 mV was determined for the purified HiPIP (Hochkoeppler A., Ferro G., Kofod P. and Ciurli, S., submitted). These thermodynamic properties suggest that electron transfer might occur between HiPIP and cyt *c*-556.

Fig. 3A shows the absorbance changes detected at 540 and 556 nm after flash-excitation of *Rf. fermentans* membranes. While at 540 nm an absorbance increase due to P photo-oxidation is observed, at 556 nm an absorbance decrease, related to cytochrome *c* oxidation, is detected. The lower trace of Fig. 3A was obtained from the signal at 556 nm corrected (see section 2) for the contribution of P^+ at the same wavelength. This trace shows that after rapid (sub ms) cytochrome oxidation, a slow re-reduction is observed. This pattern is drastically modified by addition of HiPIP (Fig. 3B). At 556 nm no absorbance change attributable to cytochrome oxidation is actually detected. Moreover, the lower trace of Fig. 3B indicates that addition of HiPIP decreases by ca. 40% the maximal extent of flash-induced cytochrome *c* oxidation (cf. Fig. 3A, lower trace), suggesting the presence of a fast (sub ms) electron transfer phase, involving the HiPIP and a *c*-type cytochrome. In order to identify the cytochrome reduced by the HiPIP, the flash-induced maximal absorbance changes over the 540–570 nm wavelength interval have been measured. Fig. 3C shows that, in the absence of HiPIP, a symmetric cytochrome oxidation spectrum, centered at 556 nm, is obtained (filled circles), which is not observed in the presence of HiPIP (empty circles). This indicates that HiPIP rapidly donates electrons to the oxidized cyt *c*-556.

The flash-induced absorbance changes over the 410–435 nm wavelength interval (cytochromes' Soret band) were analyzed (Fig. 4). The kinetics of the absorbance changes measured at 425 nm either in the absence or in the presence of HiPIP are shown in Fig. 4A and 4C, respectively. Apparently, addition of HiPIP decreases by ca. 33% the amplitude of the absorbance

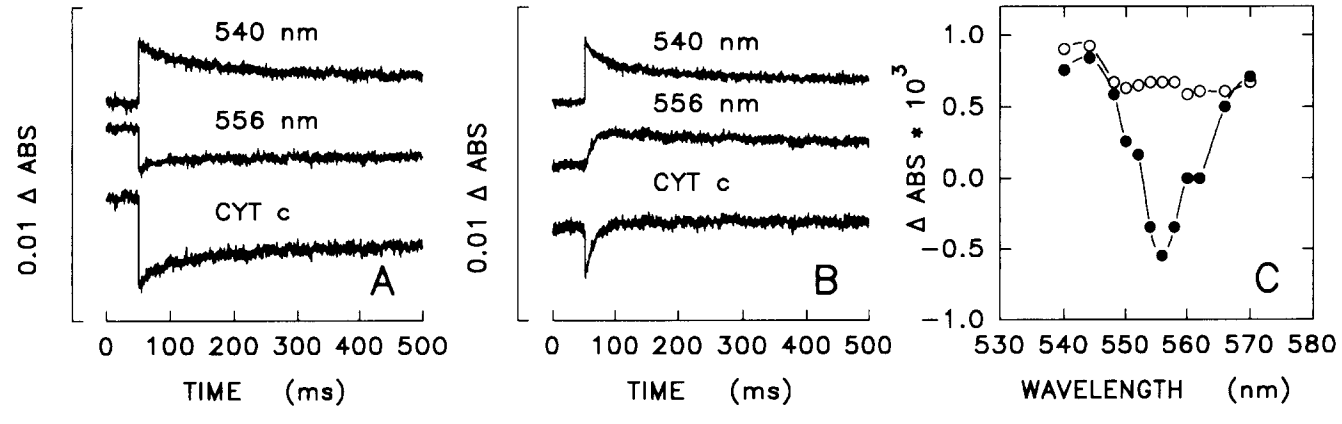

Fig. 3. Light-induced absorbance changes detected at 540 and 556 nm in the absence (panel A) and in the presence (panel B) of HiPIP. The absorbance change at 556 nm corrected for the photo-oxidized reaction center contribution is also shown as the CYT *c* trace. Panel C shows the absorbance changes detected over the 540–570 nm wavelength interval in the absence (filled circles) or in the presence (empty circles) of HiPIP. Experimental conditions: 50 mM MOPS buffer (pH 7.2), 0.22 μM RC (panels A and B), 0.12 μM RC (panel C), 21 μM HiPIP.

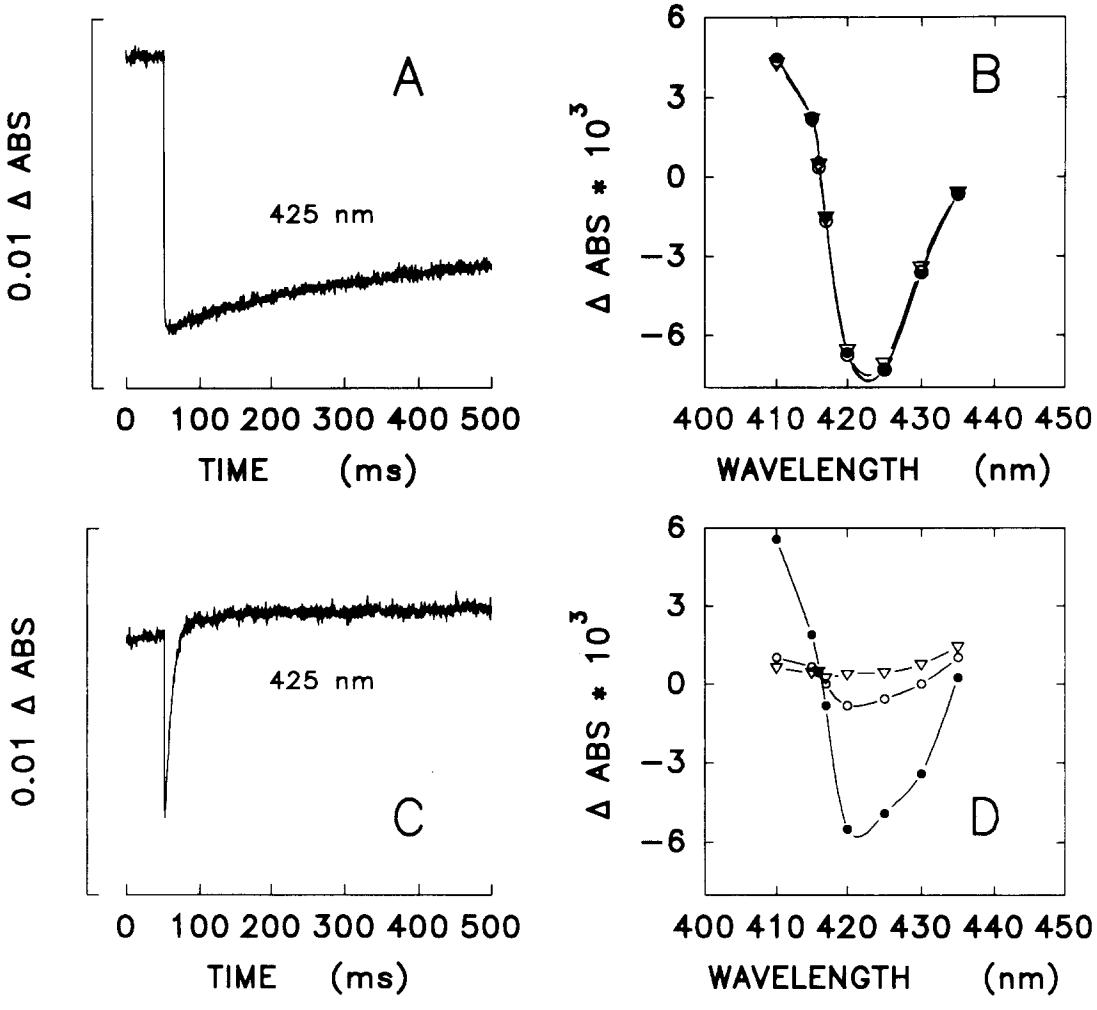

Fig. 4. Light-induced absorbance changes detected in the absence (panels A and B) or in the presence (panels C and D) of HiPIP. In panels B and D, filled circles, empty circles, and empty triangles indicate the absorbance changes detected immediately, 20 ms, and 50 ms after the excitation flash, respectively. Experimental conditions as in Fig. 3C.

change detected at 425 nm immediately after the excitation flash (see Fig. 4A and 4C), confirming that a fast (sub ms) electron transfer occurs between the HiPIP and cyt *c*-556. Moreover, addition of HiPIP leads to complete cyt *c*-556 re-reduction within 50 ms (Fig. 4B,D).

4. Conclusions

The present work demonstrates that the HiPIP isolated and purified from light-grown cells of *Rhodoferax fermentans* is competent in photosynthetic electron transfer. Our conclusion supports and extends an earlier report by Kennel et al. [9] indicating that, under continuous illumination, the HiPIP from *Chromatium vinosum* is an electron donor to a membrane-bound high-potential *c*-type haem. Here, for the first time, we have been able to obtain kinetic evidence that the HiPIP from *Rhodoferax fermentans* rapidly re-reduces the reaction center, following a single turnover excitation flash, and we have also identified the membrane-bound cytochrome (cyt *c*-556) accepting electrons from the HiPIP. The presence of a fast (sub ms) and a slow (ms) electron transfer phase between HiPIP and cyt *c*-556 has also been observed. These results, along with evidence of the lack of relevant amounts of a soluble *c*-type cytochrome in *Rhodoferax fermentans* (Hochkoeppler, A., Ferro, G., Kofod, P., and Ciurli, S., submitted), suggest that the HiPIP plays a significant role in the photosynthetic electron transfer chain of this bacterial species.

Experiments aiming to kinetically resolve the electron transfer between HiPIP and cyt *c*-556 are in progress.

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