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A membrane-bound HIPIP type center in the thermohalophile *Rhodothermus marinus*

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Abstract A HIPIP-type center was discovered in intact membranes of the thermohalophilic aerobe *Rhodothermus marinus*. In both the membrane-bound state and after detergent solubilization and partial purification, this center exhibits an almost axial EPR spectrum, with g-values at 2.13 and 2.03, similar to those of soluble HIPIP proteins isolated from purple bacteria. It has a high reduction potential, of 260 mV at pH 7.5. *Rhodothermus* HIPIP is involved in the main membrane-bound electron-transfer pathway, being reduced by NADH or succinate only in the presence of cyanide. The possible physiological function of this novel HIPIP-type center is discussed.

Key words: HIPIP; Halophile; Thermophile; EPR; Iron-sulfur

1. Introduction

The characterization of the membrane-bound electron-transfer chain from the thermohalophilic bacterium *Rhodothermus marinus* was undertaken in this study. This species was first isolated from shallow marine hot springs off the coast of Iceland [1]. Very similar strains were isolated afterwards from marine hydrothermal areas on a beach of the island of S. Miguel, Açores [2]. These microorganisms are strict aerobes, chemoorganothrophic and slightly, but strictly, halophilic, growing in 0.5–7% NaCl. They have an optimum growth temperature of 65°C and a maximum growth temperature of about 78°C.

High-potential iron-sulfur proteins (HIPIPs) have only been found in purple bacteria (Proteobacteria) of the α, β and γ subdivisions [3–5]. With the exceptions of a halophilic *Paracoccus* strain (ATCC 12084) [6] and of *Thiobacillus ferrooxidans* [7], all the other HIPIP-containing organisms are photosynthetic. HIPIPs are cytoplasmic proteins, with a minimum molecular mass of about 10 kDa, containing a $[4Fe\text{-}4S]^{3+/2+}$ center, with reduction potentials ranging from ~50 mV up to ~450 mV [4,5,8]. These centers have an $S = 1/2$ ground state in the oxidized state, yielding a characteristic quasi-axial EPR spectrum, with all g-values above 2: $g_{\parallel} \sim 2.1$, $g_{\perp} \sim 2.03$. In the reduced state, they are EPR silent ($S = 0$ ground state) [9–12]. Although discovered more than 30 years ago, and in spite of a large amount of spectroscopic and structural data available, their physiological function is still not known. It has been proposed that the HIPIPs may function as electron donors to the bacterial reaction center, substituting for cytochrome c_2 in the photosynthetic bacteria (e.g. [4]). Other possible functions have also been proposed, such as an iron-oxidizing enzyme in *Thiobacillus ferrooxidans* [7], and as an electron donor to the cytochrome *cd*-type nitrate reductase in the halophilic *Paracoccus* species [13]. In this paper we report the observation and several physico-chemical properties of a high-potential iron-sulfur protein in the intact membranes of several *Rhodothermus* strains.

2. Materials and methods

2.1. Bacterial growth

The type strain of *Rh. marinus* (DSM 4252) was obtained from the Deutsche Sammlung von Mikrooganismen. *Rh. marinus* PRQ-36 was described previously [2]. Strain PRQ-62B is a spontaneous non-pigmented mutant of strain PRQ-62. These strains were grown on Degryse medium 162 [14], with 1.0% NaCl, at 65°C with shaking in 1 l metal-capped Erlenmeyers containing 200 ml of medium. A larger scale cell growth was also performed for the strain PRQ-62B, in a 5 l Biostat fermentor.

2.2. Membrane preparation

Cells were harvested at the beginning of the stationary phase of growth, by centrifugation at $10{,}000 \times g$ for 10 min. The pellet was suspended in 10 mM phosphate buffer, pH 7.5, and sonicated for 15 min. The suspension was successively centrifuged at $10{,}000 \times g$ for 15 min and at $60{,}000 \times g$ for 1 h. The membranes were finally resuspended in 10 mM phosphate buffer, pH 7.5.

2.3. Membrane solubilization and protein purification

Triton X-100 at a final concentration of 2.0% was added to the membrane suspension. The mixture was gently stirred at 4°C for 2 h and centrifuged for 1 h at $60{,}000 \times g$. The pellet was resuspended in 10 mM phosphate buffer with 2% Triton X-100 and the procedure was repeated. The supernatants from the two extractions were mixed and applied to a DEAE-52 column, equilibrated with 10 mM Tris-HCl buffer, pH 7.6, containing 0.5% Triton X-100. The column was eluted with a linear sodium chloride gradient (from 0 to 500 mM). A fraction containing cytochromes and the HIPIP protein was eluted at about 320 mM NaCl.

2.4. Activity measurements

Oxygen consumption of membrane suspensions was measured by a Clark-type oxygen electrode using an YSI Model 5300 Oxygen Biological Monitor, equipped with a 600 μl cell. The measurements were performed at 40°C, using *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD), NADH and succinate as substrates (0.4 mM final concentration).

2.5. Spectroscopic methods

EPR spectra were measured with a Bruker ESP-380 spectrometer, equipped with an Oxford Instruments ESR-900 continuous-flow helium cryostat. Redox titrations of the membrane preparation, followed by EPR, were performed as described in [15].

3. Results and discussion

The EPR spectra of *Rhodothermus* membranes (Fig. 1A) are

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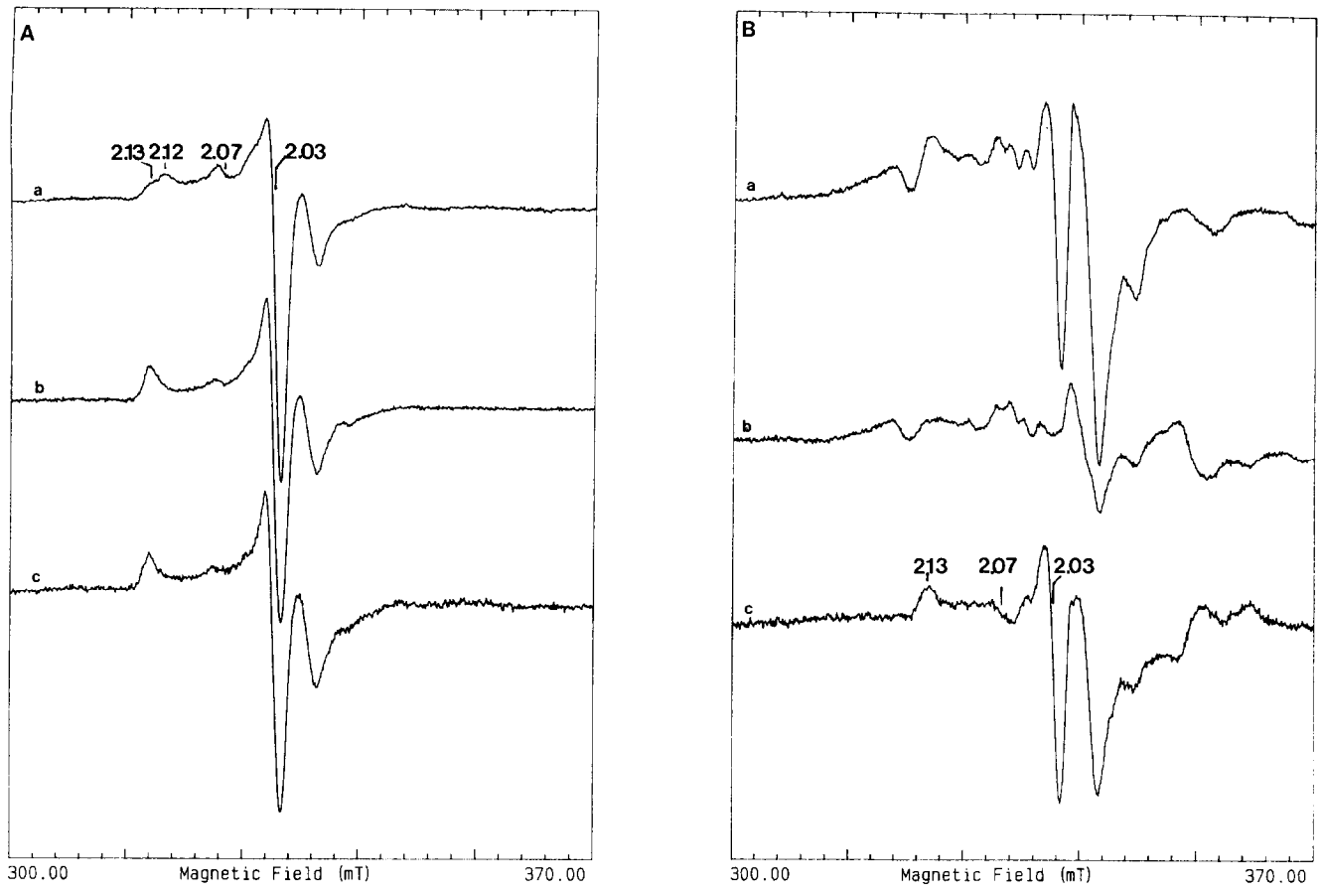

Fig. 1. (A) EPR spectra of membrane preparations from *Rhodothermus* type strain (a), PRQ-36 (b), and PRQ-62B (c). (B) EPR spectra of *Rhodothermus* strain PRQ-62B intact cells (a) and reduced with sodium dithionite (b); (c) difference spectrum, a–b. Temperature 20 K, microwave frequency 9.44 GHz (A) and 9.64 GHz (B), microwave power 2.4 mW, modulation amplitude 0.9 mT.

dominated by an almost axial signal, with g-values at ~2.13 and 2.03, observed up to 35 K without noticeable line-broadening. Other spectral components are also observed, with relative proportions depending on the bacterial strain. For the preparation from the type strain (Fig. 1Aa) two features at g = 2.13 and g = 2.12 are present, while for those from strains PRQ-36B and PRQ-62B only the signal at g = 2.13 is clearly observed. In all three preparations, a minor component with g^{med} = 2.07 is also detected. Both the overall spectral shape as well as the actual g-values of these species are remarkably similar to those observed for soluble HIPIPs (Table 1). The observed heterogeneity is reminiscent of that described for the HIPIPs isolated from *Chromatium vinosum* [9,10], *Ectothiorhodospira vacuolata* [16] and *Rhodocyclus gelatinosa* [17]. Its origin in *Rhodothermus* is not yet known. To check whether its origin was due to sample manipulation the spectrum of intact cells from *Rhodothermus* strain PRQ-62B was recorded (Fig. 1B). In order to partially eliminate the contribution of the overlapping manganese signals the cells were reduced with sodium dithionite (Fig. 1Bb) and a difference spectrum was obtained, showing essentially the HIPIP-type signal (Fig. 1Bc). Within the spectral resolution, it is clear that the spectrum obtained for the membrane preparation is very similar to that of the intact cells. The *Rhodothermus* HIPIP-type center is not observed in the soluble fractions of the *Rhodothermus* strains. The EPR spectrum of the partially purified HIPIP, after detergent solubilization, shows the same g-values and spectral line-shape as in the membrane-bound state (Fig. 2). This spectrum could be reasonably theoretically simulated assuming the presence of two components: an axial species, with g-values at 2.125 and 2.03, and a minor rhombic species with g-values at ~2.13, 2.07 and ~2.03. However, a much better reproduction of the experimental spectrum is obtained assuming the presence of three components: the last rhombic species plus two axial components, using the g_{max}-values meas-

Table 1
Reduction potentials and EPR g-values from selected HIPIPs and the HIPIP-type center in *Rhodothermus marinus*

Organism	E_o (mV)	g_x	g_y	g_z	Ref.
Rhodopila globiformis	450	–	–	–	8
Rhodocyclus gelatinosa	330	2.03	2.03	2.11	17
Chromatium vinosum	360	2.02	2.04	2.12	10
		2.04	2.07	2.13	
Paracoccus sp.	282	–	–	–	6
E. halophila HIPIP II	50	2.030	2.030	2.146	12
Rhodothermus marinus	260*	2.03**	2.03	2.13	This
		2.03**	2.03	2.12	work
		2.03	2.07	2.13	

*Reduction potential for PRQ-62B.
**Within the experimental resolution, the spectra were assumed to be axial.

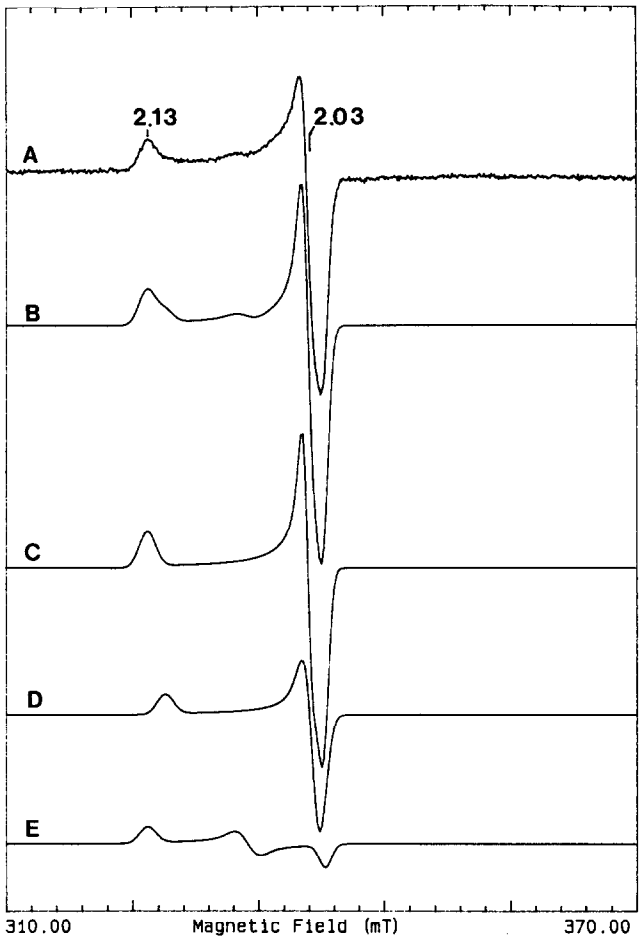

Fig. 2. EPR spectrum of the partially purified detergent-solubilized HIPIP fraction (A). The full lines in C–E are the theoretical simulations, performed with the g-values presented in Table 1, added in the ratio 1:0.4:0.15 (B). Microwave frequency 9.64 GHz. Other experimental conditions are as in Fig. 1.

ured in the membrane spectrum of the type strain (Table 1). Whether this representation of the experimental spectrum is correct remains a question of debate. The original EPR spectrum from *Chromatium vinosum* HIPIP was interpreted as resulting from three distinct species [9]. However, Dunham et al. [10] proposed an alternative interpretation of the EPR data, suggesting that the spectral heterogeneity was due both to an intrinsic, and still unexplained, protein heterogeneity (rhombic species with g_{med} at 2.07) and to protein dimerization. In these dimers the clusters from each monomer would be close enough to each other (~15 Å) so that the spin–spin interaction would lead to the spectral fine structure. The authors proposed the presence of two types of dimers, resulting in slightly distinct sets of g-values. The EPR spectrum was shown to be dependent on the rate of freezing and on the presence of sodium chloride, which favours the dimerization. If this interpretation is correct, it means that in *Rhodothermus* membranes the HIPIP is present in the form of multimers (at least dimers). An alternative explanation was proposed by Banci et al. [16]. From the NMR data on several HIPIPs these authors proposed the presence of a chemical equilibrium between at least two distinct species, differing in the electron distribution within the cluster. At present it is not possible to firmly choose which interpretation (or both) is correct and further experiments are needed to clarify this question.

The reduction potential of *Rhodothermus* HIPIP was determined by performing redox titrations of membrane suspensions, at pH 7.5, monitored by EPR and measuring the peak height at g = 2.13 and g = 2.03 as a function of the solution redox potential (Fig. 3). The data are well described by a single Nernst equation (n = 1), with a reduction potential of 260 ± 10 mV. Within experimental resolution, all the HIPIP EPR-detectable species have the same reduction potential. Preliminary redox data on the detergent-solubilized protein indicates that the reduction potential of the solubilized protein is, within ± 30 mV, similar to that of the protein in the membrane-bound state (data not shown). This value is also within the range of 50–450 mV, found for soluble HIPIPs [5,8] (Table 1).

A possible role for this center in the membrane-bound electron-transfer pathway was assessed by investigating its reduction using NADH and succinate as electron donors. *Rhodothermus* membranes contain *a*-type hemes, as deduced from the observation of a band at 602 nm in the visible spectra of dithionite-reduced samples (our unpublished data). This heme component belongs most probably to the bacterial terminal oxidase [15,18,19]. Oxygen consumption by membrane preparations, using TMPD, NADH or succinate as electron donors, was fully inhibited by adding cyanide. Only in the presence of this classical inhibitor of the terminal oxidases was a full reduction of the HIPIP-type center achieved by succinate or NADH (Fig. 4B). In its absence, only a minor extension of reduction was observed (Fig. 4A). It is worth noting that upon reduction no EPR signals typical of Rieske-type centers [20–22] was observed. The signal at g = 1.94 belongs most probably to one of the iron-sulfur centers of succinate or NADH dehydrogenases [23] and is clearly distinct from those of Rieske centers (g_y at ~1.90).

4. Conclusions

The data presented provides strong evidence for the presence of a HIPIP-type center in the membranes of *Rh. marinus*: both its EPR features and reduction potential are characteristic of

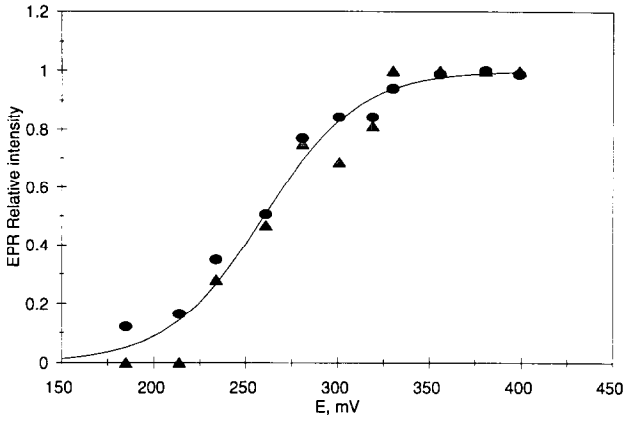

Fig. 3. EPR redox titration of *Rhodothermus* PRQ-62B membranes at pH 7.5. The data points represent the normalized peak height at g = 2.13 (△) and at g = 2.03 (○). The full line was calculated with a Nernst equation for n = 1 and E_o = 260 mV.

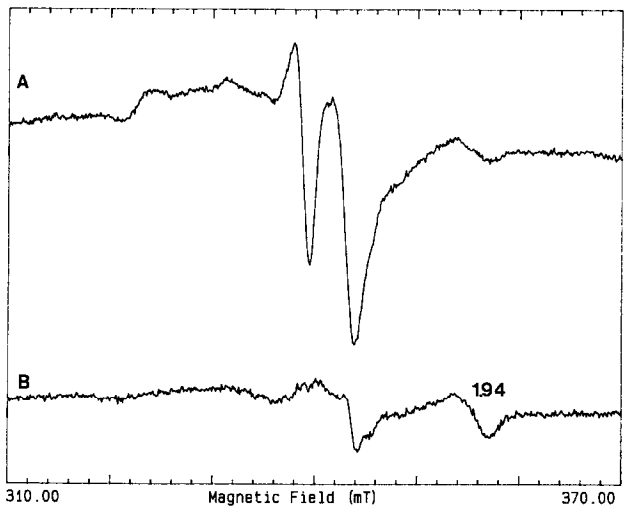

Fig. 4. EPR spectra of *Rhodothermus* PRQ-62 B membranes, reduced with 2 mM NADH in the absence (A) and presence of 1 mM sodium cyanide (B). Both samples were incubated at room temperature for 3 min. Other experimental conditions are as in Fig. 2.

HIPIPs. So far, the presence of HIPIP has been reported only in a restricted group of purple photosynthetic bacteria, in *Thiobacillus ferrooxidans* and in a halophilic *Paracoccus* strain. *Rh. marinus* was recently placed within the purple bacteria (Proteobacteria), but could not be assigned to any of the major subdivisions. This organism may perhaps represent a sixth subdivision of this phylum [24]. The observation of a HIPIP in *Rhodothermus* favours the hypothesis that this species is related to other purple bacteria, since all the organisms that have been shown to contain this protein belong to this phylum.

The unique cellular location for the *Rhodothermus* HIPIP may be related to the extreme environments where these organisms thrive. In fact, it seems that in the most extremophilic organisms investigated so far, the electron transport to the terminal oxidases is performed by membrane-bound components, without involving the usual soluble cytochromes, as in mesophilic organisms [15,18,19,25]. The functional studies performed suggest that this center is involved in the main electron flux to the terminal oxidase. Due to the apparent lack of a Rieske-type center in *Rhodothermus*, it is quite tempting to speculate that the HIPIP center could be substituting for the Rieske center, either as an electron donor to another intermediate electron transfer component or, since no soluble cytochromes were detected in *Rhodothermus*, as a direct electron donor to the terminal oxidase complex. This will remain an open question until a thorough characterization of all membrane components is performed.

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