Evidence for a novel type of iron cluster in the respiratory chain of the archaeon *Sulfolobus metallicus*

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Abstract A new type of metal centre was detected in the membranes of the thermoacidophilic archaeon *Sulfolobus metallicus*. This centre has an $S=1/2$ ground state in the oxidised form, yielding an axial EPR signal with g values at 2.035 ($g_{||}$) and 1.97 ($g_{\perp}$), optimally detected at 4.6–10 K; in the reduced form it is EPR silent (even spin). These magnetic properties point to a spin-coupled iron cluster, with a minimum of two iron ions. The centre has a high reduction potential of + 350 mV, at pH 6.5. The physiological role of this novel centre was probed through a general characterisation of *S. metallicus* respiratory chain: this archaeon contains NADH and succinate dehydrogenase activities, and cytochromes b_{562}, a_{586} and a_{600} on the oxygen reductase system. Since it is reduced in the presence of succinate, and taking into account its high reduction potential, this centre is proposed to be a functional analogue of the Rieske centres.

Key words: *Archaeon*; Electron paramagnetic resonance; Iron-sulphur; Rieske; Terminal oxidase; Thermophile

1. Introduction

Until recently, the type of electron transfer complexes found in dioxygen-reducing respiratory systems seemed to be well established, both in eukaryotes and in prokaryotes, even in spite of the multiple possible pathways and terminal oxidases found in bacteria and archaea. However, in the last decade, clearly unusual and quite divergent protein complexes have been described in prokaryotes. For example, succinate dehydrogenases from several Sulfolobales were shown to lack the trinuclear iron-sulphur cluster (centre S3) [1] and a singular soluble tetrahaemic-flavofumarate reductase was described in *Shewanella putrefaciens* [2]. Typical respiratory complexes III (quinol:cytochrome oxidoreductase) were not found in archaea, although Rieske proteins are present in several Sulfolobales [3,4]; a quite unique and novel analogue of complex III was recently characterised in the bacterium *Rhodothermus marinus* (Pereira et al., submitted). In Sulfolobales, the *soxM* and *soxABCD* complexes represent new types of oxygen reductases, most probably functionally equivalent to a quinol oxidase fused to an oxygen reductase, translocating protons through a Q-cycle-like mechanism [5–9]. A rather extreme case is the respiratory chain of the hyperthermoacidophilic archaeon *Acidianus ambivalens*, which has the simplest chain so far characterised. It contains a succinate dehydrogenase which feeds electrons into a pool of caldariella quinone, which on its turn acts both as electron donor and as redox-active element to the only terminal oxidase present, an aa_3-type quinol oxidase [10–12]. No other haem proteins, nor a Rieske protein, are present in the membranes of *A. ambivalens*.

This diversity extends also to the level of the small electron carriers, acting as functional analogues to the bacterial or mitochondrial cytochromes *c*: HiPIP in *R. marinus* [13] and in some purple bacteria [14], and possibly sulphocyanin in Sulfolobales, for which only the gene is known [8]. The large diversity of the prokaryotic world, which has only recently started to be explored more intensely, is leading to quite different views of the basic constituents of oxygen-reducing respiratory systems. For example, terminal oxidases that do not belong to the haem-copper superfamily have been described, like the *Escherichia coli bd*-type oxidase [15], plant alternative oxidases [16,17] and the flavohaem oxidase from the sulphate reducer *Desulfovibrio gigas* [18,19]. Analysis of these processes in archaea, considering that some of its members arise from deep, slowly evolving life lineages like the hyperthermophiles, has the additional advantage of allowing the study of primordial bioenergetic systems.

In the pursuit of the characterisation of other archaeal respiratory chains, the study of *Sulfolobus metallicus* was undertaken. This microorganism is a hyperthermophilic aerobic archaeon, growing between 50 and 75°C, at pH 1.0–4.5, and an obligate chemolithoautotroph that grows on elemental sulphur, with the production of sulphuric acid [20]. In this work, we report the finding of a novel type of iron cluster-containing complex present in *S. metallicus* membranes and discuss its function in the respiratory chain. For this purpose, a preliminary characterisation of *S. metallicus* electron transfer chain was also performed.

2. Materials and methods

S. metallicus cells were grown in 300-l fermenters (HTE, Bioengineering, Wald, Switzerland), using culture media and conditions described previously [21]. The membrane fraction and the dodecyl maltoside-solubilised membrane extract were prepared as in [12]; the cells were suspended in potassium phosphate buffer 50 mM, pH 6.5 and broken at 8000 kPa in an SLM Aminco KINO20 French press.

Protein concentration was determined by the modified biuret method [22] and the polarographic assays were performed on a YSI 5300 oxygen monitor at 40°C. Room temperature visible spectra were recorded in a Beckman DU-70 spectrophotometer and liquid nitrogen temperature spectra on a DW-2 Olis spectrophotometer. HPLC haem analysis was made as described in [23], using myoglobin and *A. ambivalens* haem extracts as standards for haems B and A_s, respectively. EPR experiments were performed as in [24] and in the EPR redox titrations, the compounds used as redox mediators were the same as in [12], but at a concentration of 30 μM each.

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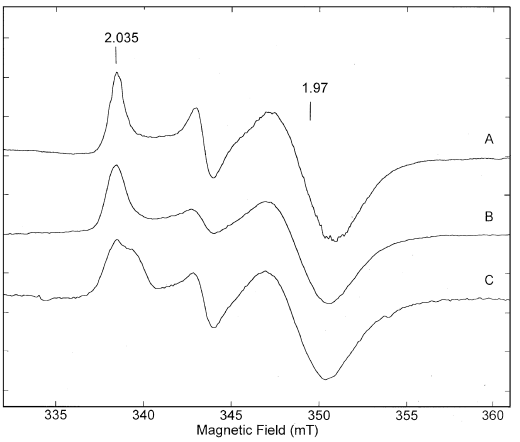

Fig. 1. EPR spectra of *S. metallicus* cells and membranes. Trace A, cells, 4 K; trace b, membranes, 4 K; trace C, membranes, 10 K. Protein concentration: 60 mg/ml. Microwave power 2.4 mW; microwave frequency, 9.643 GHz; variable gain.

3. Results

3.1. EPR spectroscopy

The low-temperature EPR spectrum of *S. metallicus* intact cells is dominated by an intense axial signal, with g values at 2.035 and 1.97 (Fig. 1, trace A). The same spectrum is observed in the membrane preparation (Fig. 1, trace B). Increasing the temperature to $\sim$10 K leads to the appearance of another resonance, discernible mainly as a shoulder at $g = 2.028$ (Fig. 1, trace C). At temperatures above $\sim$30 K, both species broaden beyond detection. These spectra were deconvoluted by selective chemical reductions, spectral subtractions and respective theoretical simulations (Fig. 2). Upon addition of hydroquinone to the native membranes the axial species is fully reduced and another set of resonances develops, with g values at $\sim$2.028, 1.90 and 1.74 (Fig. 2, trace B); by the subsequent addition of sodium succinate the intensity at $g = 2.028$ (Fig. 2, trace C) decreases. Subtraction of the spectrum of succinate reduced membranes from that of hydroquinone reduced led to the identification of the species already observed in the native membranes, with g_{max} at 2.028. By this procedure, three species were clearly identified: (i) a rhombic signal, with g values at 2.028, 1.90, 1.74 in the reduced form, characteristic of Rieske-type centres [25]; (ii) a quasi-isotropic signal, with g values at 2.028, 2.015 and 2.01, very similar to the oxidised $[3Fe\text{-}4S]^{1+/0}$ clusters of succinate dehydrogenases (centre S3) [26]; and (iii) a unique component, paramagnetic in the oxidised form, and EPR silent (even spin) in the reduced form, with g values at 2.035, 1.969, 1.969. The assignment of this set of g values to a single species was corroborated by the fact that the resonances at $g = 2.035$ and 1.969 follow the same temperature profile and the same power dependence at 4.6 and 10 K, temperatures at which a power of half saturation of $\sim$1 mW was obtained (data not shown). The relative amount of each species was determined by double integration of the respective theoretical simulations (values in Fig. 2 caption), in the reduced and oxidised forms. The intensities of the simulations were adjusted to experimental spectra obtained under non-saturating conditions (0.24 mW). By this procedure, and within experimental error, the Rieske centre and the axial centre were determined to be present in stoichiometric amounts, while the 3Fe cluster is present in $\sim$5–10% in relation to the other two species. Long incubation of *S. metallicus* membranes with succinate (Fig. 2, trace C) also results in the appearance of a minor resonance at $\sim$1.93, most likely arising from succinate dehydrogenase centre S1 ($[2Fe\text{-}2S]^{2+/1+}$ centre).

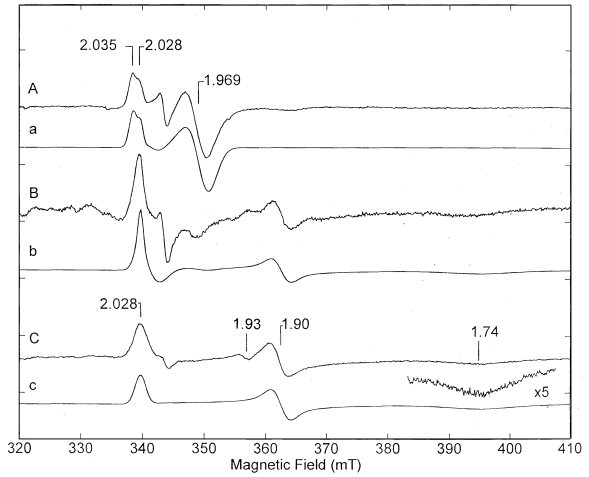

Fig. 2. EPR spectra of *S. metallicus* membranes in several redox stages. Trace A, membranes (60 mg/ml); trace B, same as A plus hydroquinone; trace C, same as B plus sodium succinate. Upper case letters refer to experimental spectra and lower case letters to theoretical simulations. Theoretical parameters: axial species, $g_{1,2,3} = 2.035$ (1.4 mT), 1.969 (3.8 mT), 1.969 (3.8 mT); Rieske, $g_{1,2,3} = 2.028$ (2 mT), 1.90 (3 mT), 1.74 (12 mT); [3Fe-4S] cluster, $g_{1,2,3} = 2.01$ (3.5 mT), 2.015 (3.5 mT), 2.027 (0.8 mT). Temperature, 10 K; microwave power 2.4 mW; microwave frequency, 9.643 GHz; constant gain.

The reduction potential of the centre responsible for the axial signal was determined by EPR redox titrations of intact and detergent-solubilised membranes, as well as of a partially purified fraction. The intensities of the resonances at $g = 2.035$ and $g = 1.969$ were measured as a function of the solution redox potential (Fig. 3). The data points for both resonances follow the same redox profile, further corroborating its assignment as a single species, and were adjusted to a monoelec-

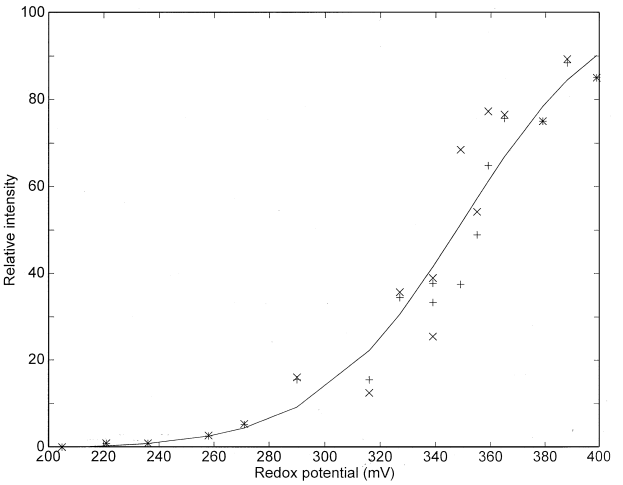

Fig. 3. EPR redox titration of the axial signal. The heights of the EPR resonances at $g = 1.98$ [+] and $g = 2.035$ [×] were measured in the spectra of the samples at each redox potential and normalised to the maximal intensity of each resonance. The experimental points refer to three independent EPR redox titrations. The line corresponds to a Nernst curve with $E = +350$ mV, $n = 1$.

tronic Nernst equation, with a reduction potential of $+350 \pm 20$ mV, a value which is in agreement with the observed reduction by hydroquinone, which has a reduction potential of +285 mV at pH 7.0, 25°C. The Rieske centre, which during the EPR titration was followed by its resonance at $g = 1.90$, poorly equilibrated with the redox mediators, even at the high concentrations used. Hence, it was only possible to estimate the value of its reduction potential as $+320 \pm 30$ mV (data not shown).

3.2. The role of the novel centre in the respiratory chain

A preliminary characterisation of *S. metallicus* respiratory chain was undertaken as a first step towards integrating the newly observed and so far unique centre, and to evaluate if other unusual metalloproteins were present in the respiratory chain of *S. metallicus*. Native *S. metallicus* membranes, as determined polarographically at 40°C, exhibit oxygen consumption driven by NADH (1 nmol O_2/min/mg) and succinate (1.25 nmol O_2/min/mg). Also, decylubiquinol leads to an oxygen consumption of 30 nmol O_2/min/mg. An inhibition of ~80% on these respiratory rates by 15 mM KCN was observed, but higher cyanide concentrations were necessary to completely block respiration. Similar to what has been observed for other archaea, such as *S. acidocaldarius* [9] and *A. ambivalens* (our unpublished observations), classical respiration inhibitors like rotenone, mixothiazol and DBMIB have little or no effect on the respiratory rates.

The visible spectra of *S. metallicus* membranes (Fig. 4) indicate the presence of A- and B-type haems, a result confirmed both by pyridine haemachrome and by haem extraction and HPLC analysis. Both methods led to a stoichiometry of 2 A:1 B haems. As observed for other Sulfolobales, the haems A are of the A_s type [23]. The redox spectrum (Fig. 4, top spectrum) exhibits multiple bands in the α region at 562 (cytochrome *b*) and at 585 and 607 nm (cytochromes *a*). For comparison purposes the α region of *S. acidocaldarius* (*Sa*) membrane redox spectrum was also included. At ~480 nm a broad band resembling those from flavoproteins is also observed in the spectrum of the oxidised membranes, which fully bleaches upon reduction. This band titrates with a reduction potential of ~120 mV, as determined from visible redox titrations (data not shown).

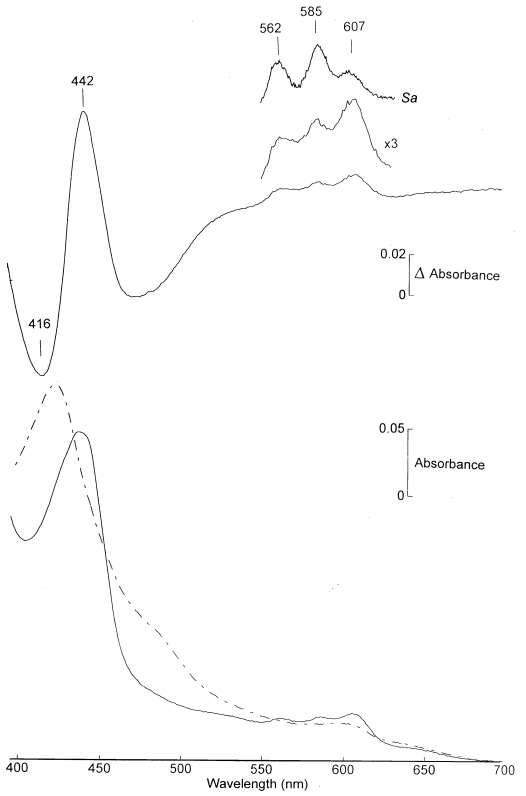

Fig. 4. Visible spectrum of *S. metallicus* membranes. Top, reduced minus oxidised spectrum of *S. metallicus* membranes. The α region of *S. acidocaldarius* membranes redox spectrum (*Sa*) is included for comparison purposes. Bottom, absolute *S. metallicus* membranes spectra: oxidised (dotted line) and dithionite-reduced (solid line). Membranes are in the presence of 0.1% dodecyl maltoside.

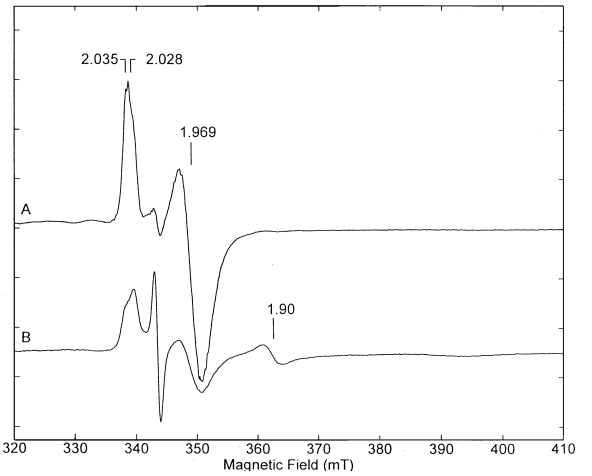

Fig. 5. EPR spectra of *S. metallicus* succinate-reduced membranes in the presence and absence of cyanide. Trace A, native membranes; trace B, membranes incubated with 10 mM cyanide (buffered at pH 7.0). Both preparations (60 mg/ml protein) were incubated for 5 min with 25 mM sodium succinate.

The physiological role of the new centre in the respiratory chain was probed by incubating *S. metallicus* membranes with succinate in the presence and absence of cyanide (Fig. 5). It is clearly observed that only by blocking the terminal oxidases (Fig. 5, trace B) is the axial centre reduced, as well as the Rieske centre, indicating their involvement in the respiratory chain.

4. Discussion

The data now presented reveal the presence of a novel and so far unique metal centre, involved in the respiratory chain of *S. metallicus*. The chemical origin of this centre, exhibiting in the oxidised form an axial EPR signal, is uncertain. Its integrity could be ascertained by the observation of the same EPR signal in intact cells and membranes. Its redox properties are reminiscent of Rieske- and HiPIP-type proteins, but its magnetic properties are unique: the EPR signal of the oxidised form has *g* values close to 2, characteristic of an $S = 1/2$ ground state; in the reduced form it is EPR silent, indicating an even spin ($S = 0,n$). The redox titration data show that these two redox states are interconverted by a monoelectronic process. In parallel mode EPR, resonances such as those associated with an $S = 2$ ground state could not be detected. The absence of resolved hyperfine structure as well as the fact that two *g* values are below 2 not only excludes most of biolog-

ically relevant transition metals but points to a spin-coupled system, with a minimal nuclearity of two ions antiferromagnetically coupled. However, the relatively small spread of the *g* values indicates a small contribution of orbital angular momentum as observed in the iron-sulphur clusters. Hence, at present, the data point to a new type of FeS cluster, of an as yet undetermined structure.

The fact that the new cluster is reduced by adding succinate, only in the presence of cyanide, strongly indicates that it is involved in the respiratory chain. Its high redox potential, with a parallel in Rieske [25] and HiPIP [13] proteins, leads to the hypothesis that it may function as an analogue of the Rieske-type proteins.

The preliminary characterisation of the respiratory chain of *S. metallicus* shows other interesting similarities and differences from other thermophilic archaea but does not reveal the presence of any other unusual metal centre. NADH and succinate lead to respiratory rates within the range determined for other archaea, namely *T. acidophilum* and *S. acidocaldarius* [27,28]. The finding of a succinate-responsive $[3Fe\text{-}4S]^{2+/1+}$ cluster in *S. metallicus* membranes further stresses the structural diversity of succinate:quinone oxidoreductases among archaea. In fact, both spectroscopic and genetic studies showed that this cluster is missing in the enzymes from the aerobically grown archaea *A. ambivalens* ([27] and Gomes et al., in preparation) and *S. acidocaldarius* [2], while it is present in *Thermoplasma acidophilum* succinate dehydrogenase [27]. The overall haem composition of *S. metallicus* is similar to that of other Sulfolobales [23], containing haems of the B and A_s types. In particular, the α-band at 562 nm suggests the presence of the so-called *soxM* oxidase [6]. A major difference with other Sulfolobales lies in the relative intensities of each α-band: *S. metallicus* has clearly a higher content of haems absorbing at 607 nm. Comparison with the redox spectra of *S. acidocaldarius* membranes (Fig. 4), and on the basis of the *soxM* and *soxABCD* oxidases [7,8], suggests that *S. metallicus* may contain a distinct relative proportion of these types of oxidases.

The presence of two functionally similar complexes in the same respiratory chain, such as a Rieske and the new axial centre, is not unique: for example, in *S. acidocaldarius* two Rieske-type proteins are present [25], one being associated with the *soxM* complex [6,25]. Work is in progress to determine the structure of this new centre.

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