

## The unusual iron sulfur composition of the *Acidianus ambivalens* succinate dehydrogenase complex

Cláudio M. Gomes <sup>a</sup>, Rita S. Lemos <sup>a</sup>, Miguel Teixeira <sup>a,\*</sup>, Arnulf Kletzin <sup>b</sup>, Harald Huber <sup>c</sup>, Karl O. Stetter <sup>c</sup>, Günter Schäfer <sup>d</sup>, Stefan Anemüller <sup>d</sup>

<sup>a</sup> Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apt 127, 2780 Oeiras, Portugal

<sup>b</sup> Institut für Mikrobiologie und Genetik, Technische Universität Darmstadt, Schnittspahnstr. 10, 64287 Darmstadt, Germany

<sup>c</sup> Lehrstuhl für Mikrobiologie der Universität Regensburg, 93053 Regensburg, Germany

<sup>d</sup> Institut für Biochemie, Medizinische Universität zu Lübeck, 23538 Lübeck, Germany

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### Abstract

The succinate dehydrogenase complex of the thermoacidophilic archaeon *Acidianus ambivalens* was investigated kinetically and by EPR spectroscopy in its most intact form, i.e., membrane bound. Here it is shown that this respiratory complex has an unusual iron-sulfur cluster composition in respect to that of the canonical succinate dehydrogenases known. The spectroscopic studies show that center S3, the succinate responsive [3Fe-4S]<sup>1+/0</sup> cluster of succinate dehydrogenases, is not present in membranes prepared from aerobically grown *A. ambivalens*, nor in partially purified complex fractions. On the other hand, EPR features associated to the remaining centers, clusters S1 ([2Fe-2S]<sup>1+/2+</sup>) and S2 ([4Fe-4S]<sup>2+/1+</sup>), could be observed. Similar findings were made in other archaea, namely *Acidianus infernus* and *Sulfolobus solfataricus*. Kinetic investigations showed that the *A. ambivalens* enzyme is reversible, capable of operating as a fumarate reductase – a required activity if this obligate autotroph performs CO<sub>2</sub> fixation via a reductive citric acid cycle. Sequencing of the *sdh* operon confirmed the spectroscopic data. Center S3 ([3Fe-4S]) is indeed replaced by a second [4Fe-4S] center, by incorporation of an additional cysteine, at the cysteine cluster binding motif (CxxYxxCxxxC → CxxCxxCxxxC). Genomic analysis shows that genes encoding for succinate dehydrogenases similar to the ones here outlined are also present in bacteria, which may indicate a novel family of succinate/fumarate oxidoreductases, spread among the Archaea and Bacteria domains. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Archaeon; Thermophile; Succinate quinone oxidoreductase; Quinol fumarate reductase

### 1. Introduction

Succinate:quinone reductase (SQR) is a membrane bound enzyme complex that operates both in the central carbon metabolism and in the respiratory

chain of aerobic organisms, catalyzing the oxidation of succinate to fumarate and the transfer of reducing equivalents to the quinone pool. In some cases, the same enzyme can also catalyze the reverse reaction, i.e., the reduction of fumarate by quinol, thus having also quinol:fumarate reductase (QFR) activity. This fact is not surprising since, in general, bacterial SQRs and QFRs have similar subunit and cofactor composition: three or four subunits, and flavin, iron-sulfur

\* Corresponding author. Fax: +351 (1) 4428766;  
E-mail: miguel@itqb.unl.pt

clusters and in some cases *b*-type cytochromes as redox active elements (for a recent review see [1]). The presence of three iron-sulfur clusters, designated as cluster S1 (a  $[2\text{Fe}-2\text{S}]^{2+/1+}$  center), S2 (a  $[4\text{Fe}-4\text{S}]^{2+/1+}$  center) and S3 (a  $[3\text{Fe}-4\text{S}]^{1+/0}$  center), makes EPR spectroscopy particularly suited for the study of these enzyme complexes, preferably in their most intact form, i.e., the membrane bound. Even if distinct SQR and QFR can be found in the same organism, as in *Escherichia coli*, the dissimilarity between the proteins has never been associated with a different FeS cluster composition but with the presence and number of *b*-type cytochromes [2]. Recently, it has been shown that SQR from archaea may have a distinct iron-sulfur composition: while the enzyme from the facultative anaerobe *Thermoplasma acidophilum* contains the three canonical iron-sulfur centers [3], the enzyme from the strict aerobe *Sulfolobus acidocaldarius* lacks the trinuclear center (center S3), which is probably replaced by a tetranuclear center [4].

Archaeal respiratory chains have been shown to contain novel protein complexes with unique features. For example, the soxM and soxABCD complexes from *S. acidocaldarius* [5–9] and *Sulfolobus* strain 7 [10] constitute new types of oxygen reductases. *Sulfolobus metallicus* contains a novel type of iron cluster in a complex that apparently is a functional analogue of Rieske centers [11]. Interestingly, although Rieske proteins are present in several archaea [11–13], a typical complex III has not yet been found.

In the past few years we have been pursuing the characterization of the membrane bound respiratory chain of thermoacidophilic archaea (Sulfolobales), particularly *Acidianus ambivalens*. This thermoacidophilic organism is an obligate chemolithoautotroph, which grows optimally at 80°C and pH 2.5. It is a facultative aerobe which, under aerobic conditions, oxidizes sulfur to sulfuric acid, while on anaerobic conditions reduces sulfur, forming hydrogen sulfide, using hydrogen as electron donor [14,15]. Our studies have shown that *A. ambivalens* has a very simple respiratory system, containing a heme protein, an aa<sub>3</sub> type quinol oxidase [16–18] to which a benzo-thiophenoquinone, caldariella quinone, acts both as electron donor and as a redox active element [18]. Quite unique proteins have been isolated from this

archaeon, such as a highly thermostable zinc containing 7Fe ferredoxin [19,20] and a novel blue protein, named ambineela [21].

Here we report the spectroscopic characterization of the membrane bound succinate dehydrogenase complex (SDH) and the *sdh* operon of *A. ambivalens*. Additionally, the membranes of *Acidianus infernus* and *Sulfolobus solfataricus* were also investigated, thus allowing for a wider view of the structural diversity of SDH complexes among archaea.

## 2. Materials and methods

### 2.1. Cell growth and membrane preparation

*A. ambivalens*, *A. infernus* and *S. solfataricus* were grown as described previously [15]. The membranes were prepared as in [19] with the exception that the cells were suspended in potassium phosphate buffer 50 mM, pH 6.5 and broken at 8000 kPa in an SLM AmincoR KINO20 FrenchR Press. For the preparation of detergent treated membranes, dodecyl-maltoside (DM) was added to the membranes in a ratio of 2 g DM/g protein and the suspension was stirred for 30 min at 4°C.

### 2.2. Enzymatic assays

Fumarate reductase activity was determined anaerobically following the oxidation of benzyl viologen, by measuring its absorbance decrease at 578 nm [22]. Succinate dehydrogenase activity was monitored by following the PMS-coupled reduction of DCPIP, measured at 578 nm, in the conditions reported in [23]. The membranes used in the assays, suspended in potassium phosphate buffer 50 mM, pH 6.5, were incubated overnight with KCN (10 mM), at 4°C on air. This treatment does not lead to solubilization of SDH (70–80% of activity remains membrane bound) and results in an efficient blockage of the terminal oxidase (as observed from the absence of substrate driven oxygen consumption in the oxygen electrode) therefore removing its interference on the determination of the SDH rate. Under these experimental conditions no significant activation was observed. The  $K_m$  and  $V_{max}$  were determined in detergent treated membranes, at 70°C. The presence of detergent min-

imizes turbidity and facilitates the access of the artificial dyes to the membrane integrated complex. Polarographic assays were made in a YSI Micro-chamber oxygen electrode at 40°C, in potassium phosphate buffer 50 mM, pH 6.5. Protein concentrations were determined by the modified microbiuret method [24].

### 2.3. EPR spectroscopy

EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with continuous-flow helium and liquid nitrogen cryostats from Oxford Instruments, as in [19].

### 2.4. Cloning, sequencing and Southern blot analysis of *SQR* operon

Total genomic *A. ambivalens* DNA was prepared by the CTAB method with CsCl purification [25]. A 5109 bp *Bam*HI-*Pst*I fragment of genomic DNA was cloned into pBluescript IISK– (Stratagene, La Jolla, Ca, USA) covering 95% of the *sdh* operon. It was picked accidentally due to weak hybridization with a probe directed against the adenylosuccinate synthetase gene (*purA*) from the same organism. Subclones were constructed by deleting different parts of the original constructs with various restriction enzymes in several steps. The nucleotide sequence of the *Bam*HI-*Pst*I fragment was determined on both strands by sequencing both the original plasmid and the subclones with standard T3 and T7 primers. Gaps in the sequence were filled in subsequently with specifically synthesized primers. All sequencing was done on a LICOR automatic sequencer (MWG, Ebersberg, Germany). Primers for inverse polymerase chain reaction (i-PCR [26]) were designed from the sequence of the original fragment in order to clone the missing part of the *sdhD* gene. A 1110 bp PCR product amplified from *Taq*I-digested and religated

genomic DNA was cloned into the pCRscript vector according to the manufacturer's instructions (Stratagene), and sequenced. The nucleotide sequence was submitted to EMBL database (accession No. AJ005961). Subunit-specific digoxigenin-labeled probes directed against the A and the C subunits were synthesized from genomic template DNA by PCR in the presence of DIG-labeled dNTPs (Boehringer Mannheim, Germany). The labeled products were used for Southern hybridization of restriction digests of *A. ambivalens* genomic DNA. The detection of the hybridized bands on nylon filters was performed according to the manufacturer's instructions (Boehringer). All amino acid sequences used in this work for sequence comparisons were extracted from the EMBL or SWISSPROT databases and Clustal W 1.6 [27] was used for sequence alignments and calculation of sequence similarities.

## 3. Results

### 3.1. Kinetic characterization

The membrane fractions obtained from aerobically grown *A. ambivalens* exhibited both succinate dehydrogenase and fumarate reductase activities. EPR and visible spectroscopy studies revealed that incubation of membranes with succinate results in electron transfer to the aa<sub>3</sub> quinol oxidase. Since caldariella quinol is the major quinone present in *A. ambivalens* [28], these experiments provide evidence for the presence of an operative succinate:caldariella quinone reductase in the membranes. A succinate driven respiratory rate of 1.8 nmol O<sub>2</sub> min<sup>−1</sup> mg<sup>−1</sup> (at 40°C) is observed, a value similar to the one obtained for the archaeon *T. acidophilum* (1.26 nmol O<sub>2</sub> min<sup>−1</sup> mg<sup>−1</sup>, at 53°C) [3]. The soluble fraction of *A. ambivalens* contained 20–30% of the total succinate dehydrogenase activity.

Table 1

Kinetic parameters determined for fumarate reductase and succinate dehydrogenase activities in *A. ambivalens* membranes

Activity	Membranes from aerobic growth		Membranes from anaerobic growth	
	$K_m$ (mM)	$V_{max}$ (μmol/min/mg)	$K_m$ (mM)	$V_{max}$ (μmol/min/mg)
Fumarate reductase	0.06	0.07	0.08	0.01
Succinate dehydrogenase	0.50	0.01	0.30	0.005

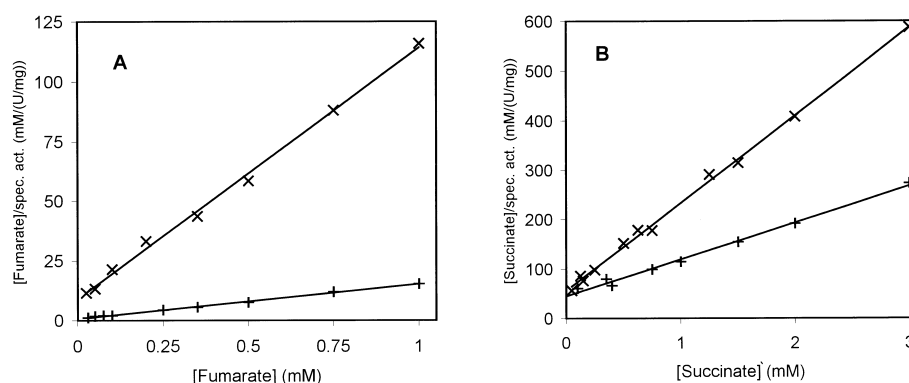


Fig. 1. Hanes plots for the determination of kinetic parameters. (A) Fumarate reductase activity; (B) succinate dehydrogenase activity. For both panels × denote assays with membranes from anaerobically grown cells and + denote assays with membranes from aerobically grown cells. See Table 1 for kinetic parameters.

The kinetic parameters for succinate oxidation and fumarate reduction, determined at 70°C for the detergent treated membranes, were calculated from Hanes plots (Fig. 1, Table 1). The affinity for fumarate as well as the maximum velocity were clearly higher than for succinate – a behavior also observed in membranes prepared from anaerobically grown cells (Fig. 1, Table 1). For reference purposes, Table 2 also lists the  $K_m$  determined for succinate and fumarate for enzymes from several sources (SQR, QFR and membranes). Although these figures were ob-

tained using different protocols and conditions, it can be observed that the data for *A. ambivalens* membranes closely resemble those from other organisms; in particular, the  $K_m$  for succinate for membrane bound archaeal succinate dehydrogenases are quite similar.

The temperature activity profile was determined for the SDH activity in membranes from aerobically grown *A. ambivalens*. As with most enzymes from thermophiles, the catalytic activity was found to be extremely temperature dependent: from 30 to 75°C

Table 2

Comparison of kinetic properties and iron-sulfur clusters of SQR/FRD complexes from several sources

Organism	Source	$K_m$ for succinate (mM)	$K_m$ for fumarate (mM)	Center S1 [2Fe-2S] <sup>1+/2+</sup>	Center S2 [4Fe-4S] <sup>2+/1+</sup>	Center S3 [3Fe-4S] <sup>1+/0</sup>	Ref.
<i>A. ambivalens</i> This work	Membranes		0.5	0.06	+	+	—
<i>A. infernus</i> This work	Membranes		nd	nd	+	+	—
<i>S. solfataricus</i> This work	Membranes		nd	nd	+	+	—
<i>S. metallicus</i> [11]	Membranes		nd	nd	+	+	+
<i>S. acidocaldarius</i> <i>Sulfolobus</i> strain 7 [40]	SDH Membranes	1.42	nd 0.28	+ nd	+ +	— +	[23] — <sup>a</sup>
<i>T. acidophilum</i> [3]	Membranes		0.32	nd	+	+	+
<i>Halobacterium halobium</i> [41]	Membranes		0.7	nd	+	+	+
<i>E. coli</i>	FR	1.0	0.017	+	+	+	[2]
<i>E. coli</i>	SDH	0.26	0.45	+	+	+	[2]
<i>Desulfobulbus elongatus</i>	SDH	0.023	0.014	+	+	+	[44]

<sup>a</sup>The EPR data presented for *Sulfolobus* strain 7 [17] are not consistent with the suggested presence of a trinuclear center.

the activity increased 45-fold, with an optimum temperature between 75 and 81°C. The overall activation energy was determined to be 75.6 kJ/mol, a value comparable to that determined for *S. acidocaldarius* SDH (59.6 kJ/mol [23]). Re-determination of the kinetic parameters of the SDH activity in this membrane preparation at 80°C showed that the  $K_m$  for succinate was not significantly altered (approx. 0.37 mM).

### 3.2. EPR characterization

The intact membrane fractions of *A. ambivalens* cells were studied by EPR spectroscopy, in several redox states (Fig. 2). In oxidized membranes from aerobically grown *A. ambivalens* only a radical type signal centered at  $g=2.00$  is observed (Fig. 2, trace A). Incubation for over 24 h with potassium ferricyanide did not change the spectrum, indicating the absence of a  $[3Fe-4S]^{1+}$  center in these membranes. Upon addition of succinate, a rhombic type signal with  $g_{med} = 1.93$ ,  $g_{min} = 1.91$  is observed (Fig. 2, trace

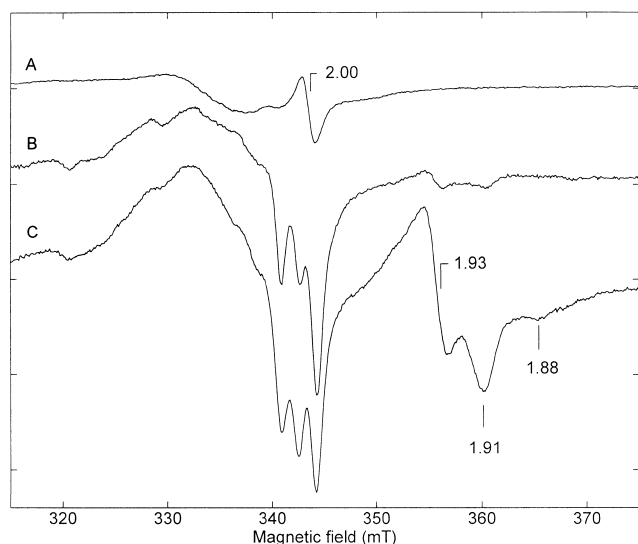


Fig. 2. EPR spectra of *A. ambivalens* membranes obtained from aerobically grown cells, in several redox conditions. Traces: A, air-oxidized membranes; B, succinate-reduced; C, dithionite reduced. Temperature: 10 K; microwave power 2.4 mW; microwave frequency, 9.643 GHz; gain:  $6.3 \times 10^4$  (trace A),  $8 \times 10^4$  (trace B),  $10 \times 10^4$  (trace C). Protein concentration: 40 mg/ml in 50 mM potassium phosphate, pH 6.5; incubation temperature: 70°C; reduced forms were produced under a nitrogen atmosphere, with 20 min incubation for the succinate reduced state.

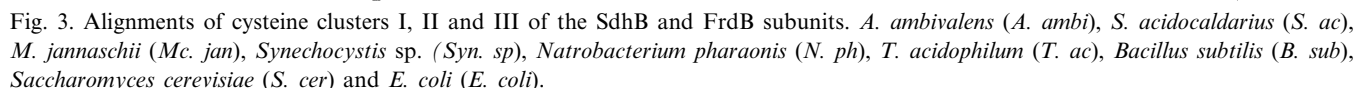
B). By addition of sodium dithionite this signal fully develops (Fig. 2, trace C), with relaxation properties typical of reduced  $[2Fe-2S]$  centers, being still observed at 100 K (data not shown). Due to these characteristics these resonances are assigned to the binuclear iron center S1. A minor component is observed at  $g \sim 1.88$ , which may be due either to the tetranuclear center S2 or to another low-potential paramagnetic center in *A. ambivalens* membranes. Resonances characteristic for Rieske-type proteins are not observed in the membranes, nor in the soluble fractions. A broad and unresolved resonance around  $g \sim 2$  is observed, which did not allow us to determine the  $g_{max}$  values for the various species.

A similar study was performed on membranes from *A. ambivalens* cells grown in the absence of oxygen (data not shown). In this case, a succinate responsive resonance at  $g=2.02$  could be observed in the oxidized state indicating the presence of a  $[3Fe-4S]$  center; upon reduction with succinate or sodium dithionite EPR signals at  $g=1.93$ , 1.91 were not observed. Instead, a set of resonances at  $g=1.92$ , 1.85 were detected. The membranes of anaerobically grown *A. ambivalens* have hydrogen uptake activity. Hence, as nickel-containing hydrogenases have a trinuclear cluster [29,30] it becomes impossible at this stage to assign the  $[3Fe-4S]$  cluster to SDH.

Two other thermoacidophilic archaea were studied by EPR spectroscopy to further assess the properties of complex II on these microorganisms: the facultative aerobe *A. infernus* and the strict aerobe *S. solfataricus*. In both organisms, spectral features identical to those observed for *A. ambivalens* SDH were found (Table 2), indicating that the enzymes from these organisms also lack the canonical center S3.

### 3.3. Cloning and sequencing of the *sdh* operon

The *A. ambivalens* *sdh* operon was cloned and both strands completely sequenced. It consists of four structural genes, whose order in the operon (ABCD) is the same as that of *E. coli* *frd* operon (QFR encoding) [31] and *S. acidocaldarius* *sdh* operon [4]. The *A. ambivalens* *sdh* operon gene product SdhA is the FAD containing subunit, by similarity towards other succinate dehydrogenase and fumarate reductase flavoprotein subunits (not shown). On the



## 4. Discussion

The EPR data clearly indicate that the trinuclear center S3 is absent, while the remaining centers are observable upon succinate and dithionite reduction. Analysis of the *A. ambivalens* *sdh* operon corroborates the spectroscopic evidence obtained for the aerobically grown cells, namely the absence of the typical signal for the trinuclear center. The subunit B cysteine cluster III, very likely representing the ligands of the  $[3\text{Fe-4S}]^{1+/0}$  cluster in other iron sulfur subunits examined so far [1], shows the presence of an additional cysteine residue (Cys211) and suggests the presence of a second  $[4\text{Fe-4S}]^{2+/1+}$  cluster, rather than the usual  $[3\text{Fe-4S}]^{1+/0}$  cluster. A similar observation has been made in the strict aerobe *S. acidocaldarius* *sdh* operon [4], and in the *frd* operons of the anaerobic methanogen *M. jannaschii* [32] and the cyanobacterium *Synechocystis* PCC6803 [33]. Together, these proteins seem to constitute a novel family of succinate/fumarate oxidoreductases, interestingly found both in archaea and bacteria.

The kinetic characterization of the *A. ambivalens* complex showed the presence of an active, reversible, membrane-bound succinate dehydrogenase. Being an obligate autotroph, *A. ambivalens* requires an efficient mechanism for CO<sub>2</sub> fixation. If this takes place via a reductive citric acid cycle (for a review see [34]), then an active fumarate reductase is needed and the observations reported here may be consistent with the physiology of *A. ambivalens*. However, it should be kept in mind that the enzymatic activities were determined with artificial dyes and not with the endogenous quinones as electron donors or acceptors. Also, there is no clear evidence that such a pathway is operating in *A. ambivalens*. In fact, it has been recently found in the related archaeon *Sulfolobus bri-*

*erleyi* that CO<sub>2</sub> fixation is achieved by a modified 3-hydroxypropionate cycle [35] – a pathway in which interestingly a fumarate reductase is also involved [35–37]. Whatever the case, in vivo, the switch between the succinate dehydrogenase/fumarate reductase activities may possibly be controlled by the energetic state of the cell and by the internal transient concentrations of the respective substrates.

From the data so far gathered, it may be concluded that there is a considerable structural diversity concerning the type of FeS clusters present, among archaeal succinate/fumarate oxidoreductases (Table 1), which is not related to growth mode in respect to oxygen or to autotrophy or heterotrophy. Also, so far there is no evidence, except for *T. acidophilum* [3,38] for the involvement of *b*-type hemes in these enzymes. The absence of the [3Fe-4S] cluster has strong functional implications as it has been proposed and is generally accepted that the trinuclear cluster is directly involved in electron donation to quinones [1,39]. This implies that an alternative mechanism is operating in these organisms.

It remains to be shown whether the enzyme expressed under anaerobic conditions is identical to that expressed under aerobic conditions. The fact that the EPR resonances of center S1 are not observed in the membranes of cells grown in the absence of oxygen is in agreement with the lower  $V_{\max}$  determined for this preparation, which suggest a lower level of relative expression under anaerobic conditions.

In conclusion, it becomes again clear that bioenergetic strategies are much more diverse than generally considered, not only in archaea [11] but also in bacteria [42,43], and that as more phylogenetically distant microorganisms are studied new respiratory complexes will be unraveled.

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