The respiratory system of *Sulfolobus acidocaldarius*, a thermoacidophilic archaebacterium

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The respiratory properties, cellular ATP content and absorption difference spectra of *Sulfolobus acidocaldarius* (DSM 639) have been investigated. In contrast to earlier postulates regarding *Thermoplasma acidophilum* [(1984) Syst. Appl. Microbiol. 5, 30–40], *S. acidocaldarius* seemed to depend energetically on respiration-coupled phosphorylation. Its ATP content strictly depended on respiratory activity. Its membrane is capable of proton pumping and presumably contains a branched electron transport system. The latter is composed of at least 2 types of cytochromes, an *a*- and presumably an *o*-type, while *c*-type cytochromes could not be detected. It appears possible that one of the terminal oxidases is directly reduced by the so-called caldariellaquinone, found in the same organism [(1983) Syst. Appl. Microbiol. 4, 295–304].

Archaebacterium *Thermoacidophile* *Electron transport* *Oxidative phosphorylation*

1. INTRODUCTION

In recent years archaebacteria have gained much interest from evolutionary aspects. Studies regarding 16 S rRNA sequences [3–5], ADP-ribosylation of elongation factor 2 (EF-2) [6], tRNA synthetases [7], subunit structures of DNA-dependent RNA polymerases [8,9], cell wall structure [10], and membrane structure [11–13] revealed homologies as well as divergences between eucaryotes, eubacteria and archaebacteria. Very little is known, however, about the energy-conserving systems of thermoacidophilic archaebacteria. For *Thermoplasma acidophilum* an exclusively fermentative pathway was postulated [1], though this organism is an obligate aerobic and generates a protonmotive force sufficient for ATP synthesis [14]. Nevertheless, oxidative phosphorylation has not yet been detected in thermoacidophilic archaebacteria. We examined the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* (DSM 639) which grows optimally at pH 2–3 and at temperatures between 70 and 80°C [15]. Because of the low pH optimum of growth *Sulfolobus* has to maintain a large pH gradient [16] which most likely is energetically linked to cell respiration. In the following we studied the effects of various substrates on endogenous respiration and cellular ATP content. Furthermore, we tested the influence of various respiratory inhibitors and examined the redox behaviour and types of cytochromes.

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Abbreviations: HOQNO, 2-heptyl-4-hydroxy-quinoline *N*-oxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); BAL, British Anti-Lewisite (2,3-dimercaptopropanol); EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline; FCCP, carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone; 2,4-DNP, 2,4-dinitrophenol

2. MATERIALS AND METHODS

S. acidocaldarius (DSM 639) was kindly provided by Dr W. Zillig (Martinsried). The cells were grown at 70°C under vigorous gyratory shaking in 2–5 l Erlenmeyer flasks containing Brock's medium [15], supplemented with 0.1% yeast extract (Difco) and 0.2% sucrose. After about 36 h the cells were

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harvested by centrifugation for 5 min at 5000 × *g*, washed once in buffer containing 25 mM glycylglycine, 1 mM K-phosphate, pH 3.5, and resuspended in the same buffer. Final protein concentration of the stock suspension for respiratory experiments was about 40–60 mg/ml. For special purposes the cells walls were disrupted by sonication of an aliquot of the stock solution with a Branson sonifier for 30–120 s. After centrifugation at 12 000 × *g* for 5 min, the supernatant was used as broken cells. Purified membranes were prepared at 4°C in a buffer containing 50 mM malonate, 1 mM EDTA, pH 5.5, as described [17]. After sonication the sample was centrifuged at 9500 × *g* for 15 min. The supernatant was spun down at 150 000 × *g* for 1 h and the pellet washed 3 times. Final protein concentration of the membranes was about 20–30 mg/ml. Respiration was measured polarographically with a Clark-type electrode using freshly harvested cells. Spectroscopic studies were performed with a Sigma ZWS-II dual-wavelength spectrophotometer, equipped with a split-beam device. For membrane preparations, the buffer was supplemented with 100 mM KCl. Pyridine hemochrome spectra were measured according to Williams [18] with 2% desoxycholate present. The reduced state was generated in all cases by adding a few grains of sodium dithionite; the oxidized state by using an oxygen-saturated medium. ATP was determined by a luciferase method [19]. For complete denaturation and release of ATP, it was necessary to use a detergent containing mixture of 20% trichloroacetic acid and NRB (Abimed), 1 : 1 (v/v). All chemicals were purchased from Merck, Boehringer and Sigma.

3. RESULTS

S. acidocaldarius cells showed respiration with endogenous substrates. The rate of respiration could be enhanced by succinate, isocitrate and glyoxylate, oxalacetate and fumarate, as well as by gramicidin D, an uncoupler of oxidative phosphorylation in mitochondria [20] (table 1). Other substrates, like amino acids, sugars, sugar phosphates, mono-, di- and tricarboxylic acids, did not stimulate the rate of endogenous respiration.

Succinate stimulated endogenous respiration in a pH-dependent manner (pH 2.5–4.5); around its pK_2 the stimulation vanished; presumably succinate respiration reflects differences of penetration properties of the bianionic and the monoanionic species of the molecule. Other stimulating substrates listed in table 1 did not show a significant pH dependence of respiration; the rate of endogenous respiration was influenced only slightly in pH range tested.

Table 1

Substrates and effectors stimulating respiration of *S. acidocaldarius* cells

Conditions	Respiration rate
Endogenous respiration	13.2 ± 2.9
Succinate (3.8 mM)	29
Isocitrate (3.8 mM) + glyoxylate (3.8 mM)	16.9
Oxalacetate (3.8 mM)	17.2
Fumarate (3.8 mM)	21
Gramicidin D (3.8 μg/ml)	25.1

Values given in nmol O_2/min per mg protein. Respiration was measured at 60°C in a buffer containing 25 mM glycylglycine, 1 mM K-phosphate, pH 3.5

NADH did not increase the respiratory rate of whole cells. However, after sonication of the cells it could restore oxygen uptake to values well above those of intact cells. Therefore NADH must be considered the main respiratory substrate. Glyoxylate (3.8 mM) further increased NADH-dependent respiration 1.4-fold.

The uncoupler FCCP did not stimulate cellular respiration, which might be due to its unfavourable pK value. DNP led to a slight acceleration, whereas gramicidin D enhanced the respiratory rate by a factor of 1.9. Furthermore, endogenous respiration was accelerated by a rapid pH shift from 3.5 to 7.2, increasing respiration rate 7.5-fold over an interval of 10–15 s. Afterwards, however, only 20% of the endogenous respiration persisted.

Endogenous respiration was strongly temperature dependent. Although the results indicate a further doubling of respiratory rate at 70–80°C, the measurements were usually performed at 60°C for technical reasons.

In table 2 the effects of various inhibitors of mitochondrial and bacterial respiration on oxygen uptake of *Sulfolobus* are shown. Cyanide and azide exerted only partial inhibition, revealing that 40% cyanide-insensitive respiration persists. Po-

Table 2

Effect of inhibitors on cellular respiration of *S. acidocaldarius*

Conditions	Respiration rate	% inhibition
Endogenous[a] respiration	13.2 ± 2.9	0
+ cyanide (1.8 mM)	5.3	60
+ azide (0.6 mM)	6.6	50
+ tributyltin-Cl (1.5 μM)	0	100
+ mersalyl (0.2 mM)	0[b]	100
+ erythrosin B (76 μM)	6.6	50

[a]The following compounds did not influence respiration at all: rotenone (1.9 μM), antimycin A (1.9 μM), HOQNO (14.5 μM), DTNB (95 μM), EEDQ (76 μM) orthovanadate (0.2 mM), BAL (is oxidized chemically in the reaction vessel)

[b]Full inhibition established after 3 min

Rates are given in nmol O_2/min per mg protein; respiration was measured at 60°C in a medium containing 25 mM glycylglycine, 1 mM K-phosphate, pH 3.5

tent inhibitors were tributyltin and mersalyl, whereas erythrosin B caused only a 50% inhibition. The classical bc_1 inhibitors inhibited neither endogenous respiration nor NADH respiration of broken cells.

As shown in fig.1, a certain ATP level of about 3–5 nmol/mg protein was maintained during endogenous respiration. Sudden anaerobiosis, induced by dithionite, caused an immediate and drastic drop of ATP. The initial ATP content could be restored after a subsequent oxygen pulse (H_2O_2/ catalase). After inhibition by cyanide, the ATP content did not decrease by the same degree as for total anaerobiosis (fig.1b). Moreover, *Sulfolobus* is capable of proton extrusion driven by aerobic electron transport. This could be demonstrated with whole cells by oxygen-induced acidification of a weakly buffered neutral medium. In a typical experiment 70 mequiv. H^+/min per mg protein were extruded (fig.2).

Spectroscopic investigation of membrane preparations revealed the presence of membrane-bound cytochromes. Reduced-minus-oxidized difference spectra show a peak at 562 nm indicating a *b*-type cytochrome. The peak at 587 and 605 nm may be attributed to an *a*- and/or *o*-type cytochrome (fig.3). The presence of an *o*-type cytochrome is

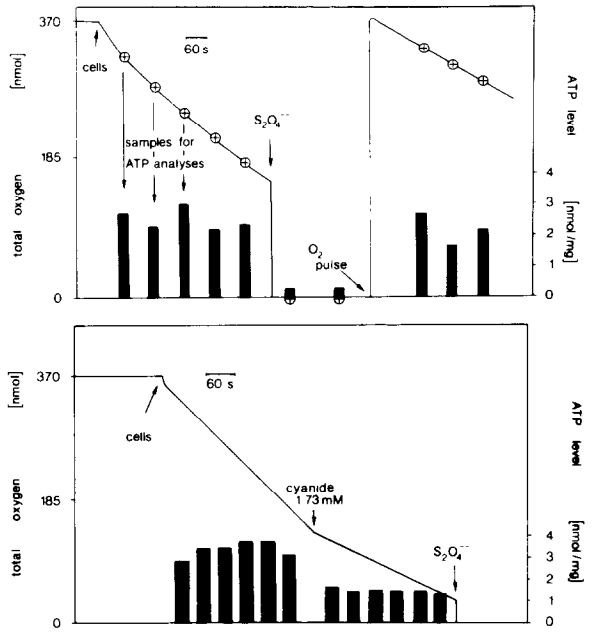

Fig.1. (Top) Oxygen uptake (trace) of *S. acidocaldarius* cells and cellular ATP levels (bars) during endogenous respiration, after anaerobiosis, and after a subsequent oxygen pulse. The buffer contained 25 mM glycylglycine, 1 mM KH_2PO_4, pH 3.5, *T* 60°C; total volume 2.62 ml; total cell protein added 2.7 mg, (Bottom) Oxygen uptake and ATP levels during normal and cyanide-resistant respiration; total cell protein, 3.3 mg; other conditions as above.

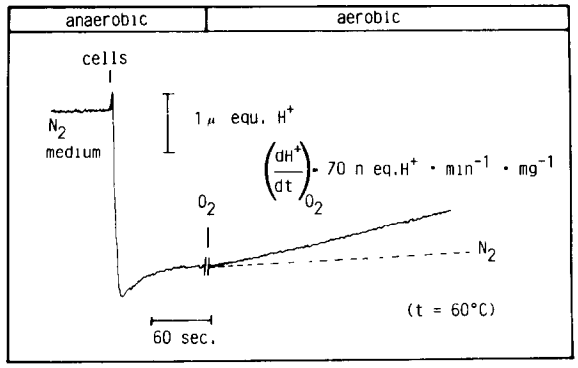

Fig.2. Respiration-dependent proton extrusion. *Sulfolobus* cells were incubated at 60°C under N_2 in a medium containing 9.8 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.48 mM KH_2PO_4 at an initial pH of 6.9; where indicated, the mixture was flushed with oxygen; H^+ liberation was monitored with a microglass electrode (reference calomel); electrode voltage was monitored with a Keithley 610 C electrometer with a compensatory difference amplifier and a Nicolet 4094 A storage oscilloscope.

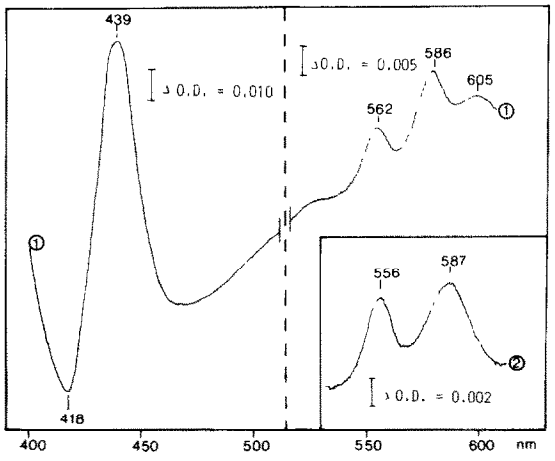

Fig.3. Reduced-minus-oxidized difference spectrum of *S. acidocaldarius* membranes (1). (Inset) Pyridine hemochrome spectrum of dissolved membranes (2). Reduced-minus-oxidized spectra were taken in a buffer containing 50 mM malonate, 200 mM KCl, 1 mM EDTA, pH 5.5; membrane protein, 2.9 mg/ml; the sample was reduced by a few grains of dithionite. Pyridine-hemochrome spectra were recorded in KOH/pyridine (2:1, v/v) with 2% deoxycholate present; the sample was reduced by dithionite.

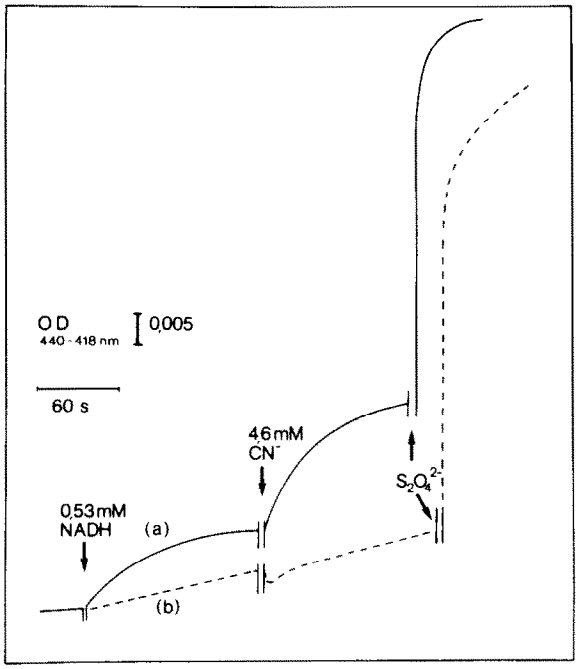

Fig.4. Reduction of cytochromes in *S. acidocaldarius* membranes by NADH; effect of temperature. (a) 50°C, (b) 22°C. Measurements in a buffer containing 50 mM malonate, 200 mM KCl, 1 mM EDTA, pH 5.5; membrane protein, about 3 mg/ml; the sample was fully reduced by a few grains of sodium dithionite.

further corroborated by a typical peak at 417 nm in the carbon monoxide-reduced minus reduced spectrum of detergent solubilized particles (not shown). Difference spectra of pyridine hemochromes of solubilized membranes show pronounced maxima at 566 and 587 nm (inset, fig.3), the former indicating a *b*- or *c*-type cytochrome, the latter an *a*-type cytochrome. The relative intensities of both maxima suggest a 1 : 1 ratio of 2 types of heme. A *c*-type cytochrome could not be detected so far, either spectroscopically or chemically; thus the pyridine hemochromes obviously indicate the presence of *a*- and *b*-type hemes only. Cytochrome *c* seems to be lacking in *S. acidocaldarius*. Final resolution, however, requires further separation of the single hemoprotein complexes from the membrane.

Reduction of cytochromes by NADH could easily be registered by dual-wavelength spectrophotometry (fig.4). Even at 50°C the observed reduction rate was rather slow, whereas at room temperature it was negligible. In accordance with the cyanide-resistant respiration NADH was able to reduce only about 40% of membrane-bound cytochromes (cyanide present). Full reduction required subsequent dithionite addition.

The absence of rotenone- and antimycin A-induced inhibition of NADH respiration was also confirmed in spectroscopic experiments. Both inhibitors influenced neither the degree nor the rate of cytochrome reduction under aerobic conditions. Therefore, a bc_1 complex typical of eubacteria or higher organisms is unlikely to be present in *S. acidocaldarius*.

4. DISCUSSION

The thermoacidophilic archaebacterium *S. acidocaldarius* shows endogenous respiration, which can be enhanced by metabolically occurring substrates. Respiration seems to be totally linked to the membrane fraction. The tight connection of cellular ATP content and respiration makes it unlikely that *S. acidocaldarius* generates ATP exclusively by a fermentative pathway. The coupling

of respiratory activity and proton conduction is further supported by the effect of uncouplers like 2,4-DNP and gramicidin D as well as by the proton pumping properties under aerobic conditions. Therefore, presumably a chemiosmotic way of oxidative phosphorylation is responsible for energy conversion.

The existence of a partially cyanide-insensitive respiration clearly indicates a branched respiratory chain, which contains at least 2 types of membrane-bound cytochromes. One is evidently identified as an *a*-type cytochrome, whereas the other cannot yet be definitely assigned as an *o*- or *b*-type; the occurrence of branched electron transport and spectral properties of membrane preparations, in fact, are in favour of an *o*-type cytochrome as the alternative terminal oxidase. Typical inhibitors of the bc_1 comnplex are ineffective; the existence of a *b*-type cytochrome, however, cannot be excluded. *c*-type cytochromes are not found at all. Thus, a bc_1 complex is apparently lacking.

Because caldariellaquinone, a thiophene-containing condensed quinone/quinole system, has been detected in membranes from *S. acidocaldarius* [2] a direct electron transport from the latter to a cytochrome *a* containing terminal oxidase might take place, an analogous pathway as recently suggested for *Escherichia coli* [21].

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