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Review

Bioenergetics of the archaebacterium *Sulfolobus*

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Abstract

Archaea are forming one of the three kingdoms defining the universal phylogenetic tree of living organisms. Within itself this kingdom is heterogenous regarding the mechanisms for deriving energy from the environment for support of cellular functions. These comprise fermentative and chemolithotrophic pathways as well as light driven and respiratory energy conservation. Due to their extreme growth conditions access to the molecular machineries of energy transduction in archaea can be experimentally limited. Among the aerobic, extreme thermoacidophilic archaea, the genus *Sulfolobus* has been studied in greater detail than many others and provides a comprehensive picture of bioenergetics on the level of substrate metabolism, formation and utilization of high energy phosphate bonds, and primary energy conservation in respiratory electron transport. A number of novel metabolic reactions as well as unusual structures of respiratory enzyme complexes have been detected. Since their genomic organization and many other primary structures could be determined, these studies shed light on the evolution of various bioenergetic modules. It is the aim of this comprehensive review to bring the different aspects of *Sulfolobus* bioenergetics into focus as a representative example of, and point of comparison for closely related, aerobic archaea.

Keywords: Archaea; Hyperthermophile; Electron transport; Terminal oxidase; ATPase; Energy conservation; (*Sulfolobus*)

Contents

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone; DCCD, *N,N'*-dicyclohexyl-carbodiimide; 2,4-DNP, 2,4-dinitro-phenole; DCPIP, 2,6-dichlorophenole-indophenole; PMS, phenazine methosulfate; NEM, *N*-ethyl-maleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; *p*-HMB, *p*-hydroxy-mercuri-benzoic acid; pmf, proton motive force; Tm, midpoint temperature of thermal transition; TCS, tetrachlorosalicylanilide; TTFB, 4,5,6,7-tetrachloro-2'-trifluoromethyl-benzimidazole.

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1. Introduction

Sulfolobus is one of the best investigated archaebacterial organisms and in certain respects has become the standard example for extreme thermoacidophilic archaea. The broad interest in such organisms results from their unusual growth conditions which have attracted a large number of scientists as well as of biotechnologists. Archaea share the general property to thrive in extreme biotopes characterized by high temperature, high pressure, high salt concentration, extremely high or low pH, or a combination of these criteria. Though a variety of these extremophilic microorganisms was known since long ago and has been subject to biochemical investigations for several decades, it was only in the seventies when it was recognized on the basis of 16s ribosomal RNA sequences that a number of extremophilic microbial genera and orders belong to a separate urkingdom of life within the prokaryotic domain of the phylogenetic tree [1]. Three primary kingdoms have been defined which were assigned as archaea, bacteria, and eukarya [2]. Besides their congeniality on the level of ribosomal RNA structures a number of other biochemical features is unique to the archaeal domain and has been frequently used as taxonomic marker: on the one hand the specific composition of their surface layer [3–5] which does not contain peptidoglycan, on the other the structure of ribosomes [6–8] and of their RNA-polymerases [9,10]. In addition the archaeal plasma membrane contains archaetypical di- and tetra-ether lipids [11–14] not found in any other organisms. Compiled reviews on the various aspects have been published repeatedly over the past few years [5,14–18].

Although archaea are prokaryotes, their phylogenetic distance to eukarya in several respects appears closer than to the bacterial domain. It is noteworthy

in this context that archaea in fact exhibit properties reminiscent of eukaryotic organisms as for example the subunit structure of RNA polymerases [19–22] or the presence of introns in ribosomal RNA genes [23–27]. It even has been speculated that the eukaryotic cytoplasm may have evolved from premordial archaea [28] and that eukaryotes originated from a fusion event between archaea and bacteria [29,30].

The kingdom of archaea presumably is populated by many more species than we presently know. Due to their extraordinary environmental requirements, many species might not survive usual sampling procedures. Actually, new species are constantly being detected [31] from hot deep-sea steam vents, from acidic solfataric fields, or from saltlakes. In general three major groups can be distinguished on the basis of their metabolic and energy conserving capabilities. One of these are the obligate anaerobes, the methanogenic archaea, comprising chemolithotrophic as well as organotrophic species; among them the barophilic genera can exhibit optimum growth temperatures above 100°C [32,33]. Together with methanogenic genera the extreme halophiles are sharing the same main branch of the phylogenetic tree but in general they are aerobic organisms. This does not exclude, however, that several species can use other electron acceptors than molecular oxygen as for example nitrate [34]. In addition, several strains are able to use light as an auxiliary energy source at low oxygen tension; light energy is transformed into proton motive force by the rhodopsin photo-cycle in their purple membrane [35–37]. The extreme thermoacidophiles are forming a separate branch of mainly oxygen respiring genera like the *Sulfolobaceae*; among these one finds chemolithotrophic and organotrophic species, or also sulfur reducers [38,39], which facultatively may live aerobically, or anaerobically.

In the following, the reader's attention will be focused on the energy transduction pathway of the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. This organism has been chosen because no other thermoacidophilic archaeon has been studied in such detail as yet with respect to respiration driven energy conservation. Many details of its ATPases, phosphotransferases and its electron transport chain have been elucidated within the past few years. These will be discussed also in comparison to closely related species and to other archaea. Special attention will be directed towards unusual electron transport complexes and their function, and possible evolutionary implications are discussed.

2. The genus *Sulfolobus*

The genus *Sulfolobus* has first been described by Brock [40] comprising a number of bacteria which live at extremely high temperature and very low pH. Originally classified as sulfur-oxidizing bacteria, we know now that several of them can grow much faster on organic substrates than chemolithotrophically. The genus includes the species *S. acidocaldarius*, *S. solfataricus*, *S. brierleyi*, *S. shibatae*, and *S. metallicus*; the species called '*Sulfolobus* sp. *strain 7*' is likely to be a *S. solfataricus* strain. Fig. 1 on a partial phylogenetic tree illustrates the position of the Sulfolobales. The genus *Sulfolobus* is closely related to *Desulfurolobus* and *Acidianus* (which are strongly related among each other) and more distantly to *Metallosphera* and *Stygioglobus*. Other branches on this tree are the genera *Thermoproteales*, *Pyrodictiales* and *Desulfurococcales*, which together with the above are forming the major orders of the 'crenarchaeota' [41].

The natural habitats of Sulfolobales are usually found in geothermal areas where elemental sulfur is abundant, especially in terrestric solfataric springs. These can be mildly (pH 4–6) or strongly acidic (pH 0.5–3) and are rich in sulfate. The temperature may range from 60 to nearly 100°C. Aerobic species are found in a layer of about 15 to 30 cm below soil surface. The limiting growth temperatures for *Sulfolobus acidocaldarius* are 60° and 90°C, respectively, with optimum growth at 75–80°C. Below that in anaerobic layers species performing reductive sulfur metabolism exist. Some of these are facultative aerobes like *D. ambivalens* and *A. infernus*.

The morphology of Sulfolobales is irregular and lobe-shaped with cell diameters from 0.2 to 2 μm. They have no typical periplasmic space between the plasma membrane and a firmly attached surface layer, exhibiting even anchoring protrusions into the former [42–44]. This glycoprotein surface layer usually forms an extremely regular and *quasi*-crystalline structure

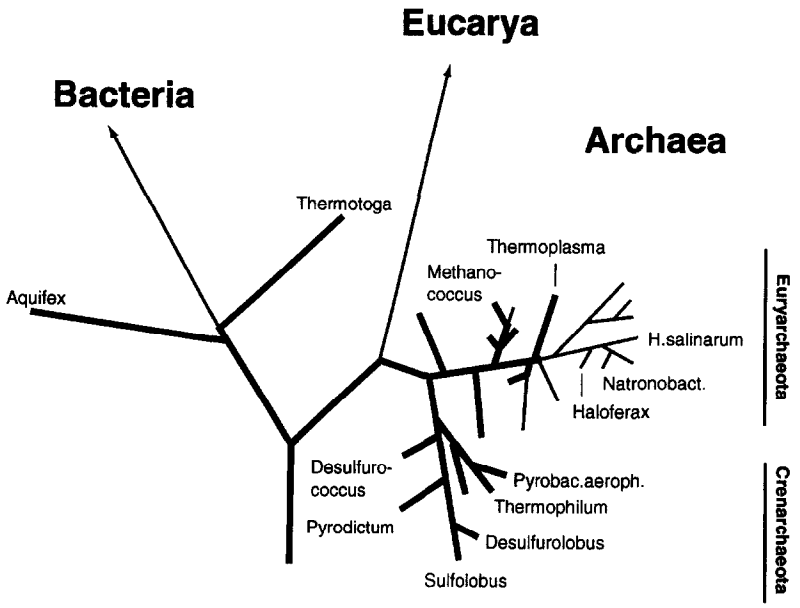

Fig. 1. Simplified phylogenetic tree emphasizing the archaeal branch. The tree is drawn according to Refs. [56,76]; bold traces indicate the domain of hyperthermophilic organisms.

and contributes to the stability of the cells under their extreme environmental conditions. It can be isolated without losing its two-dimensional crystal-like shape [45]. Nevertheless, in case of *Sulfolobus*, the firm cell wall has pore-like openings on top of dome-shaped membrane vaults covering areas of a pseudo-periplasmatic compartment [44].

All obligate aerobes among the cren-archaeota belong to the genus *Sulfolobus*. The general exergonic reactions driving metabolism of these organisms are given in Eqs. (1)–(4).

$$H_2 + \tfrac{1}{2}O_2 \rightarrow H_2O \quad (1)$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (2)$$

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4 \quad (3)$$

$$\text{organic-[H]} + O_2 \rightarrow H_2O + CO_2 \quad (4)$$

Though *Sulfolobus* has been asigned as 'opportunistic heterotroph', several species show the highest growth rates in fact under heterotrophic conditions metabolizing organic carbon sources (sucrose, glucose, glutamate, other mono- and disaccharides, peptides, amino acids [40]) according to Eq. (4). Some members of the genus are even strict heterotrophs. Autotrophic growth has been described for *S. brierleyi* [46] assimilating CO_2 during sulfur oxidation. This is also the typical aerobic growth condition for *D. ambivalens* according to Eq. (2). Oxidation of S^0 or sulfidic ores (Eq. (2), (3)) is always connected to formation of sulfuric acid as the final product. Very slow autotrophic growth of *Sulfolobus* and *Acidianus* has been reported even by hydrogen oxidation [32] (Eq. (1)).

Oxidants other than molecular oxygen can be used by some genera of the crenarchaeota branch (Fig. 1) performing sulfur-, sulfate-, or nitrate-respiration. Examples are the genera of *Thermoproteus*, *Pyrodictium*, *Desulfurococcus*, *Archaeoglobus*, and *Pyrobaculum* [33,39,47]. These strict anaerobes derive energy from reactions (5)–(6)

$$2H + S^0 \rightarrow H_2S \quad (5)$$

$$8H + SO_4^{2-} \rightarrow H_2S + 4H_2O \quad (6)$$

The species of *Acidianus* and *Desulfurolobus* even prefer sulfur-respiration (Eq. (5)) to oxygen respiration (Eq. (2)), assimilating CO_2 in both cases and requiring extremely acidic conditions (pH 0.7–1). Thus, not all members of the order Sulfolobales are obligate aerobes, except for the genus *Sulfolobus*; none of the species can grow simply by fermentation.

3. Energy metabolism of *Sulfolobus*

Metabolic capabilities of the genus *Sulfolobus* have been investigated in many details and a large number

of enzymes from central pathways of energy metabolism has been isolated, characterized or sequenced. However, drawing a homogenous picture is hampered by the fact that the information has partly been obtained from *S. solfataricus* and partly from *S. acidocaldarius*. Although both species are closely related, significant differences exist and generalization of results has to be taken with precaution. Most metabolic and enzymological studies have been performed with *S. solfataricus* which can be handled more conveniently in the laboratory and grows optimally under moderate acidity (pH 3.5). Nevertheless, the following paragraph on essentials of energy metabolism is treating *Sulfolobus* in general terms, including both species.

Under aerobic conditons *Sulfolobus* can grow heterotrophically using various monomeric or polymeric carbohydrates as carbon source and ammonium salts as nitrogen source. It can also grow on glutamate as the single carbon- and nitrogen source. Oxidative degradation of substrates drives ATP-synthesis, which appears to result mainly from respiration coupled ADP-phosphorylation [48]. ATP generation by substrate-level phosphorylation is unlikely [49]; due to the lack of hexokinase and fructose-6-phosphate kinase an Embden-Meyerhof glycolytic pathway is excluded. The modified Entner-Doudoroff pathway which has been shown for Halobacteria [50] would allow formation of 1 ATP/glucose. It operates also in *S. solfataricus* with another modification by which 2-keto-3-desoxy-gluconate is directly cleaved by an aldolase into glyceraldehyde and pyruvate [51]. This would establish a non-phosphorylative pathway as shown in Fig. 2 if glyceraldehyde-3-phosphate cannot be formed. Radiorespirometric investigation [52] of *Sulfolobus* could not distinguish between an ATP-forming and a non-phosphorylative pathway. However, by isotope chase experiments in our laboratory [53], support for a completely non-phosphorylative glucose degradation to pyruvate was obtained with *S. acidocaldarius*. That means 0 ATP/glucose; only by further pyruvate degradation via an oxidative citric acid cycle 1 ATP(GTP) would be obtained via substrate-level phosphorylation.

Although a futile ATP-cycle seems to occur in this metabolic pathway (Fig. 2), its single reactions must be seen in view of other physiological processes like gluconeogenesis. In fact, glycogen has been found as

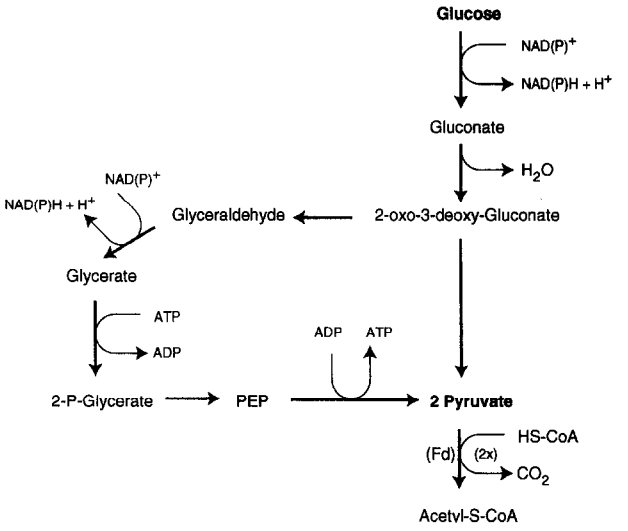

Fig. 2. Scheme of the modified, non-phosphorylating Entner Doudoroff pathway [51] as used in *Sulfolobus acidocaldarius* for conversion of glucose to pyruvate [53].

a metabolic energy store in *Sulfolobus* [54], interestingly associated with a polyphosphate kinase bound to the glycogen particles. The role of this enzyme in energy metabolism of *Sulfolobus* is still unclear.

Actually, the formation of glycogen and the fact that under certain conditions *Sulfolobus* can grow autotrophically [55] require both, a gluconeogenetic pathway, and an energetically efficient CO_2 fixation. None of these pathways has been elucidated completely for *Sulfolobus*. It has been speculated that CO_2 assimilation may occur via a reductive citric acid cycle [46,52,55] as it has been demonstrated for the strict anaerobe *Thermoproteus* [56]. As reviewed elsewhere [49], all essential enzymes of the citric acid cycle were indeed detected in *Sulfolobus*. However, the origin of necessary reducing equivalents remains unsolved. Although the simultaneous operation of assimilatory and oxidative pathways appears contradictory, no other energy source than respiratory chain phosphorylation can be responsible for anabolic metabolism under autotrophic or gluconeogenetic conditions.

Independent of the energy source the pathway of gluconeogenesis in *Sulfolobus* is not established. It remains to be investigated whether or not the modified, non-phosphorylative Entner-Doudoroff pathway can be reversed. Surprisingly, even though a degradative Embden-Meyerhof pathway does not operate,

essential enzymes of its triose-phosphate section were found in *Sulfolobus* and may even be organized in a cotranscribed gene cluster [57,58] like glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate-kinase. Their significance for energy metabolism remains unclear, however, as far as other enzymes to formulate an uninterrupted pathway are still unknown. This is surprising in comparison to methanogens and halophilic archaea where gluconeogenesis via a reversed Embden-Meyerhof pathway has been shown [59,60]. Earlier the possibility has been discussed [49] that the Entner-Doudoroff- and the Embden-Meyerhoff-pathway originally developed in evolution as catabolic and anabolic reaction paths, respectively. Even then, an opposite mutual regulation would be indispensible in order to rule out an energetic futile cycle. If, in contrast, two parallel degradative pathways could exist in *Sulfolobus*, a nonphosphorylative and a phosphorylative Entner-Doudoroff reaction involving glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate-kinase, again mutual regulation would be required. No such regulatory principles in archaea have been discovered as yet.

Actually, it has to be clearly established by future investigation whether or not even closely related *Sulfolobus* species really display uniformity in this respect. Moreover, the expression of distinct enzyme patterns of energy metabolism under the influence of different growth conditions has not yet been critically studied.

Sulfolobus utilizes pyruvate formed from sugars by oxidation via the citric acid cycle [53]; all enzymes of the cycle are present [61] in archaea.

However, the energetics of pyruvate utilization in archaea are unique as the formation of the thioester-bond of acetyl-CoA is coupled to the reduction of ferredoxin instead of NAD^+. Obviously all archaea contain pyruvate:ferredoxin-oxidoreductases (reviewed in Ref. [62]) which sequentially abstracts two electrons from the hydroxyalkyl-ThPP intermediate [63] in thermoacidophiles and halophiles via two iron-sulfur clusters which are reoxidized by archaebacterial ferredoxin (Fig. 3). This mechanism is generally used for oxidative decarboxylation of 2-oxoacids [64,65] in archaea. It was hypothesized to operate also reversibly in a reductive tricarboxylic acid cycle during CO_2 fixation of chemolithitrophically

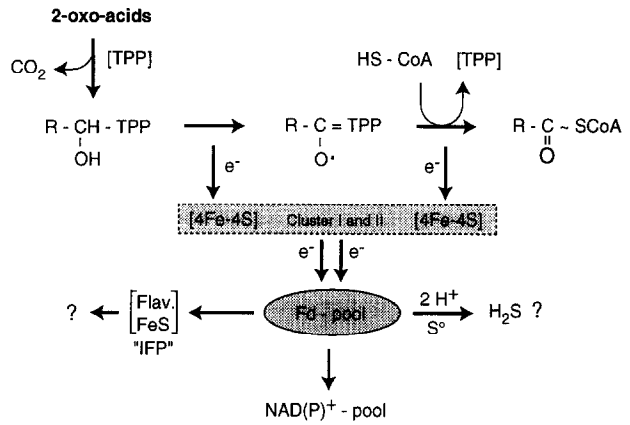

Fig. 3. General mechanism of 2-oxo-acid:ferredoxin oxidoreductases operating in archaea [62,63]. For *Sulfolobus* two [4Fe-4S] clusters are assumed to function as primary single electron acceptors. The ferredoxin-pool represented by *Sulfolobus*-ferredoxin [169] serves as secondary cytosolic hydrogen acceptor. Electrontransfer to elemental sufur has been suggested as a possibility for anaerobic sulfur-dependent archaea; the respective enzyme is still uknown. The iron-sulfur flavo protein (IFP) has been isolated [66]. For further explanation see text and Section 6.2.

growing *Sulfolobus* [66]. The endogenous iron-sulfur clusters of the enzyme may be of the [4Fe-4S] type, as concluded from analogy to the halobacterial enzyme [66,67].

Formation of acetyl-CoA and oxidation of 2-oxoacids in archaea might reflect mechanisms of early evolution providing reducing equivalents on a potential negative enough to drive hydrogenase catalyzed reactions (E_0-450 mV) as well as sulfur reduction ($S^0/S^{2-}E_0$-270 mV) and $NAD(P)^+$ reduction (E_0-320 mV); it can thus connect catabolic metabolism to anaerobic as well as to aerobic electron transport pathways. In *Sulfolobus*, acetyl-CoA oxidation via the citric acid cycle generates reducing equivalents on the level of ferredoxin, NADH, and Caldariella-quinone (E_0 + 100 mV) by the function of succinate dehydrogenase [68]. $NAD(P)^+$ and ferredoxin are connected by the function of a ferredoxin:NAD-oxidoreductase [Th. Hettmann, personal communication, and [69]] (cf. Section 6.2).

While aerobic energy metabolism of acetyl-CoA in *Sulfolobus* proceeds via citrate to CO_2, other glucose metabolising archaea (*Th. acidophilum*) were reported to utilize acetyl-CoA for the production of

ATP with concommitant excretion of acetate even under aerobic conditions [70]; the reaction is catalyzed by acetyl-CoA synthase (ADP utilising), but normally contributes only in anaerobic hyperthermophiles to energy conservation by fermention [62,71].

4. Whole cell energetics

Aerobic extreme acidophiles exhibit a near neutral cytosolic pH [72] and thus have to withstand a large pH gradient, depending on the outside pH; in case of *Sulfolobus*, values between 1.5–2.5 are compatible with normal growth, while related organisms grow even between pH 0.5–1. If a proton-coupled ATP-synthase is present (see below), acidophiles can generate ATP on expense of this large gradient if appropriate charge compensating mechanisms are active. On the other hand efficient proton pumps are required to keep the cytosol near neutral, and to continuously create new cytosolic space from acidic environmental space during bacterial growth.

Previous whole cell experiments with *Sulfolobus acidocaldarius* have clearly shown that both, ATP-synthesis and proton extrusion are coupled to cellular respiration [73]. Prolonged anaerobiosis causes cell death and lysis. With cells respiring on endogenous substrate ATP content decreases within 20 s after withdrawl of oxygen supply [74] and is restored by immediate re-airation. The effect of DCCD revealed the operation of a proton coupled ATP-synthase [61]. It has been described that growth inhibiton by 'classical' respiratory chain inhibitors (rotenone, antimycin) or by DCCD and uncouplers (2,4-DNP) is rather sluggish or does not occur [75]. This has multiple reasons; one is the insensitivity of the archaebacterial respiratory complexes against these agents (see below), the other the unfavourable pK of most uncouplers which is significantly above the optimum growth pH of *Sulfolobus*; further, the membrane permeability of *Sulfolobus* towards these compounds may be low as well as their stability at very low pH. Gramicidin was reported to inhibit growth at 10^{-7} M [75]. In our hands gramicidin-D caused also a rapid decline of cellular ATP level as did respiratory inhibition by azide.

From the fact that only about 40% of the respiration was sensitive to cyanide (1 mM), a branched electron transport chain was proposed already from early whole cell experiments [74].

Study of cellular proton extrusion is experimentally limited at ambient pH below 2–3; thus, most reported experiments were conducted at higher medium pH (3.5–6.0) and extrapolations to optimum growth conditions for *Sulfolobus* have to be made. Oxygen-pulse experiments with cells transitorily kept anaerobic have been performed in two ways; either by initial rate measurements, or by steady-state flux determinations [48,76]. In the latter case H^+/O ratios of 3 were determined, whereas by initial rate determinations much higher values of 6–8 were found [16,77] when stopped flow techniques were used. Notably, 20–100 nmol DCCD/mg cells increased the amplitude and the rate of proton extrusion and thus suggested a classical proton-motive energy transduction to operate in *Sulfolobus*. Assuming a minimum value of $3H^+$ to be consumed per molecule ATP formed by the synthase, the reported H^+/O ratios would account for 1–2 ATP formed per 1/2 mol O_2. Specific energy transduction sites with respect to the respiratory chain could not be concluded from whole cell experiments.

Proton extrusion is sensitive to the uncouplers TTFB, TCS or gramicidin-D. The highest reported rates of proton extrusion are about 400–700 nmol $\cdot$ $min^{-1} \cdot mg^{-1}$ measured at 60°C. No data at optimum growth temperature of *Sulfolobus* (75–80°C) are available. It is important to note that respiration-linked proton pumping of cells is critically influenced by the potassium concentration (up to 20 mM). This is surprising because, in its natural habitat, *Sulfolobus* grows under low salinity [40]. Moreover, highest proton fluxes are observed only when $[NH4]^+$ is omitted or reduced below the concentration for optimum growth. It appears possible that the isosteric ammonium ion might inhibit an essential potassium translocator which in a combined operation of primary proton pumps and secondary potassium flow enables the cells to maintain a moderate internal pH balancing for the membrane potential generated by massive proton extrusion. However, so far a potassium translocator in *Sulfolobus* membranes has not been described. Also a primary K^+ pump could not be detected [W. Meyer and G. Schäfer, unpublished experiments].

Table 1
Influence of uncouplers and DCCD on the components of the proton-motive force and cellular ATP level of *Sulfolobus acidocaldarius*

Condition	-Z ΔpH (mV)	ΔΨ (mV)	Δp(mV)	[ATP] (nmol/mg)
Control (pH_o 3.5)	−134	−19	−153	4.5–5.5
+TTFB (6.7)	−107	+54	−53	0.6–0.8
+TCS(6.7)	−74	+69	−5	n.d.
+Gramic.-D (3.4)	−80	+57	−23	n.d.
+Gramic.-D(30)	−67	+63	−4	0.5–1.5
+DCCD (180)	−90	−55	−145	0.7
Control (pH_o 6.1)	−96	+2	−94	n.d.
acid pulse (pH_o 3.4)	−210	+39	−171	n.d.

Data and experimental details from Refs. [61,76]; figures in parentheses indicate the amounts of ionophores as (nmol/mg protein). pH_o = outside pH; TCS = tetrachlorosalicylanilide; TTFB = 4,5,6,7-tetrachloro-trifluormethyl-benzimidazole; DCCD = dicyclohexl-carbodiimide; n.d. = not determined. In the second experiment (means of 3 independent determinations) the cells were equilibrated for 10 min at pH 6 and 60° prior to the pH jump.

Active proton pumping by whole *Sulfolobus* cells has also been confirmed with the exogenous reductant ascorbate/TMPD [15] and oxygen pulses. In the presence of valinomycin for dissipation of the membrane potential, a H^+/O ratio of 2–3 has been measured in line with the assumption of electron transport-linked pumping. The data do not allow an attribution of pumping to specific components of the respiratory chain; neither is the pathway of electrons clear in these experiments. It is difficult to exclude that endogenous reducing equivalents contributed to the observed effect. Details of proton pumping will be discussed in Section 8, below.

According to Mitchell's notion of the proton motive force [pmf] [78]

$$\Delta p = \Delta\mu H^+/F = \Delta\Psi - 2.3RT\,\Delta pH\,[mV]$$

in the absence of a significant membrane potential, a pH difference of 4 to 5 as observed with obligate acidophiles [72] would account for a pmf of 265 to 335 mV at 70°C. In reality such high values could not be measured in *Sulfolobus*. In independent studies we

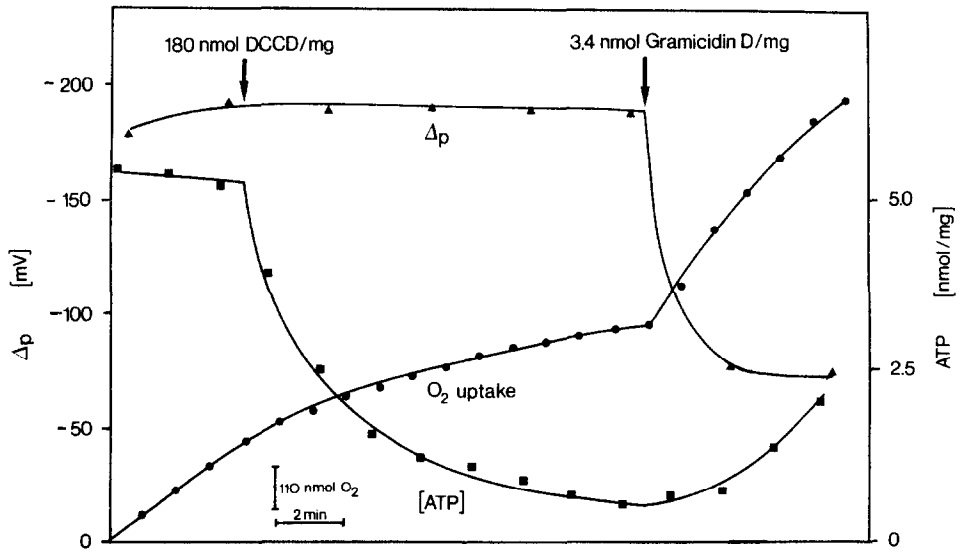

Fig. 4. Demonstration of respiratory control by the effect of DCCD and gramicidin in whole cells of *Sulfolobus acidocaldarius* (DSM 639). Δp indicates the proton motive force across the plasma membrane. Data are taken from Lübben and Schäfer [61] and represent the first decisive evidence for the operation of a H^+-coupled ATP synthase in *Sulfolobus*.

found repeatedly values of 140–160 mV under steady-state conditions at an external pH of 3.5 (45–60°C); the pH gradient of 2–3 was complemented by a small membrane potential negative inside. Whether or not at lower outside pH an inside positive membrane potential contributes to balance the proton charges remains to be established. One may speculate that the assumed potassium translocator operates in that way only at low ambient pH. Of course, a non-energized translocator could maximaly reduce the membrane potential to zero. For reversal of the potential an electrogenic K^+ uptake may play an important role in *Sulfolobus* or a K^+-H^+ symport as reported for *Bacillus acidocaldarius* [79].

Table 1 gives an example for the sensitivity of the pmf in *Sulfolobus* towards uncouplers and gramicidin. The interesting observation is the persistance of a pH gradient even under conditions where no pmf exists anymore. This reflects a protective mechanism of obligate acidophiles preventing overacidification of the cytosol and loss of viability during cell starvation; the possible contribution of a Donnan potential developing after massive proton influx has been discussed in Ref. [80]. Dynamics of energy conservation in *Sulfolobus* as shown in Fig. 4 support the function of a chemiosmotic proton cycle [61] by demonstrating the decline of ATP at high pmf in the presence of DCCD, while the uncoupler gramicidin causes a rapid increase of respiratory rate with a concommitant collapse of pmf but without complete loss of the ΔpH (cf. Table 1).

5. Modules of high energy phosphate transfer

Energy stored in the electrochemical gradient of protons, $\Delta\mu H^+$, across the plasma membrane of *Sulfolobus* is obviously utilized by a DCCD-sensitive ATP-synthase (see Fig. 4) reminiscent of the well-investigated F_0F_1-type ATPases of bacteria and eukarya. This is an integral membrane complex with a peripheral protein moiety representing the large catalytic head piece, connected to an ion channel by a stalk operating as a conformational energy transducer. It is general conjecture that also archaeal ATP synthases have a corresponding spatial structure.

Further molecular modules of the energy transduction pathway on the level of phosphotransferases are the adenylatekinase, buffering the cellular energy charge, and the cytosolic pyrophosphatase as a thermodynamic sink in coupled activation processes. All three enzyme categories of *Sulfolobus* will be reviewed in the following [1].

5.1. The ATP synthase

Isolated plasma membranes of *Sulfolobus* exhibit an ATPase activity with two widely separated pH-optima of pH 2.5 and pH 6.5, respectively [75,81,82]. These reflect the operation of two independent enzymes with the 'acid ATPase' activity revealing an unspecific pyrophoshatase activity (see below) while the ATPase activity at pH 6.5 belongs to the ATP-synthase complex. The ATPase isolated from *Sulfolobus* sp. *strain 7* [81] (M_r 360 kDa) is activated by sulfate, sulfite or bicarbonate; it is inhibited by NBD-Cl, or nitrate (63% at 20 mM), but not by azide, vanadate, DCCD, or NEM. The membrane-bound ATPase of *S. acidocaldarius* (DSM 639) as well as its isolated form [83,84] is activated by sulfite and in its presence displays a pH optimum of 6.3; the ATPase is also insensitive to azide or DCCD, oligomycin, bafilomycin, or vanadate; it is inhibited by nitrate, *p*-HMB, Mersalyl, and NBD-Cl. The enzyme can be released from the plasma membrane by pyrophosphate treatment [85] and in this form contains 4 polypeptide subunits of 65, 51, 20, and 12 kDa and has a total molecular mass of 380 kDa. The number of isolated subunits reported for solubilized ATPases from archaeal membranes varied between 2 and 7 [86–88]. Presumably this is an accidental finding highly dependent on the isolation conditions and the membrane properties; especially from extreme thermophiles the protein complexes may either display cold-lability and/or certain polypeptides may remain on the membrane. Table 2 compares the subunit composition of *Sulfolobus* ATPase with

[1] Previous communications on '*S. acidocaldarius*' by the group of T. Oshima et al., T. Wakagi et al., and M. Yoshida et al. were conducted with a *Sulfolobus* species '*strain 7* and/or *98-3*' which are very likely not *S. acidocaldarius* but represent a *S. solfataricus* species. All data from our group were obtained with the type strain *S. acidocaldarius* (DSM 639). This explains some of the discrepancies in the literature by confusion of strain terminology until 1994.

Table 2
Subunit composition of *Sulfolobus* ATPase in comparison to other archaeal ATPases

Source	M_r^{app}	M_r of subunits					Ref.
S. acidocaldarius	380	65	51	20	12	–	[84]
S. sp. *strain 7*	390	**66**	**51**	**25**	**13**	(7)	[81,126]
S. solfataricus	370	63	48	24	–	–	[102]
H. salinarum	320	**64**	51	–	–	–	[107,109,266]
H. saccharovorum	350	87	60	29	20	–	[267,268]
H. volcanii	n.c.	**64**	**52**	–	–	–	[111]
H. mediteranei	n.c.	53	49	49	22	12	[269]
M. barkeri	420	**62**	**49**	–	–	–	[270,271]
M. thermophila	540	67	52	37	28	22	[272]
M. tindarius	445	67	52	20	–	10	[106]
M. mazei (Gö1)	> 400	**63**	**51**	**41**	**24**	**12**	[88]
E. coli	382	**56**	**52**	**32**	**22**	**11**	[273]
B. taurus	371	**55**	**51**	**30**	**15**	**5.6**	[274]

The data refer to preparations of the peripheral catalytic portion of membrane ATPase. Molecular masses printed in bold face are derived from the respective DNA sequence; other masses are calculated from migration in SDS gels or from gel exclusion chromatography. '–' respective polypeptide not found; data in parentheses = gene detected but polypeptide not present in preparation. For comparison, data of the two best investigated F_1-ATPases are included on the bottom. n.c. = data not communicated.

Table 3
Catalytic properties of solubilized membrane ATPase from two *Sulfolobus* species

Parameter	*S. acidoc.* DSM 639	*S. sp. strain 7*
M_r^{app}	380	360
Subunit stoichiom.	A_3B_3GD	A_3B_3GD
Substr. specificity	ATP > GTP > ITP > CTP > UTP	GTP > ATP
Rel. activity (%)	100 77 72 37 31	–
Activating cations	$Mn^{2+} >= Mg^{2+}$	Mg^{2+}; –
Anions	SO_3^{2-}	SO_3^{2-}, SO_4^{2-}, HCO_3^{2-}
pH optimum	6.3	5 and 8.5
T-optimum	75–80°C	85°C
Activ. energy E^a	67 kJ/mol	–
Tight nucleotides	1 ADP/mol	–
K_m [ATP-Mg]	180–200 μM	–
n nucleot. sites	6	–
K_{d1} [ADP]	0.2 μM	–
K_{d2} [ADP]	1.8 μM	–
K_m [2-N_3ATP-Mg]	300–350 μM	–
K_{d1} [2-N_3ADP]	1.5 μM	–
K_{d2} [2-N_3ADP]	170 μM	–
Predomin. labelled	subunit A	–
n labels to inact.	3 (extrapolated)	–
n_{max} labelled inhibitors	6	–
	NO_3^- (8 mM)	NO_3^- (20 mM)
	NBDCl (0.22 mM)	NBDCl (1 mM; 90%)
	p-HMB (0.05 mM)	
Insensitive to	N_3^-	N_3^-
	o-vanadate	o-vanadate
		p-HMB, NEM

'–' indicates no data published. Figures in parentheses after inhibitors indicate $[c]_{i50}$ values.
Data are collected from Refs. [61,84,85,99] and [75,81,126]; '–' indicates no data published; figures in parentheses after inhibitors indicate $[c_{i50}]$ values. *Sulfolobus* sp. *strain 7* most likely is a strain of *S. solfataricus*.

preparations from other archaea, and selected examples of bacteria and eukarya.

The molecular properties of the solubilized ATPase from *S. acidocaldarius (DSM 639)* are summarized in Table 3. This is the only *Sulfolobus* ATPase of which data on nucleotide binding and exchange are available. Interestingly the ATPase has 6 nucleotide binding sites; three high affinity and three very low affinity sites [87], reminiscent of typical F_1-ATPases as for example from beef heart or *E. coli* (for review, Refs. [89–93]). Cooperativity of binding between 3 of the sites seems to exist; the degree of cooperativity could be determined with limited precission, however. One has to notice that most of the techniques to study the binding change mechanism and kinetics of nucleotide exchange of ATPases are not applicable at the working temperatures of hyperthermophilic enzymes; on the other hand, these proteins normally acting at $> 75°C$ when transferred to room temperature may undergo conformational changes severely disturbing their ligand binding properties. Of the three tightly binding nucleotides on *Sulfolobus* ATPase, one becomes unexchangable at room temperature [94,95]. It has also been demonstrated that by 2-N_3-ATP mediated photoaffinity labeling the enzyme is irreversibly inhibited; 2-N_3-ATP can covalently bind to all 6 nucleotide sites [96]. The analog serves as a substrate ($K_m = 400\ \mu M$) and competes with ATP for binding. However, it is inserted into both, A- and B-subunits with a preference for the A-polypeptide which in archaeal ATPases is considered the catalytic subunit [2].

Titration of inhibition by 2-N_3-ATP labelling extrapolates to full inhibition when 3 sites/ATPase molecule are labelled; extensive labelling of all 6 sites is possible [96]. Whether or not a rotating or alternating site mechanism is operating as in classical F_1-ATPases [91,97,98] is open because a clear differentiation between catalytic and non-catalytic site labelling was not achieved.

The finding of 6 nucleotide binding sites is in agreement with the determined subunit stoichiometry [85] A_3B_3 of the large polypeptides, which is substantially supported also by the appearance of the *Sulfolobus* (DSM 639) ATPase in high resolution electron micrographs. The large subunits show a characteristic pseudohexagonal arrangement of 6 peripheral globular masses and a smaller central mass, as confirmed for all typical F-type ATPases [99] reflecting the alternating arrangement of α/β subunit pairs exemplified with the chloroplast enzyme [100]. These basic structural elements of F-type ATPases were confirmed by the recently achieved atomic resolution structure of F_1-ATPase from beef heart [101].

A third ATPase was reported from *Sulfolobus* obtained from membranes of the strain *S. solfataricus strain 98/2* [102]. Though with regard to molecular mass (M_r 370 kDa), pH-optimum, NBD-Cl- and nitrate-sensitivity, the ATPase shares most properties with the other two *Sulfolobus* enzymes [81,85], only 3 constituent polypeptide subunits were identified. Differences in anion sensitivity and inhibition by NEM suggest that indeed the three enzymes from various *Sulfolobus* species are largely analogous (and strongly homologous, see below), but reveal individual differences which, however, should be considered irrelevant for their catalytic function.

Despite obvious similarities to F_1-ATPases, the differences of primary structure [103] and altered inhibitor sensitivies suggested a close relation of *Sulfolobus* ATPase to vacuolar V-type ATPases. The essential questions are, however, whether or not the ATPases have an F-type analogous function as ATP-synthases, and what is the structure of the membrane spanning F_0-analogous proton conductor.

The first question can be positively answered. A clear correlation has been found between DCCD inhibition and cellular ATP content (Fig. 4). Moreover, DCCD was shown to inhibit proton backflow into *Sulfolobus* cells after an oxygen pulse; the rate constant is diminished in a concentration-dependent manner, while the initial rate of proton extrusion is increased [76]. In addition a titration of ATP content by increasing ^{14}C-DCCD concentrations was accompanied by successive incorporation of DCCD into a 7-kDa polypepdtide [61] with striking similarity of its amino acid composition to the F_0-subunit c from *E. coli*, or of the thermophilic bacterium *PS3*. From a *Sulfolobus* species '*strain 9*' a gene was cloned encoding the respective subunit of the ATPase [104].

[2] Instead of α, β, γ... as for F-type ATPases, the subunit polypeptides of archaeal ATPase are commonly named A, B, C,.. etc.

After cleaving a 22 residues long presequence from the DNA-derived primary structure, an alignment with the chemically determined partial sequence from the *S. acidocaldaius* (DSM 639) subunit-c [61] shows an 82% identity. The putative DCCD binding glutamate residue is located at the very same position within the hydrophobicity profile as in the respective subunits of known F_0F_1-ATPases [105] and therefore is likely to assume an identical position also within the membrane architecture. Together, the data strongly suggest that the *Sulfolobus* ATPase indeed acts as an ATP-synthase with a perfect functional analogy of the DCCD binding subunit to those from known F_0F_1-complexes. Also from *M. tindarius* a small hydrophobic subunit could be labelled in membranes [106], and from gene analysis an essentially homologous 7 kDa polypeptide is also predicted for *Methanosarcina mazei* [88].

Nevertheless, opposing opinions on the classification of archaebacterial membrane ATPases are based on their chimeric properties shared with both, F-type and V-type ATPases [87]; therefore archaeal ATPases became also named as 'A-type' ATPases [107–109]. The relation to V-type ATPases is documented by the high degree of sequence similarity of the large subunits A and B to those of vacuolar ATPases and has been established when the first genes for *Sulfolobus* ATPase were cloned and sequenced [103,110]. In principle it was also confirmed for other archaeal genera including halophiles [109,111] and methanogens [112]. The ambivalent position between F-, and V-type ATPases is further documented by immunological cross-reactivities. An antiserum against the B-subunit of *S. acidocaldarius* (DSM 639) cross-reacted with the β-polypeptides of F-type ATPases from bacteria, eukaryotic mitochondria, or chloroplasts [84], while antisera against the A-subunit from another *Sulfolobus* species and against V-type ATPase showed mutual cross-reactivities with archaeal and eukaryotic V-type ATPases [113,114]. In addition, labelling studies with NBD-Cl as well as sequence homologies identify the large A-subunit as the catalytic subunit in archaeal ATPases/-synthases (reviewed in Ref. [49]). Regarding the catalytic mechanism, it appears likely that also typical V-type ATPases share a multi-site catalysis with F-type ATPases [115] in harmony with the nucleotide binding data of *Sulfolobus* [87].

Typically F_0F_1-like is the size of the DCCD binding polypeptide as a constituent of the proton path. The respective components of vacuolar ATPases are much larger (16–19 kDa) than mature DCCD binding polyppetides from archaea [61,117] presumably as a result of duplication of an ancestral gene [116].

Though the above whole cell experiments with *Sulfolobus* and a series of studies with ATPase from halophiles [118–122] emphasizes F_0F_1 analogous functions of V-type analogous ATPases in archaebacterial membranes, precaution from generalization appears advisable until further data have been accumulated. On the one hand the discovery of a V-type ATPase in the (eu)bacterium *Th. thermophilus* might change the evolutionary picture [123], on the other a DNA fragment homologous to a classical F_1-type ATPase β-subunit has been found in an archaebacterial genome [124]; however, the respective gene product has not been identified as yet. Moreover, from differential inhibitor studies the simultaneous occurrence of an A-type and an F-type ATP-synthase in a *Methanosarcina* species has been recently reported [125] with the A-type enzyme coupling H^+ transport to ATP synthesis while the F-type ATPase is Na^+-coupled. Preparative and genomic separation of both activities has not been achieved yet but will be extremely important for the understanding of the evolution of ATP synthases. Actually, a possible coexistence of V- and F-type ATPases/-synthases has already been proposed for halobacteria previously [119,120].

Only for *Sulfolobus* (strain 7; presumably a *S. solfataricus* strain) and very recently for *M. mazei* the genomic organization of ATPase subunits is known [88,126]. In *Sulfolobus* the genes are organized in an operon but are arranged in a different manner as compared to *E. coli* as a typical example of F_0F_1-ATP synthases [127,128]. A total of 6 polypeptides is encoded in the order δ, α, β, γ, ϵ, and c; the genes α, β, and c encode the large subunits A, B, and the small DCCD binding subunit, respectively. The other genes (δ, γ, ϵ) encode hydrophilic proteins with 13, 25, and 7 kDa, respectively. The gene product of γ presumably corresponds to the fourth subunit found in solublilized *Sulfolobus* ATPase; the function of the other polypeptides is unknown. No similarity of the latter to F_0F_1-type ATPases could be found. Especially, the hydrophobic

equivalents to the a- and b-subunits of the proton conducting F_0 moiety of bacterial ATP synthases [129] are missing. Thus, the molecular structure of the membrane residing F_0-analogous part of the *Sulfolobus* ATP-synthase remains unsolved at present. This holds equally for all other archaeal organisms; in fact, neither from halobacteria nor from methanogens an ATPase-operon has been fully sequenced, and therefore the total number of polypeptides of other archaeal ATP-synthases cannot be deduced.

Unfortunately all attempts to isolate an integrated F_0F_1-analogous complex from *Sulfolobus* failed (and were obviously abandoned) due to difficulties to resolve the rigid structure of the *Sulfolobus* cell membrane without desintegration of the complex.

5.2. Adenylatekinase

Adenylatekinases are essential to sustain life functions and may be regarded as truly 'archaic' enzymes; the activity serves as a fundamental catalyst for the operation of an energy buffer within the adenine nucleotide pool [130]. This is facilitated by the low specificity for nucleotide triphosphates as phosphate donors.

The enzyme from *Sulfolobus* was the first adenylate kinase from archaea to be purified to homogeneity [131] and to be fully sequenced [132]. The 22 kDa polypeptide is likely to form a catalytic dimer in solution and exhibits a temperature optimum of about 90°C. The pH optimum of 5.5–6.0 reflects the cytosolic pH of *Sulfolobus*. It is absolutely specific for AMP as phosphate acceptor with nearly identical K_m values for ATP, ADP, and AMP of $0.6–0.7 \times 10^{-3}$ M. Distinct from mammalian enzymes, its sensitivity towards the inhibitor diadenosine-5,5′-pentaphosphate is very low ($c_{I50} > 300\ \mu M$).

Sequence comparisons of the 'archaic' adenylate kinase with the catalytic subunit of the respective ATP-synthase were of interest due to a previous proposal predicting an adenylate kinase-like structure within the catalytic core of the latter [133], implying a common evolutionary origin. Such similarities could not be found, however, and are also disproved by the atomic 3-D structure of the bovine F_1-ATPase [101]. Similarity dendrograms of adenylate kinases and phylogenetic trees based on their amino acid sequence clearly split off the adenylate kinases from the clusters of F- or V-type ATPases, even if only the region of nucleotide binding domains are aligned.

Interestingly a slightly closer similarity of *Sulfolobus* adenylate kinase to eukaryotic enzymes than to prokaryotic enzymes was recognized within a second homology box [132] besides the consensus motif common to all adenylate kinases, respectively to adenine nucleotide binding proteins exhibiting the so-called P-loop [134].

The N-terminal sequences of four other archaeal adenylate kinases from deep sea methanogenes were recently determined; all are members of the genus *Methanococcus*. Fig. 5 compares the canonical P-loop motif of adenylate kinases, demonstrating that this location next to the N-terminus is conserved throughout all ur-kingdoms. The motif is known to serve as part of the catalytic fold hosting the pyrophosphate moiety [135,136]. Archaea display two significant deviations from the consensus **GxPGxGK*G*T**: one is the replacement of the glycin adjacent to the conserved lysine by a second hydroxylated residue of either serine (in *Sulfolobus*), or threonine (in the methanogens); the other is the absence of this very lysine in the methanogens which is highly conserved in all other adenylate kinases including the archaeon *Sulfolobus*. As it represents an essential residue within the phosphate binding region the lack of lysine might be compensated for by another basic residue from

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S.acidoc. -------MKIGIVTGIPGVGKSTVLAKVKEILDNQGINNKIINYGDFMLAT
E.coli    -------MRIILL-GAPGAGKGT---QAQFIMEKYGIPQ--ISTGD-MLRA
P.denitr. ------AINIILL-GPPGAGKGT---QARRLIDERGLVQ--LSTGD-MLRE
S.cerev.  -MSSSESIRMVLI-GPPGAGKGT---QAPNLQERFHAAH--LATGD-MLRS
My.capr.  -------MNIMLL-GAPGCGKGT---QAEQLVNKLNFIQ--VSTGD-LMRK
Sus scr.  MEEKLKKSKIIFVVGGPGSGKGT---QCEKIVQKGYYTH--LSTGDLLRAE
H.sapiens MEEKLKKTKIIFVVGGPGSGKGT---QCEKIVQKYGYTH--LSTGD-LLRS
Mc.volta. -----MKNKVVVVTGVPGVG-STTSxQLAMDNLRKEGVNYKMVS....
Mc.therm. -----MKNKLVVVTGVPGVG-GTTIT......
Mc.jann.  -----MKNKVVVIVGVPGVG-STTVxNKAIEELKKE....
Mc.igneu. -----MKNKVVVITGVPGVG-GTTxLQKTIEKLKEE....
```

Fig. 5. Alignment of the N-terminal sequences of *Sulfolobus* adenylate kinase [131,132] with partial sequences from methanogenic archaea [262] and selected examples from bacteria and eucarya. The essential signature of the P-loop [134] contributing to the nucleotide binding site is conserved throughout all phylogenetic domains. Data other than *Sulfolobus* or *Methanococcus* are derived from the EMBL data bank. From the 4 *Methanococcus* species only the above N-terminal stretches are known. The abreviations assign the following organisms: *S. acidocaldarius* (DSM 639), *E. coli*, *P. denitrificans*, *Sacch. cerevisiae*, *Mycoplasma capricolum*, *Sus scrofa* (pig), *Homo sapiens*, *Methanococcus voltae*, *-thermolithotrophicus*, *-jannashii*, and *-igneus*.

outside the P-loop domain in adenylate kinase of the methanogens.

Despite identical functions and comparable molecular size the over-all sequence similarities between archaeal, bacterial, and eukarial adenylate kinases are only limited; this is also expressed by immunological data [132]. A polyclonal antiserium against *Sulfolobus acidocaldaius* adenylate kinase neither cross-reacts with other archaeal, nor with bacterial and eukaryal cell extracts; even not with the close relative *Sulfolobus solfataricus*.

The function of adenylate kinase in *Sulfolobus* is depicted in Fig. 4 (bottom right) where the ATP-level is partially restored after completely arresting its synthesis by oxidative phosphorylation. The increase in ATP, however, is only transitory, demonstrating that its fermentative generation is not possible in *Sulfolobus acidocaldarius*. The data are supported by analysis of all parameters contributing to the energy charge [61].

Recently we succeeded in crystalizing the *Sulfolobus* enzyme; the crystals diffract to a resolution of 2.6 Angström [H. Bönisch, C. Vonrhein, G. Schäfer, G.E. Schulz (1996), unpublished] and exhibit the space group $P2_12_12_1$ with $73 \times 146 \times 172$ A as assymetric unit. Its extreme thermostability has been investigated by various spectroscopic techniques including FTIR [137], demonstrating that the T_m of thermal denaturation is above 100°C. Nevertheless, the long-term heat tolerance of the enzyme ($T > 80$°C) is much less than the doubling time of *Sulfolobus* cells, suggesting a rather high turnover of the protein in vivo.

5.3. Pyrophosphatases

Bioenergetic mechanisms indispensibly involve the hydrolytic cleavage of pyrophosphate as an equilibrium trap to pull various metabolic activation reactions in the direction of product formation.

$$NTP + X \rightleftharpoons NMP - X + PP_i \quad (1')$$

$$PP_i + H_2O \rightleftharpoons 2P_i \quad (2')$$

Reaction (2′) is catalyzed by cytosolic inorganic pyrophosphatases. In fact, deletion of the gene for inorganic pyrophosphatase is incompatible with life as shown with *E. coli* cells [138]. The importance of inorganic pyrophosphate as a possible premordial energy source has been discussed in a number of reviews [139–141]. Actually, inorganic pyrophosphate is capable of functioning as a biological energy donor in various microbial phosphorylation reactions [142,143]. It may serve as an energy store in the form of inorganic polyphosphates which have also been detected in archaebacteria [144]. In *Sulfolobus acidocaldarius* polyphosphate kinase has been detected, though the metabolic role of polyphosphate has not been further elucidated [54]. Besides an inorganic energy store, it may mainly serve as a store of phosphate in the low salinity habitat of *Sulfolobus*. The actual concentration of inorganic pyrophosphate in *Sulfolobus* is not known; though the concentration appears to be usually low, bacterial cells may contain up to about 500 mM [145].

Sulfolobus exhibits a membrane-bound as well as a cytosolic pyrophosphatase activity. The respective enzymes have been characterized from two species, *S. acidocaldarius* [146,147] and *Sulfolobus* sp. *strain 7* [148,149].

(a) Membrane residing pyrophosphatases have been identified long ago from *Rh. rubrum* as energy converters coupling pyrophosphate bond formation with the electrochemical proton potential cross the plasma membrane [150,151]. Also from plant and yeast vacuolar vesicles H^+-translocating pyrophosphatases were described (reviewed in Refs. [139,152] Phil. Rea TIBS Sept.92). The membrane associated pyrophosphatase of *Sulfolobus*, however, is not involved in ion translocation. The enzymes from two *Sulfolobus* species were identified as ecto-enzymes with extremely low pH-optima of 3 [153], and 2.5 [149], respectively; their molecular mass was reported as 35 kDa (strain 7) and 70 kDA (*Sulfolobus* DSM 369). The latter enzyme is a homooligomere of a 17–18 kDA polypeptide whose N-terminal sequence has been determined [146]. Both enzymes require no divalent cations for activity and exhibit a broad unspecificity against pyrophosphate bonds, also hydrolyzing ADP and ATP. Actually, their presence in membranes accounts for the observed second pH-optimum of membrane bound ATPase activity at pH 2.5–3 [75,82]. A physiological function in mediating phosphate supply from pyrophosphate is unlikely in the natural habitat of Sulfolobales; also, a presumed induction by PP_i could not be demonstrated [153].

However, from our observation on inhibition of *Sulfolobus* cell growth by the antibiotic bacitracin [146], it becomes likely that the enzyme plays a significant role in bioenergetics and mechanism of cell wall biosynthesis acting as a dolichol pyrophosphatase. It could be shown that bacitracin indeed inhibits membrane PPase activity [W. Meyer and G. Schäfer, unpublished (1993)].

(b) The cytosolic energy dissipating pyrophosphatase was first characterized from *Sulfolobus strain 7* as homotetramer of a 21-kDA polypeptide. The enzyme exhibits an extraordinary stability against denaturating agents; its pH optimum is well within the cellular pH of 6.5 [149]. The enzyme from *S. acidocaldarius* (DSM639) has similar catalytic properties and an absolute requirement for Mg^{2+} cations. It is a homotetramer of a 19.365 kDA polypeptide, the gene of which could be cloned and sequenced [147]. A distinct pattern of invariant amino acids has been identified to be strictly conserved in cytosolic inorganic pyrophosphatases [154]. All these amino acids essential for binding of Mg-ions and pyrophosphate [154] are present in their respective distance and positions in the *Sulfolobus* enzyme. The sequence can be aligned very well with those from the aerobic archaeon *Th. acidophilum* [155], also determined in our laboratory, and the thermophilic bacterium *Th. thermophilus* [156]. Despite alignments almost without gaps, the finding is surprising that no immunological cross-reactivity of the antiserum against the *Sulfolobus* PPase was found; not even against other archaea, except for a weak signal with *S. solfataricus*. It reiterates the situation with adenylate kinase from *Sulfolobus*. Based on the known 3-D-structure of PPase from *Th. thermophilus*, a model of *Sulfolobus* PPase was constructed which by energy minimization yielded a stable structure extremely close to the former. Though this theoretical structure has to be verified by X-ray crystaloghaphy, it illustrates how diverse primary structures may produce analogous folding patterns and enzymatic properties. Crystalization of the *Sulfolobus* pyrophosphatase has already been successfully performed [Th. Schäfer and G. Schäfer, unpublished (1996)].

The phylogenetic origin of cytosolic and membrane-bound pyrosphophatases is apparently different as is the catalysis of pyrophosphate hydrolysis [154]. The primary structure of the cytosolic *Sulfolobus* enzyme is the 'oldest' example with regard to the universal tree of organisms and thus may reflect an image of the 'ur'-PPase.

6. Redox and electron transport components

6.1. Oxygen respiration

Though all essential components of the electron transport system of *Sulfolobus* were identified and most have been characterized in great detail no homogeneous data-set on direct respiratory measurements exists. Especially there is only poor information on the turnover of membrane-bound electron transport in terms of oxygen consumption. In addition, available data from various laboratories have been determined under widely different conditions with respect to temperature and pH. Of course, one reason is experimental difficulties occuring with measurements near the optimum living temperature of *Sulfolobus* (75–85°C); chemical side reactions of electron donors, autoxydation, and thermal instability are severe obstructions superimposing enzymatic catalysis. The following data are normalized to 60°C assuming standard temperature dependence for calculation.

Early reports on NADH oxidation rates [75] of *Sulfolobus* sp. *strain 7* plasma membranes, determined at 30°C, would account for 8–16 nmol O_2/min/mg protein, an extremely low value. Also a cyanide-and azide-sensitive cytochrome-*c* oxidase activity of 0.15 μmol/min/mg (30°C) was reported which would account for 0.336 μmol O_2/min/mg (60°C). But later reports give a calculated activity of membranes of only 82 nmol O_2/min/mg [157]; in all cases rates were determined spectrophotometrically and not by oxymetry. The activity with horse heart cytochrome is surprising and presumably artifactual because naturally *Sulfolobus* does not contain any *c*-type cytochromes. The activity has also been observed with *Sulfolobus acidocaldarius* membranes [73] but disappears with progressive purification of the terminal oxidase [158].

Resiratory rates of isolated membranes with succinate (5 mM at 60°C) are 10–15 nmol O_2/min/mg [159].

Our measurements with intact cells revealed a steady-state respiration on endogenous substrate of

13.5 ± 2.9 nmol O_2/min/mg (60°C) and 'burst' respiration – the initial rate after oxygen pulses to anerobic cells – of 25–32 nmol O_2/min/mg [74,76]. In harmony with observations by others [75,160], it was established that the *Sulfolobus* respiratory system as a whole differs from known pro- and eukaryotic respiratory chains by its insensitivity against specific inhibitors of complex-I and complex-III. As a remarkable difference between related *Sulfolobus* species respiration of *Sulfolobus* sp. *strain 7* was shown to be totally cyanide- and azide-senitive, whereas *Sulfolobus acidocaldarius* shows only partial inhibition even at about 5 mM cyanide [74]. Also full reduction of membrane residing cytochromes could not be achieved by this concentration of cyanide. It was concluded that *S. acidocaldarius* membranes contain a branched electron transport pathway with differential inhibitor sensitivity of terminal oxidases.

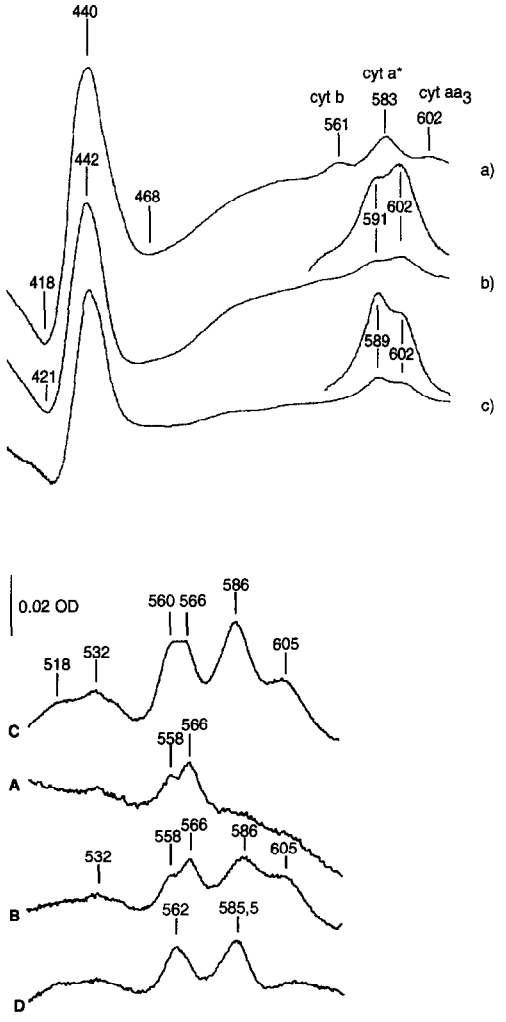

Fig. 6. Difference spectra identifying the hemoproteins in membanes from various Sulfolobales. Top: reduced *minus* oxidized difference spectra of (a) *S. solfataricus*, (b) *Desulfurolobus ambivalens*, and (c) as (b) plus 1 mM cyanide; α-bands of (b) and (c) also amplified 5×; from Schäfer et al. [161]. Bottom: α-band spectra of *S. acidocaldarius* (DSM 639) membranes as produced by differential reduction. (A) ascorbate reduced *minus* oxidized; (B) as (A) plus cyanide (1 mM); (C) fully reduced by dithionite; (D) difference spectrum (C *minus* B), indicating the reduction of cytochrome b^{562} and cytochrome a^{587}-II by full anaerobiosis (for discussion see Section 6.7).

In line herewith are the spectral characteristics of membranes from different *Sulfolobus* species. Fig. 6 demonstrates a significant difference in relative abundance of *b*-type and *a*-type cytochromes between *S. acidocaldarius* and *S. solfataricus* (which is representative for *Sulfolobus* sp. *strain 7*) also indicating typical differences in absorption maxima in the Q-band region. The distantly related *D. ambivalens* exhibits practically no *b*-type cytochrome and shows an unusual appearance of its a-type terminal oxidase bands. Of special interest is the differential reducibility (cyanide sensitivity) of hemes in *S. acidocaldarius*. An unusual *b*-type cytochrome (cf. Section 6.7) can already be reduced by ascorbate; cyanide causes reduction of a large fraction of *a*-type cytochromes; only by anaerobiosis (dithionite) full reduction is achieved. Subtraction of spectra at different reduction states reveals the existence of an additional *b*-cytochrome and of two species of cytochromes obviously contributing to the absorption at 586–587 nm [161]. Neither protein-chemical nor spectroscopic evidence was obtained for *c*-type cytochromes to occur in *Sulfolobus*, whereas it can be present in other archaeal genera like halobacteria or methanogens [15]. The presence of NADH- and succinate-dehydrogenases ensued from respiratory experiments; other components, like blue copper proteins [162], were predicted on genetic basis. In the following the constituent components of electron transport will be reviewed individually.

6.2. Ferredoxin

Ferredoxins are present in all archaeal genera and orders. In certain sulfur metabolizing archaea the

enormous abundance of ferredoxin allows its direct EPR-spectroscopic investigation in whole cells [163]. Ferredoxins assume unusual functions in archaea as they serve as coenzymes for dehydrogenases which in higher organisms use pyridine nucleotides (cf. Section 3). Besides 2-oxoacid:ferredoxin oxidoreductases [63–65,164] aldehyde:ferredoxin oxidoreductase [165,166] and glyceraldehyde-3-phosphate dehydrogenase [167] are other examples. This may be a hint to the use of iron-sulfur clusters as premordial redox systems in early evolution as has been proposed theoretically [168].

Ferredoxin from *Sulfolobus acidocaldarius* has been isolated and sequenced from autotrophically [67] or heterotrophically growing [169] cells. It contains 7 iron atoms in a 10.9 kDa polypeptide which is hosting two iron-sulfur clusters, one [4Fe-4S] and one [3Fe-4S] cluster. Ferredoxin from *Sulfolobus* sp. *strain 7* has been crystalized [170]; though diffraction to a resolution of 2 Å was reported, no 3-D structure has appeared as yet. Also from other archaeal ferredoxins no 3-D structures are known; only partial structures have been resolved by ^{1}H-NMR of 3Fe- and 4Fe-clusters in single cluster type ferredoxins from hyperthermophilic archaea and *Th. maritima*, respectively [171–173]. By ^{1}H-NMR analysis of ferredoxin from the sulfur metabolizing archaeon *D. ambivalens*, a member of the *Sulfolobaceae*, evidence was provided for a close proximity of both iron-sulfur clusters indicating structural similarity to other dicluster ferredoxins [173,174].

Ferredoxins of both investigated *Sulfolobus* species, *S. acidocaldarius* and *Sulfolobus* sp. *strain 7*, share many properties with that from *D. ambivalens* [163], such as the redox potentials of the $[3Fe\text{-}4S]^{1+/0}$ and the $[4Fe\text{-}4S]^{2+/1+}$ clusters; the potentials of the latter were found to be extremely low (< -500 mV). Regarding ferredoxin from *Sulfolobus* sp. *strain 7* 1 aspartate and 7 cysteins as possible ligands to the FeS clusters were postulated on a sequence basis and from EPR spectroscopy [175]. Cyclic voltametry revealed a half-reduction potential of -280 mV for the $[3Fe\text{-}4S]^{1+/0}$ cluster and -530 mV for the $[4Fe\text{-}4S]^{2+/1+}$ cluster.

More recent physicochemical analysis of ferredoxin from *S. acidocaldarius (DSM639)* revealed three redox couples in cyclic voltametry at pH 6.4 and 0°C [176]; half reduction potentials of -275, -529, and -660 mV were found. These potentials shifted in parallel by 20–30 mV to more positive on raising the temperature to 60°C. Using sensitive techniques of film voltamography and controlled electrolysis identified the couples of -275 and -529 mV as one-electron processes while the -660 couple is consistent with a two-electron reaction analogous to that observed in some other proteins containing [3Fe-4S] clusters [177]. By combination of EPR- and MCD-spectroscopy two redox-dependent protonation equilibria were detected with $pK_a = 5.81$ and $pK_a = 8.9$, respectively. The former corresponds to the transition between the reduced species $[3Fe\text{-}4S]^0$ and $[3Fe\text{-}4S]^0H^{1+}$ and, interestingly, exactly meets the intracellular pH of *Sulfolobus* [61,76], suggesting a physiological role. This protonation equilibrium is strongly reflected by the shape of the low-temperature MCD spectra. It has been speculated that it may directly affect the electron transfer properties of ferredoxin and its interactions with partners in the electron transfer chain [176]. Thus, the $[3Fe\text{-}4S]^{1+/0}$ cluster is likely to serve as the physiological electron acceptor in *Sulfolobus* ferredoxin as also supported by studies with *Sulfolobus* sp. *strain 7*, indicating that only the [3Fe-4S] cluster is reduced under steady-state electron turnover with pyruvate dehydrogenase at physiological pH [175]. Analogous observations were reported for the [3Fe-4S] cluster of *D. ambivalens* ferredoxin [163].

In contrast, the physiological function of the [4Fe-4S] cluster is still under debate; it might assume merely a structural role as proposed for endonuclease-III of *E. coli* [178]. At very low potentials ($\sim$ -700 mV), a spin-spin interaction between the two paramagnetic centres ($[3Fe\text{-}4S]^0$ and $[4Fe\text{-}4S]^{1+}$) can be observed, indicating a very short inter-cluster distance as also concluded for *D. ambivalens* ferredoxin [174].

The electron acceptor for the reduced *Sulfolobus* ferredoxin is not identified. From *Sulfolobus* sp. *strain7*, however, a ferredoxin reoxidizing iron-sulfur flavoprotein 'IFP' has been described [66] as a dimer, composed of three polypeptides (87, 32, and 22 kDa), containing two FMN ($E_m = -57$mV) and two plant-ferredoxin-type [2Fe-2S] centres ($E_m = -260$ mV; $g_{xyz} = 2.91;1.95;1.90$). From the redox potentials an electron path was proposed as ferredoxin → IFP [2Fe-2S] → FMN. Details of its physiological function re-

main to be elucidated. It has to be mentioned that also ferredoxin:NAD(P) oxido-reductase activities have been found in soluble fractions of *Sulfolobus acidocaldarius* cells (Hettmann and Schäfer, unpublished) [69]. These are connecting the reducing equivalents of the large ferredoxin pool to biosynthetic pathways as well as to respiratory electron transport via the NAD(P) pool. Isolated 'IFP', however, did not react with pyridine nucleotides [66] and thus may be a fragment of a still unresolved electron transport pathway.

6.3. NADH dehydrogenase

Though membranes of *Sulfolobus* show some NADH-oxidizing activity, an integrated NADH-dehydrogenase complex could not be demonstrated as yet. As a general experience the NADH oxidizing capacity obviously dissociates easily from the membrane by various mild treatments (pH changes, salinity changes, low temperature, etc.). The remaining activity is completely insensitive towards rotenone, amytal, piericidine, antimycin-A (author's observation, unpublished), but can be totally inhibited by cyanide or azide in *Sulfolobus* sp. *strain 7* membranes [75], whereas only partial inhibition is achieved by 1–3 mM cyanide in *S. acidocaldarius* (DSM 639) membranes [74]. Under anaerobic conditions the cytochrome pool in the membranes is completely reduced by NADH. Thus, the latter experiments clearly suggest an electron-flow from NADH through the respiratory carriers to the terminal oxidases. However, a NADH:coenzyme-Q reductase activity was never demonstrated directly. Though *Sulfolobus* does not contain any *c*-type cytochrome, an artificial NADH:cytochrome-*c* reductase activity of purified membranes from *S. acidocaldarius* can be measured (unpublished observations of the author, 1987). This activity is also insensitive to typical complex-I inhibitors.

From *Sulfolobus* sp. *strain 7* a NADH-dehydrogenase was purified and characterized which uses 2,6-dichlorphenol-indophenol or ferricyanide as an electron acceptor. This activity is largely found in the soluble fraction; the minor membrane-bound activity is completely released by 0.5 M KCl [179]. The purified enzyme consists of two ~ 50 kDa subunits and has a native M_r of 95 kDa; it was clearly identified as a flavoprotein containing presumably 2 FAD/mol. At present one has to assume that it represents the entry point for reducing equivalents from NADH into the respiratory chain as supported by its very weak activity also with caldariella quinone [179]. Very likely, additional subunits necessary for full quinone-reductase activity may be left in the membrane during dissociation of the peripheral flavoprotein moiety. However, no sequence- or genetic data supporting this hypothesis have become known so far.

From our studies on inhibition of cell respiration by acridone carbonic-acid derivatives [179a], it is concluded that *Sulfolobus* has a NDH-II NADH-dehydrogenase not capable of energy coupling. Support comes from the complete absence of all EPR detectable FeS-clusters typical for energy transducing NDH-I complexes (Anemüller, 1995, personal communication). Moreover, none of the available hybridization probes against components of pro- or eukaryotic NDH-I genes produced a signal with DNA from *Sulfolobus acidocaldarius* (Steinmüller, 1994, personal communication).

In a comparative review [161] it is concluded that archaeal plasma membranes in general are lacking a complex-I equivalent NADH dehydrogenase; the conclusion has to be validated, of course, by further experiments.

6.4. Succinate dehydrogenase

Respiration of *Sulfolobus acidocaldarius* cells can be stimulated by succinate [74], and purified membranes show succinate oxidase activity (11–13 nmol O_2 min^{-1} mg^{-1}; 60°C) which is partially cyanide-resistant [159]. Treatment with 20 mM pyrophosphate releases a soluble succinate:DCPIP/PMS-dehydrogenase, an activity which is also found in supernatants of *Sulfolobus* cell disruption, suggesting that a peripheral catalytic portion of an SDH complex is easily dissociable. By detergent extraction a succinate dehydrogenase complex composed of 4 polypeptides can be prepared from *S. acidocaldarius* membranes. The native molecular mass of ~ 141 kDa corresponds to an equimolecular stoichiometry of the subunits a, b, c, d of 66 kDa, 31 kDa, 28 kDa, and 12.8 kDa, respectively. The size of the large subunits corresponds to those of succinate dehydrogenases

from eukarya and bacteria [180]. The complex contains acid labile sulfur and iron, and the 66 kDa polypeptide carries 1 covalently bound FAD causing a broad 445 nm absorption band in (oxidized-reduced) difference spectra. The thermostable enzyme has a temperature optimum of 81°C and a pH optimum at 6.5 coinciding with the intracellular pH of *Sulfolobus*. No cytochrome is present in the complex. With caldariella quinone, the putative natural electron acceptor in the membrane, only about 1% of the activity with dyes is obtained; this might result from damage of one of the iron-sulfur clusters which can be identified by EPR spectroscopy. As expected for SDH the resonances of the S1, S2, and S3 clusters types were clearly identified in the solubilized complex and in membranes [181]. Only the S1 [2F2-2S] and S3 [3F3-4S] clusters are reducible by succinate, while the typical S2 [4Fe-4S] signal is produced by dithionite.

An antiserum against the FAD bearing 66 kDa subunit cross-reacts with 66–77 kDa polypeptides from membranes of *Sulfolobus solfataricus* and *Thermoplasma acidophilum*. Interestingly the membrane of the latter contains unusually high amounts of SDH activity allowing to investigate the succinate dehydrogenase complex by EPR spectroscopy in situ. All characteristic iron-sulfur clusters S1 [2Fe-2S], S2 [4Fe-4S], and S3 [3Fe-4S] were also identified as constituent components [182].

Very recently the genes of the *Sulfolobus acidocaldarius* (DSM 639) succinate dehydrogenase complex were localized and cloned (S. Jansen, 1996, personal communication), identifiying the 31-kDa polypeptide (subunit b) as the iron-sulfur protein; the gene organization of the operon resembles known prokaryotic succinate dehydrogenases.

Also from *Sulfolobus* sp. *strain 7* a complex-II equivalent has recently been purified and character-

Table 4
Properties of Complex-II

Parameter	*Sulf. acidocald.* (DSM 639) [68,181]	*Sulf.* sp. *strain 7* [183]	Beef heart mitochondria [275]
n subunits	4	4	4
M_r	138	148	131
M_r subunits	66; 31; 28;12.8	66; 37; 33; 12	74; 26; 15.8; 14.9
Flav. nmol/mg	4.6	5.6	5.9–6.2
λ_{max} nm	455 (ox-red)	445 (66kD pept.)	–
Fe cont. nmol/mg	102	83	52–54
S^0 cont. nmol/mg	150	–	48–50
Cytochrome *b*	none	none	1.5–2
K_m (succ.)	1.4 mM	0.28 mM	20 μM
K_m (DCPIP)	65.4 μM	89 μM	–
K_m (Q^{cal})	–	60 μM	o.5 μM (Q_2)
V_{max} μmol/min/mg	7.8 (55°)	13 (50°)	54 (accept.)
	–	14 (Q_1)	–
	–	1.8 (Q^{cal})	–
Turnover [s^{-1}]	154 (81°)	–	150
EPR: (S-3) oxid.	g = 2.02	g = 2.02	g = 2.01
	g = 2.08 (satel.)	–	–
(S-1) succ. reduced	g_z = 2.05	g_z = 2.03	g_z = 2.03
	g_y = 1.935	g_y = 1.94	g_y = 1.93
	g_x = 1.904	g_x = 1.90	g_x = 1.91
pH optimum	6.5	6.5–6.8	–
E^a kJ/mol	59–64	–	–
K_i malonate	3.1 mM	–	–
Oxaloacetate	o.28 mM	–	–
TCBQ	1.5 μM	–	–

Comparison of succinate dehydrogenase (complex-II) from two *Sulfolobus* species with the mitochondrial complex-II.

ized. It essentially parallels the complex from *S. acidocaldarius* (DSM 639) with subunits of 66, 37, 33, and 12 kDa and does not contain cytochrome [183]; the 66 kDa subunit contains covalently bound flavin; the 37 kDa polypeptide is supposed to carry the three FeS clusters found in the holo-enzyme. The latter complex, like that from *S. acidocaldarius*, can use DCPIP as electron acceptor without any other mediator but has the advantage to transfer electrons also to caldariella quinone (see below) with significant rates. This enabled the functional reconstitution in detergent micells of a succinate oxidizing respiratory chain together with the terminal Q-oxidase complex of *Sulfolobus* sp. *strain 7* [160]. Thus, it is suggested that succinate dehydrogenase complexes of *Sulfolobus* represent fully functioning complex-II equivalents in the respiratory chain.

From EPR monitored reduction of the iron-sulfur centres by succinate it is concluded that centres S1 and S3 are directly involved in intramolecular electron transfer [183] similar as in *S. acidocaldarius* and the *T. acidophilum* complex of which the half-reduction potentials have been determined (+68 mV and +60 mV, resp.) [182]. Together with a stable radical feature in the archaeal complex-II, a 'linear-sequence model' for electron flow is proposed for *Sulfolobus* sp. *strain 7* [183]. Interestingly, this might explain the strong inhibition of *Sulfolobus* succinate dehydrogenase by 2,3,5,6-tetrachloro-benzo-quinone [159], a compound which forms a stable radical under the applied conditions. The function of the low potential S2 centre (−210 mV in *T. acidophilum* [182]) remains to be clarified. A close proximity of S1 to S2, as envisaged by spin-spin interaction [182], may affect the rate of electron transfer between FAD and the quinone pool, however. In Table 4 the properties of Sulfolobus complex-II are compiled in comparison to mitochondrial SDH complexes.

6.5. The quinone pool

Respiratory quinols are functioning as lipid soluble hydrogen stores and redox mediators which in most known respiratory chains are present in large excess. An exception are sulfur-reducing respiratory pathways [184] which appear to be independent of quinones.

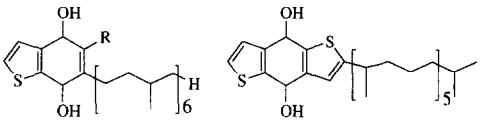

Fig. 7. Structure of the reduced thiopheno-benzo-quinones acting as respiratory quinols in *Sulfolobus* plasma membranes. left: R = H: Sulfolobus quinol, R = CH_3-: Caldariella-quinol; right: tricyclic quinol.

The unique isoprenoid quinones of *Sulfolobus* and related archaea from the sulfur metabolizing acidophilic branch are derivatives of benzo-[b]-thiophen-4,7-quinone. All are bearing a C_{30} isoprenoid saturated side chain. First described in a strain called '*Caldariella acidophila*' [185], the novel 'caldariella quinone' was detected in membranes of all other Sulfolobales as a major constituent [186]. As shown by the structures in Fig. 7, a few variants of the core quinone were described [187,188] of which the tricyclic quinone (2-polyprenyl-benzo(1,2-b;4,5-b')dithiophene-4,8-quinone) is present in traces only, while Sulfolobus-quinone and caldariella-quinone may vary with growth temperature and other growth conditions [187]. The quinones represent 0.28–0.38% of cell dry weight. Caldariella quinone varies between 60–99% of total quinones in *S. solfataricus*. Interestingly, on transition from aerobic to anaerobic growth the ratio between caldariella- and Sulfolobus-quinone is reversed in *D. ambivalens*.

The electrochemical properties were investigated in detail only of caldariella quinone extracted from *S. acidocaldarius* [158]. Stabilized in Triton micelles at ambient pH 6,5 and 25°C, it has a half-reduction potential of +100–106 mV. The oxidized to reduced transition induces in difference spectra a minimum at 351 nm and a maximum at 325 nm with an isosbestic point at 340 nm ($\epsilon 351-341 = 1.77 \times 10^3$ M^{-1} cm^{-1}). Using these wavelengths the activity of caldariella quinol oxidases can be conveniently measured. However, at temperatures above 60°C and pH above 6.5–7, a considerable autoxidation rate is observed.

6.6. Rieske FeS proteins

All respiratory chains of higher organisms contain Rieske iron-sulfur protein as constituent of their complex-III, the cytochrome-*c*:coenzyme-Q-reductase

[189,190]. Rieske FeS clusters are unique [2Fe-2S]-clusters different from the ferredoxin-type cluster by their *g*-factor ($g_{av} = 1.9–1.91$) and their positive redox potential between +150 to +300 mV. The first hint to a Rieske-type cluster in *Sulfolobus* was found by EPR investigation detecting the indicative g = 1.89 signal in a crude membrane preparation from *Sulfolobus acidocaldarius* (T. Ohnishi and G. Schäfer, 1990, unpublished). Convincing evidence was provided later, demonstrating in the plasma membrane a true respiratory Rieske FeS-cluster with $g_z = 2.031$, $g_y = 1.89$, and $g_x = 1.725$ (rhombicity 0.37) [16,191]. The redoxpotential at 80°C of +325 mV shows a significant pH dependency between pH 6.7 to 8.2, and the FeS centre is reducible by ascorbate, NADH or succinate in respiratory inhibited membranes. It differs from known Rieske centres as the band shift normally produced by the inhibitor DBMIB is absent. Since *Sulfolobus* does not contain *c*-type cytochromes, and thus no *bc*1 complex, novel functional associations had to be assumed.

In membranes of *Sulfolobus* unequivocal evidence for the occurrence of two redox dependent protonation equilibria of the Rieske centre was reported with pK^{ox} values of 6.2 and 8.5 [192]. Both pK values are lower than that reported for the 'classical' Rieske FeS protein from bovine heart [193] and other bacterial sources [194]. A physiological role may be envisaged as the lower pK_a falls close to the interacellular pH of *Sulfolobus*. From spin quantification an abundance of the Rieske centre > 1/cytochrome aa_3 emerged which can now be explained by the unique case of the existence of two different respiratory Rieske proteins in *Sulfolobus acidocaldarius* [195,196].

A Rieske protein purified from *S. acidocaldarius* membranes to homogeneity (now termed SoxL gene product, see below) displays *g*-values of the [2Fe-2S] cluster of $g_{xyz} = 1.768$, 1.895, and 2.035, respectively [197]. In contrast to other purified Rieske proteins, it exhibits ubiquinol:cytochrome-*c* reductase activity in absence of any *b*-type cytochrome with a pH optimum at high temperature close to the cyosolic pH of *Sulfolobus* [197]. This activity is observed with various *c*-type cytochromes and cytochrome-*f*. Its N-terminal sequence differs, however, from that of another *Sulfolobus* Rieske protein identified as component of the alternate terminal oxidase complex,

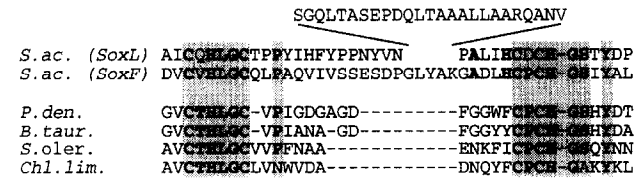

Fig. 8. Alignment of the iron-sulfur cluster binding sites of both *Sulfolobus* Rieske [2F e-2S] proteins (SoxL and SoxF) with selected examples of Rieske proteins from 'classical' bc_1- or b_6f complexes. For details see [196] and text. Abbreviations assign: *Sulfolobus acidocaldarius* DSM 639 (S. ac.); *Paracoccus denitrificans* (P. den.); *Bos taurus* (bovine mitochondrial enzyme); *Spiol oleracea* (S. oler.); *Chlorobium limnicola* (Chl. lim.).

SoxM, of *Sulfolobus* [198,199]. Since the gene-derived sequences are available [161,162] definite proof exists for two different Rieske FeS proteins in the plasma membrane of an individual archaeal organism [194]. In Fig. 8 a partial alignment of the sequences around the FeS-cluster forming sites is given. The mutual identity of both Rieske proteins is only about 29% based on alignment of their total sequences. The two gene loci are different in that the SoxF Rieske protein is coded for within the gene cluster of the alternate oxidase [162] while the gene for the SoxL Rieske protein is not associated with genes of an oxidase complex [161]. Its functional association in vivo has still to be shown. However, both Rieske FeS proteins are constitutively expressed in heterotrophically growing *Sulfolobus acidocaldarius* but may have different biosynthetic pathways as the SoxL Rieske is bearing a leader peptide which is cleaved off in the mature protein [196,197]. The molecular masses of both (SoxF 27 kDa, SoxL 25 kDa) are exceeding those from other pro- or eukaryotic sources due to larger loops between the cluster forming sites and sequence insertions as well as C-terminal extensions.

The EPR-spectroscopic and redox properties of the SoxF Rieske protein have not been studied in such detail as for SoxL. Since their equilibration with redox mediators might be different in situ, the data with integrated membranes deserve reinvestigation with regard to assignment of the reported protonation equilibria while the redox potentials apparently are extremely similar.

Recently from another *Sulfolobus species* (presumably a *S. solfataricus*) strain also a Rieske [2Fe-2S] EPR signal has been characterized as present in a

Table 5
Synopsis of cytochromes

Type of cytochr.	Source organism	M_r [kDa]	Gene	Heme	Spectral features	E′(mV)	Function and remarks
$b^{558/566}$	*S. acid.* (DSM 639)	66 (PAGE) = native 55 (PAGE) = deglycosalted 50.7(DNA)	*gac*A	1 B 6cLS	λ_{max} 558,566, 427 nm (red.-oxid.); λ_{min} 562 nm low-T: 552, 562 (526, 534) nm $E^{562}/E^{558} = 1.96$	+375	unknown highly gylcosylated no CO-reactivity imidazole induces single λ_{max} = 560 nm and CO-compound (λ_{max} 418, λ_{min} 560)
b^{562}	*S. acid.* (DSM 639)	45 (PAGE) 87.1 (DNA)	*sox*M	1 B 1 A_s	λ_{max} 562, 605 nm (red.-oxid.); 433, 596 nm (CO/red.-red.)	n.d.	component of SoxM terminal oxidase homologous to fusion of subunit I + III of heme/Cu oxidases
b^{562}	*Sulf.* sp. *strain 7*	37 (PAGE) -	n.a.	1 B 6cLS	λ_{max} 562 nm (red.-oxid.) EPR g_z 2.90	+146	assumed component of terminal oxidase 'supercomplex'; no CO reactivity
a^{587}-I	*S. acid.* (DSM 639)	39/64(PAGE) 62.8 (DNA)	*sox*C	2 A_s 6cLS	λ_{max} 586,5 nm (red.-oxid.)	+200 +260	component of SoxABCD quinol oxidase; homologous to *apo*-Cyt *b*; one heme His/Met liganded
a^{587}-II	*S. acid.* (DSM 639)	37 (PAGE) 56.7 (DNA)	*sox*G	2 A_s	λ_{max} 586 (592) nm (red.-oxid.)	+20 +100	component of SoxM terminal oxidase presumably His/His liganded
a^{583}	*Sulf.* sp. *strain 7*	unknown -	n.a.	1 A_s	λ_{max} 583 nm; sh. 570 nm (77 K) (red.-oxid.) composite Soret at 442 nm	+270 ($n = 1$) EPR: g_z 2.67	component of putative oxidase 'supercomplex'; assumed to be single heme His/Met liganded.

aa_3	*S. acid.*	38 (PAGE)	*sox*B	2 A_s	λ_{max} 441, 604/606	+220	component of SoxABCD quinol-oxidase homologous to subunit-I of heme/Cu oxidases
	(DSM 639)	57.9 (DNA)		6cLS 5/6cHS 1 Cu	nm (red.-oxid.); oxid. 421, 597 nm red. 439, 601 nm (CO/red.-red.) 429, 594 nm; λ_{min} 445 nm	+365 EPR: +200 +370	
aa_3	*Sulf.* sp.	40 (PAGE)	n.a.	2 A_s	λ_{max} 442,603 nm	+117	catal. subunit of putative oxidase 'super complex'; has quinol- and low *c*-oxidase activity
	strain 7	-		1 Cu	(red.-oxid.); λ_{max} 429, 595 nm λ_{min} 444 nm (CO/red.-red.); EPR HS g_z 5.98 LS g_z 2.90	+325	
aa_3	*D. ambival.*	40 (PAGE)	*dox*B	2 A_s	λ_{max} 422, 603 nm	+235	catal. subunit of quinol oxidase; other subunits of complex: 45,28,20 kDa
	(DSM 3772)	65 (DNA)		1 Cu	(red.-oxid.); λ_{max} 428, 590 nm λ_{min} 442 nm (CO/red.-red.) EPR g_z: 6cLS 2.88 5/6cHS 6.06	+330	
b^{562}	S.solfat.	-	n.a. B and		λ_{max} 441, 462, 583		unresolved components of respiratory chain; CO reactivity of aa_3 analogous pigment
aa_3	(DSM 1616)	-		A_s type	603 nm (red.-oxid.) in membranes.	n.d.	

Collective list of properties and functions of individual cytochromes from *Sulfolobus* and *D. ambivalens*. Data were compiled from Refs. [15,90,157,158,160,162,198,202,204,211,214,216,265].

membrane residing terminal oxidase 'super complex' [160] with *g*-values of 2.02, 1.89, and 1.79 in the ascorbate reduced complex, respectively; a function similar to Rieske centres in complex-III has been proposed. Data on its redox potential or primary structure were not reported, however.

These Rieske-FeS proteins differ from a water-soluble presumably cytosolic Rieske-type protein extracted from *Sulfolobus* sp. *strain 7*, named Sulredoxin [200]. The latter also contains a [2Fe-2S] cluster with EPR signals at $g_{zyx} = 2.01$, 1.91, and 1.79 in dithionite-reduced form. The average *g*-value falls close to that of respiratory Rieske proteins but is distinct from that of plant type ferredoxins ($g_{av} = 1.96$). The molecular mass of 12.15 kDa is less than half of that of respiratory Rieske proteins; it was speculated, however, that it might be derived from the latter [200]. From reducibility by ascorbate a fairly positive redox potential was roughly estimated. Its functional association has not been clarified. With respect to molecular mass and redox potential, it might function as a ferredoxin-like Rieske-type protein like those found in soluble bacterial dioxygenases.

6.7. The cytochromes

Present literature documents general agreement that *c*-type cytochromes are not found in Sulfolobales. As summarized in a recent review on archaebacterial cytochromes [15], only heme B and heme A have been identified as the prevailing iron porphyrin systems; neither heme O nor heme D have been detected. However, heme A of *Sulfolobus* has an extension of the isoprenoid side chain by one isopren unit, assigned as heme A_s; the corresponding extensions were found on the O-type hemes of *Thermoplasma* and *Pyrobaculum* [201]. Interestingly, heme A_s also occurs in cytochromes of the eubacterial thermophile *Thermus thermophilus* [201]. This organism also exhibits a close sequence similarity of its terminal oxidases to archaea though by 16s-RNA and other criteria it is clearly a member of the bacterial kingdom.

Table 5 gives a comprehensive data collection on cytochromes of *Sulfolobus*; individual properties are covered in the following.

6.7.1. Cytochromes b

Cyt-b^{558}: Only in *Sulfolobus acidocaldarius* (DSM 639) a *b*-type cytochrome with a split Q-band absorbance peak with maxima at 558/566 nm in the reduced form has been found [202]; at low temperature (77 K) a blue-shift of ~ 4 nm is observed. This high potential cytochrome is expressed only under microaerobic conditions in significant amounts. Its previous functional assignment as a respiratory pigment has to be revised, however. A formerly observed CO binding reactivity is obviously artificially induced by exposure to imidazole, indicating an easily displaceable weak ligand. Recent investigation identified this cytochrome as a highly glycosylated (25% of M_r) mono-heme protein containing one 6cLS heme B. The gene has been cloned and is associated with two other reading frames in an operon, but no sequence similarities to any entry in data banks were found; the derived amino acid sequence displays three histidines which may contribute to heme binding [203]. Only two strongly hydrophobic membrane anchoring segments are present at the N- and C-terminus, respectively, but do not contain the putative heme binding region. The majority of the sequence resembles a large globular protein. At present its function is still totally obscure.

Cyt-b^{562}: Other *b*-type cytochromes have absorbances at 560–562 nm and are definitely associated with electron transport complexes in *S. acidocaldarius* and *S. solfataricus*. The cytochrome b^{562} of *Sulfolobus* sp. *strain 7* is only partly characterized as a 37-kDa (SDS PAGE) subunit of a putative electron transport 'super complex' and is proposed to interact with a Rieske FeS protein also present in the same preparation [160]. Its redoxpotential of +146 mV is in the range of *b*-type cytochromes in bc_1 complexes, but the redox titration shows only 1 heme centre. It is reducible by succinate [160,204]. No sequence data are available as yet.

In contrast the cytochrome b^{562} band of *S. acidocaldarius* (DSM 639) has been clearly assigned to the SoxM polypeptide of the alternate terminal oxidase [15,198]. As a homolgue to a fusion between subunits I + III of typical heme/Cu oxidases, it displays 19 putative transmembrane helices with the canonical heme binding motifs at the correct sequence positions. One of the sites is occupied by a low-spin heme B, suggesting a *ba*3-type configuration within

the larger terminal oxidase complex as reviewed in Ref. [162] and discussed below.

6.7.2. Cytochromes a

Sulfolobales exhibit an extraordinary and otherwise unobserved cummulation of *a*-type cytochromes in their terminal oxidases. The occurrence of the most prominent absorbance peak at 583–587 nm initially was supposed to result from a cytochrome 'a_1' [74] analogous to *A. aceti* [205,206]. As in the latter case [207] it was identified, however, as component of a novel terminal oxidase complex [208]. Actually, two genetically different cytochrome a^{587} species are expressed in *S. acidocaldarius* (DSM 639) exhibiting identical absorbance spectra but different redox potentials.

Cyt-a^{587}: Cytochrome a^{587}-I (cf. Table 5; occasionally also called cyt-a^{586}) was identified as the product of the *sox*C gene [208] and is hosting two low-spin hemes A_s. The primary structure mimics a cytochrome *b* as found in classical bc_1 complexes [209,210] with the heme complexing histidines in transmembrane helices 2 and 4. EPR and MCD spectroscopy revealed, however, one heme A_s to be His/Met-liganded [211] which might be responsible for the rather high recently determined redox potentials (+220 mV) [212]. The previously reported low redox potential [16] was determined in preparations free of SoxABCD complex; at that time it was not known that two pigments with identical absorbance maxima exist in the *Sulfolobus* membrane. The low potential (~ +100 mV) has now to be attributed to cytochrome a^{587}-II (cf. Table 5).

The primary structure of cytochrome a^{587}-II also has strong similarity to *apo*-cytochrome *b* [162]. It is the product of the *sox*G gene and shares considerable homology with cytochrome a^{587}-I causing cross-reactivity with the antiserum against a fragment of SoxC [198]. Thus, it was concluded that an ancient gene duplication in archaea gave rise to two different very similar *b*-type cytochromes [162]. In contrast to cytochrome a^{587}-I both heme centres have to be bis-His liganded as derived from the primary sequence. Their redox potentials lie between +20 to +100 mV.

Cyt-a^{583}: *Sulfolobus solfataricus* and *Sulfolobus* sp. *strain 7* membranes contain cytochromes *a* with absorbance maxima at 583 nm [161]. It is tempting to assume analogous functions to cyt-a^{587}; but no information exists as to whether two different species are expressed also in these organisms. As constituent of the proposed respiratory 'super complex' [160] in *Sulfolobus* sp. *strain 7*, a redox potential of +270

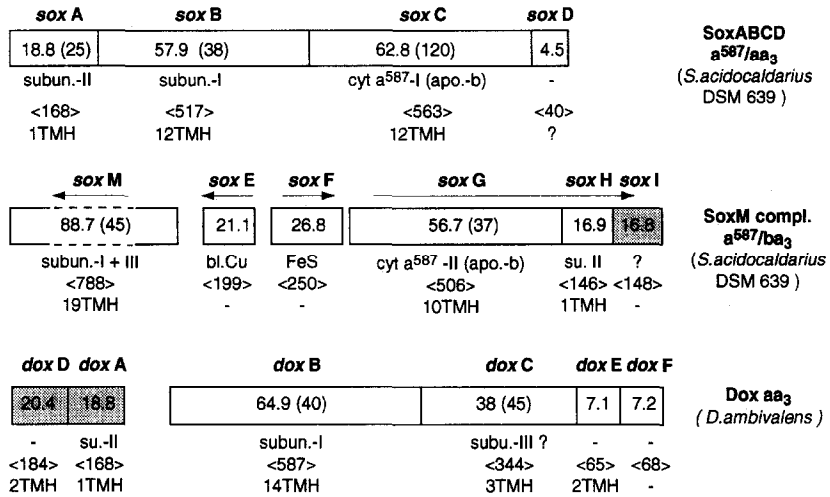

Fig. 9. Genomic organization of terminal oxidase complexes from Sulfolobales. Boxes below single genes are symbolizing the size of the gene with indication of the calculated molecular mass of the gene product (M_r in SDS PAGE in parentheses); attribution, number of aminoacids (⟨ ⟩), and the number of putative trans membrane helices (TMH) of each gene product are indicated below the boxes; arrows (when present) indicate the direction of transcription when genes are organized on opposite strands; doxD and doxA (stippled boxes) are located separately, distant from the remaining genes of the cluster of the *D. ambivalens* oxidase [161,220]; data for *S. acidocaldarius* are from [162,208].

mV has been reported assuming only a single heme centre for cytochrome a^{583} which would significantly deviate from the analogy to both diheme cytochromes a^{587}. The molecular mass of cytochrome a^{583} is not known. From tentative assignments of EPR spectra of the multi-component complex a His/Met ligandation of the heme centre was proposed and a cytochrome *c*-like function in electron transport was concluded.

Cyt-aa$_3$: Cytochrome aa_3 was the first *Sulfolobus* cytochrome described in isolated form [158,213]. It has an appararent molecular mass of 57.9 kDa (38 kDa in SDS PAGE) and exhibits high quinol oxidase activity with caldariella quinole as the optimum substrate. From CO difference spectra the presence of an a_3 heme centre was demonstrated, supported by EPR spectra revealing a high-spin ($g_y = 6.03$) feature; a low-spin ($g_z = 3.02$) feature corresponds to heme *a*. Redox titration of the high-spin a_3 centre gives a bell-shaped curve indicative of a classical binuclear heme-a_3/Cu_B centre [214] with spin coupling in the Cu(II) state. Initially assigned as a 'single subunit' or 'single entity' terminal oxidase of the aa_3 type subsequent reports clearly reveal this preparation as the terminal oxidase moiety of the SoxABCD complex in *S. acidocaldarius* [208], i.e., the product of the *sox*B gene. The heme centres of this minimal form of a terminal oxidase have unusual features in Resonance-Raman spectra [215,216], suggesting no or only very weak hydrogen bonding of the formyl moieties in both hemes. The RR data confirm the EPR derived heme coordination but, moreover, reveal a coordination equilibrium between a hexa-coordinated and a penta-coordinated high-spin configuration which cannot be resolved in any other known cytochrome aa_3. It is confirmed by RR spectroscopy of the integrated SoxABCD complex [217]. In the latter a nearly identical environment of the formyl-CO in all four heme A_s in the reduced state of the complex is found leading to a superposition of all C = O stretching vibrations at 1658 cm^{-1}.

Spectroscopic evidence also indicates the presence of cytochrome aa_3 in membranes of *S. solfataricus* and *D. ambivalens* (Fig. 9). From the proposed oxidase 'super complex' of *Sulfolobus* sp. *strain 7* the 40 kDa protein band is assigned to cytochrome aa_3 [160,218] containing the CO reactive binuclear centre characterized by an EPR high-spin signal at $g_y = 5.98$ and a low-spin feature with $g_z = 2.90$. The aa_3 moiety can be isolated as a single subunit form like in case of *S. acidocaldarius* [158] resembling most of its general properties. Further structural comparison has to await primary sequence data.

Cytochrome aa_3 from *D. ambivalens* (Table 5), the latest addition to the superfamily of heme/Cu-oxidases, is the catalytic subunit-I of the terminal quinol oxidase and reveals all typical optical and EPR spectroscopic features [219]. Its abundance in membranes of aerobically growing cells permits direct EPR spectroscopy in vivo. It shows high oxidation rates with caldariella quinol and has also TMPD oxidase activity which is suppressed in preparations containing significant amounts of a 45-kDa subunit which copurifies irregularly, but by genetic analysis is clearly assigned as a component of the intact oxidase complex [161,220]. The gene-derived primary structure of the 65 kDa aa_3-polypeptide (40 kDa in SDS PAGE) suggests a total of 14 transmembrane helices; it contains the heme coordinating histidine motifs in an otherwise extreme poorly conserved sequence pattern (see also Section 9).

7. Terminal oxidase complexes

All terminal oxidases of *Sulfolobus* proved to be much more complex than expected from the initially described 'single entity' quinol oxidase [213]. A preparation from *Sulfolobus* sp. *strain 7* was first shown to purify as a complex containing additional pigments (cyt-a^{583}) besides cytochrome aa_3 [157], a property also shared by other *Sulfolobus* oxidases. Its high cytochrome *c*-oxidase activity, however, might be considered as artifactual due to possible detergent perturbations because cytochrome *c* is not a natural substrate in Sulfolobales.

Two terminal oxidase complexes from *S. acidocaldarius* (DSM 639) could be structurally and functionally resolved due to complete resolution of the operons encoding the components of the SoxABCD and the SoxM complex, respectively [162,198,208,221]. Also from *Desulfurolobus ambivalens* a terminal oxidase complex has been genetically and protein-chemically resolved [219,220] and is thought to represent the most ancient form of an aa_3-type terminal oxidase. Structural data and gene organizatin of these complexes are compiled in Fig.

9. The respiratory system of *Sulfolobus* sp. *strain 7* was isolated as a functional 'super complex' [160,183,204]; resolution into functionally integrated subcomplexes by genetic analysis is still open.

The operon encoding the SoxABCD oxidase from *S. acidocaldarius* (DSM 639) was shown to comprise four reading frames [208]. The product of soxA is a homologue to subunit-II of cytochrome *c*-oxidases, however, lacking the typical binuclear $[Cu^{2+}\text{-}Cu^{1+}]_A$ binding motif. This is in line with its function as a quinol oxidase; cytochrome *c* is not oxidized. All genes are cotranscribed, including a small hydrophobic polypeptide of unknown function (*sox*D) and cytochrome a^{587}-I (*sox*C). This component is a structural analog to di-hem *b* cytochromes. The sketch of Fig. 10 illustrates the proposed functional organization of the complex asccording to Ref. [208]. The interesting hypothesis was put forward that the complex might exert proton pumping in a bc_1-like mechanism as reviewed in Section 8. However, so far neither data on differential quinone-binding sites nor on site-specific inhibitors as known for the bc_1 Q-binding sites [210,222,223] were presented. Interestingly, preparations of the complex routinely exhibit a $g = 2$ stable radical signal eventually indicative of a tightly bound caldariella quinone (author's observation), but direct analytic proof has to be given. At 70°C the integrated complex has an almost 3-fold higher turnover ($> = 1300\ s^{-1}$) than the 'single entity' cytochrome aa_3 [161]. The rapid oxidation-reduction kinetics of the SoxABCD complex as well as its CO binding properties have been directly measured in *Sulfolobus* membranes [224] and are similar to that of beef heart mitochondrial cytochrome *c*-oxidase with $k = 5.5 \times 10^4\ M^{-1}\ s^{-1}$ and $K_a = 5 \times 10^4$. The same experiments provide evidence for a joint redox behaviour of cytochrome aa_3 and cytochrome a^{587}-I in *Sulfolobus* membranes.

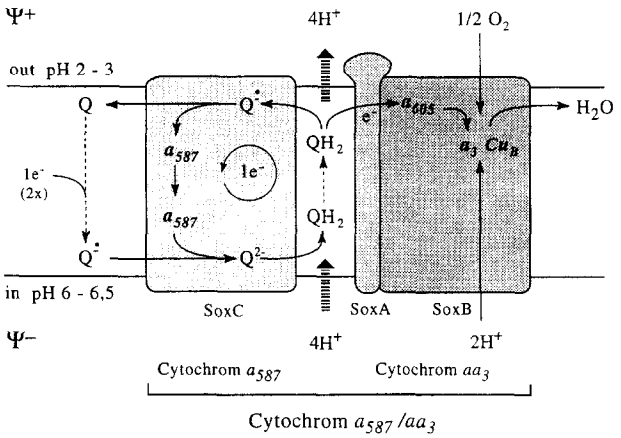

Fig. 10. Hypothetical functional oganization of redoxcenters in the 4-heme terminal quinol oxidase (SoxABCD) of *Sulfolobus acidocaldarius* exerting a Q-cycle as proposed in [208]; the small subunit D is omitted for simplicity.

Compared to SoxABCD the catalytic activity of purified SoxM complex is poor. It copurifies together with cytochrome a^{587}-II and a Rieske FeS protein [198]. In our hands only a negligible quinol oxidase activity can be measured compared to SoxABCD, as well as some minor TMPD- and cytochrome *c*-oxidase activity. The latter is frequently observed in crude preparations from *Sulfolobus* membranes and may result from an unspecific interaction of reduced cytochrome c with the Rieske protein (cf. Section 6.6). This points to the probability of another hitherto unisolated natural reductant. As such, sulfocyanin may operate, a homologue to azurin-like blue copper proteins which was predicted as the likely product of the *sox*E gene [162]. In contrast to SoxABCD oxidase the genes of the SoxM complex are organized in two adjacent clusters in opposite directions (Fig. 9). Both gene clusters have to be transcribed, however, to build the integrated complex which somehow mimics a functional fusion of classical complexes-III and -IV. Especially the product of *sox*H is of interest because the translated amino acid sequence reveals the signature of a Cu_A binding site with structural homology to subunit-II of cytochrome *c*-oxidases [162]. The presence of Cu_A remains to be demonstrated, however. Its presence would contradict the issue that the SoxM-ba_3 is a simple quinol oxidase. *sox*F codes for the second Rieske FeS protein and *sox*G for cytochrome a^{587}-II. Spectral properties of the entire complex may vary with preparation procedures; in contrast to originally reported Q-bands of the ba_3 moiety at 592 + 605 nm [15,198], new preparation methods show a band at 586 and a shoulder at 604 nm [212] in accordance with spectra of intact membranes.

The terminal quinol oxidase of the related thermoacidophile *D. ambivalens* is an exception as it does not contain additional cytochromes besides aa_3 [219]. It purifies as a four subunit complex (20, 28, 40, and 45 kDa approx.) and contains 1 Cu/mol. The

integrated complex oxidizes caldariella quinol with high activity and is strongly inhibited by specific inhibitors of Q-oxidases [225]; significant activity with TMPD as donor is associated only with preparations partially deficient in the 45-kDa subunit. Its genomic organization has been resolved revealing split gene clusters [161,220] as indicated in Fig. 9. The product of *dox*B (65 kDa) is a subunit-I homologue with respect to the heme binding motifs. But significant differences to other oxidases of the heme/Cu-family are found. An analog to subunit-III is missing; it is replaced by the rather hydrophilic DoxC 38 kDa polypeptide (45 kDa approx.) with no known homologue. Both subunits are co-transcribed together with a small polypeptide. The product of the distantly located *dox*A gene is an analog to subunit-II with respect to size and hydrophobicity profile but has no further homology; like in other quinol oxidases its primary sequence is deficient of any metal binding motifs.

8. Respiratory chains and proton pumping

8.1. Electron pathways

Because specific inhibitors of intermediate electron transport are obviously not functioning with respiratory systems of *Sulfolobus*, any hypothesis on the sequential order of redox components forming a respiratory chain has to rely (1) on their standard potentials, (2) on structural associations in functional complexes, and (3) on fast redox kinetics. The only data of the latter kind available are for *Sulfolobus* membranes, indicating a rapid and simultaneous oxidation/reduction cycle of aa_3 and a fraction of a^{587}, whereas the heme *b* components react much slower under the applied conditions [224].

Based on the two preceding criteria the conclusion appears justified that in *Sulfolobus* membranes an energy transducing complex-I is absent but both, NADH and succinate, are oxidized by respective quinone reductases filling the pool of caldariella quinone with hydrogen.

Reoxidation of the latter occurs in *D. ambivalens* by the only possible catalyst the aa_3-type Q-oxidase complex. In *S. acidocaldarius* caldariella quinol is reoxidized by the SoxABCD complex while the function of the SoxM complex – which has almost no Q-oxidase activity – is still open. However, anticipating that the genetically predicted sulfocyanin is expressed but accidentally escapes present SoxM oxidase preparations, and assuming a redox potential as for halocyanin [226–228], respiratory chains are proposed here as outlined in Fig. 11.

Though SoxM appears as a homogeneous co-purifying complex, inclusion of sulfocyanin into the upper branch in Fig. 11 would functionally split the complex into two segments with the high potential ba_3-moiety as a terminal oxidase and the low potential part resembling a complex-III like function; sulfocyanin would act as an intermediate carrier-like cytochrome *c*. Inclusion of a Rieske (SoxL) FeS protein also into the lower branch in Fig. 11 is a tentative suggestion based on the finding that SoxL Rieske is readily reoxidized by the aa_3 complex [197]. Of course, additional hitherto unidentified components may be necessary. In both models cytochromes a^{587}, which indeed have cytochrome *b* analogous primary structures and hydrophobicity profiles, would assume a cytochrome *b*-like function. The lower reduction potential of a^{587} in SoxM (cf. Table 5) as compared to its analog in the SoxABCD complex fits into the model.

Electron transport in *S. solfataricus* may be organized similarly. However, a scheme suggested for the proposed supercomplex of *Sulfolobus* sp. *strain 7*

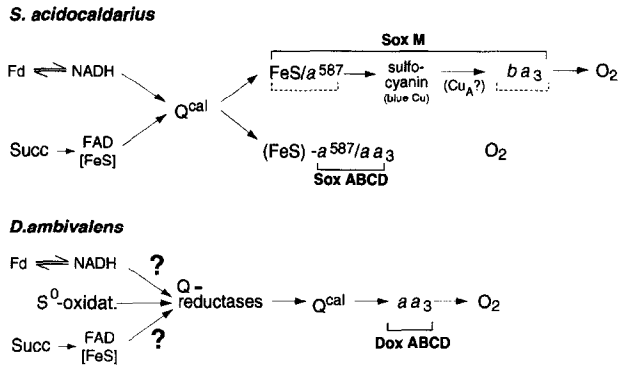

Fig. 11. Proposed organization of electron transport components in the respiratory chains of *S. acidocaldarius* (DSM 639) and *D. ambivalens*. The terminal oxidase complexes are printed in bold. The dashed cramps illustrate the possibility of two functional units within the integrated SoxM complex. Individual functions of the components and possible association of proton pumps are described in the text and Table 5.

[204] appears largely speculative as a monoheme *b*-cytochrome is postulated to be involved in a proton translocating Q-cycle [160]; its reduction potential rather shows similarity to the diheme cytochrome a^{587} from the SoxABCD complex.

8.2. Proton pumps

Respiration driven proton extrusion by *Sulfolobus* has been demonstrated [15,16,76,221] (cf. Section 4), but a final decision on the number of pumps needs further investigation. Due to the lack of a complex-I pumps can be associated only with the Q-oxidizing branches of the respiratory chain(s). Conclusive data have been obtained only with two experimental systems. First, by reconstitution of the 'single entity' aa_3 oxidase, the generation of a proton motive force was demonstrated [229], leaving open, however, whether or not superstoichiometric proton translocation was measured, or if only chemical protons for water formation were asymmetrically consumed inside the vesicle lumen; H^+/e^- ratios could not be determined. It is a general problem with reconstituted Q-oxidases to find impermeable reductants in systems with partially randomly oriented pumps. Then, very recently, by core constitution of the SoxL Rieske protein with the integrated SoxABCD complex in archaeal lipids, definite proof was obtained that the latter complex is a proton pump [212]. Due to a reversible redox equilibrium between the SoxL Rieske and cytochrome c^{2+}, this system functions as an 'artificial' cytochrome *c*-oxidase. With reduced cytochrome *c* as an impermeable reductant a $H^+/e^- >= 2.2$ is measured. The result is discussed in support of a Q-cycle-like mechanism with indispensable involvement of cytochrome a^{587}-I according to Ref. [208].

Pumping by the aa_3 moiety itself is considered unlikely in *Sulfolobus* for two reasons. On the one hand, an even higher stoichiometry is expected if in addition to a Q-cycle the aa_3 (subunit-I) would pump protons by a mechanism as suggested from detailed mutagenesis data with subunit-I of other proton pumping oxidases [230–232], and strongly supported by recently available 3-D structures [233,234]. On the other hand, several essential amino acid residues indispensably necessary to establish the proton pathway for this mechanism are not conserved in cytochrome aa_3 from *Sulfolobus acidocaldarius* (DSM 639) as discussed in Ref. [161].

On the same basis cytochrome aa_3 from *D. ambivalens* is unlikely to be a proton pump, whereas the terminal ba_3-oxidase of the SoxM complex bears the structural signatures of a proton pump in the primary sequence of its subunit-I.

9. Evolutionary aspects of bioenergetic modules

The study of archaeal protein sequences from energy transducing pathways is of significant importance for the construction of protein-based phylogenetic trees and has been of great help in shining light on the development of energy metabolism. While the general evolution of 'kingdoms' and 'domains' is ruled by 16sRNA analysis, protein-based trees demonstrate the development and propagation of functional modules.

Since chemiosmotic energy conservation has been found as the universal principle in existing organisms, its components are of specific interest as evolutionary distance markers. As such phosphotransferases and electron transport systems are ideal targets.

Though cellular life is incompatible without adenylate kinases and pyrophosphatases, the number of known sequences is too low for a highly resolving phylogenetic tree. In general it was found that sequence homologies are comparatively low for both series of enzymes [154] besides the conservation of catalytic and substrate binding residues. Crystal structures [156,235] and modeling studies [147], however, support the view that analogous spacial structures and functional properties can be achieved with widely different amino acid patterns if the catalytically essential residues are conserved at appropriate positions. This is what evolution obviously did. Actually, for both adenylate kinase and pyrophosphatase, the sequences of *Sulfolobus* represent the lowest branch in the respective trees, with large distances to bacteria but a somewhat closer relation to eukarya [132,147,155].

A very detailed tree can be constructed for the main subunits of ATPases/synthases due to the large number of organisms analysed. An uninterrupted line of evidence has been accumulated that both polype-

tides originated from duplication of one ancestral gene [236–240], one of which lost the property to code for a catalytically active polypeptide. By a number of deletions and extensions not only was their size modified, causing an interchange of catalytic and non-catalytic subunits, but so was their further propagation into F-type and V-type ATPases [241]. Archaebacterial ATPase sequences (A- and B-subunits) are closely related to each other [109] as well as to vacuolar type ATPases. Since sequences are known now for *Sulfolobus* [126], *Methanobacterium* [112] and *Halobacteria* [109,111], a rooted tree provides evidence for a parallel evolutionary path of both polypeptides from the split into A- and B-subunits through the later endosymbiosis events [109]. From this, it emerges that already in early stages of evolution the $\alpha\beta$-, resp. AB-association was maintained as the central functional unit. The evolution of components of the proton channel may have occurred independently [116]. Whether or not a split into V-type and F-type ATPases occurred prior to division into a bacterial and an archaeal/eukaryal kingdom, as suggested by some recent sequence data [123,124,242], is difficult to decide; simply because there is (are) only one (two) example(s). An explanation by horizontal gene exchange has to be proven. For a detailed discussion see Ref. [241].

Regarding terminal oxidases the recently published 'respiration early' hypothesis [243] is challenging the classical theory that oxygenic photosynthesis predates the evolution of respiratory enzymes [244]. A series of conclusive arguments in favour of an earlier occurrence of terminal oxidases has been put forward which is substantially supported by primary structures of the *Sulfolobus* cytochromes [245]. Essentially the data are based on comparison of subunit-I sequences, suggesting the presence of all functional modules already in an ancestor preceding the archaea-bacteria split. Strong evidence is based on the distant evolutionary relation to a cytochrome oxidase from nitrogen-fixing bacteria thriving under microaerobic conditions [246] as well as on the NorB subunit of the strictly anaerobic nitric oxide reductase [247]. Both enzymes share the 12 transmembrane helix motif and an identical pattern of the heme binding histidines, identifying them as members of the heme-copper oxidase superfamily [248,249]. In fact, several archaea are adapted to microaerobic conditions which may have prevailed already under early geochemical conditions prior to generation of high atmospheric oxygen tensions [250,251]. Also denitrification has been reported to function in archaea [86,252–256]. Moreover, copper is the essential co-factor in respiratory terminal oxidases as well

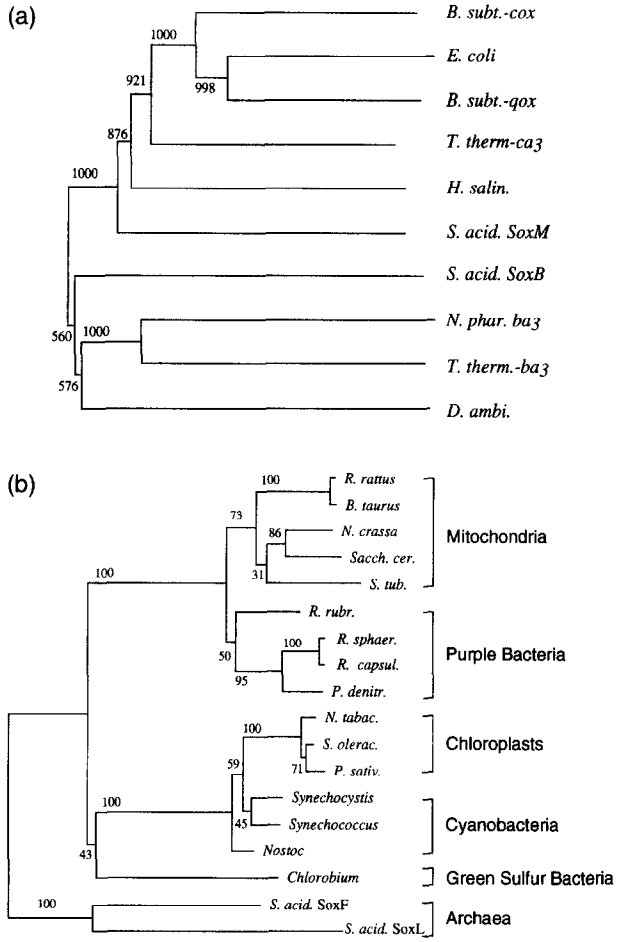

Fig. 12. Protein based pylogenetic trees. (a) Tree of subunit-I from terminal oxidases including only procaryotic organisms demonstrating the early split into two branches; both branches comprise archaeal and bacterial members. The sequence from *D. ambivalens* has the shortest distance to the hypothetical root. The FixN-oxidase [263] sequence served as outgroup. (b) Tree of resiratory Rieske iron-sulfur proteins including sequences from all three domains: archaea, bacteria and eucarya. Ferredoxin-like Rieske-type proteins were used as outgroup. Trees were constructed with the CLUSTAL-W program package [264]. Abbreviations: B.s. = *Bac. subtilis*; E.C. = *E. coli*; T.th. = *Therm. thermophilus*; H.h. = *Halobact. halobium* (*salinarum*); S.ac. = *Sulfolobus acidocaldarius*; N.Ph. = *Natronobact. pharaonis*; D.a. = *Desulfurolobus ambivalens*; 'cox' and 'quox' signify cyt-*c* or quinol oxidases of an organism.

as in denitrifying oxidoreductases [253,257]. Subunits-II of nitric oxide reductase and terminal oxygen reductases also share the binding sites for Cu_A which are absent in quinol oxidases like that from *Sulfolobus*. The inferred issue that cytochrome *c*-oxidases precede quinol oxidases in evolution [249] is difficult to resolve, however. In summary, the basic architecture of respiratory terminal oxidases may be older than their present electron acceptor, molecular oxygen. An additional argument is that oxygenic photosynthesis is not found in archaea and only occurs in bacteria and plastides of endosymbiontic origin.

A phylogenetic tree (Fig. 12a) including the recently determined sequences of *D. ambivalens* [220] and *Natronobacterium pharaonis* (M. Engelhard et al., personal communication) not only supports the above hypothesis, but also emphasizes that an early gene duplication presumably generated two homologous types of subunit-I polypeptides which were unequally distributed between archaea and bacteria. This may explain the appearance of *T. Thermophilus* ba_3 in the same cluster with *N. pharaonis* and *D. ambivalens* aa_3. The two subunit-I sequences of *Sulfolobus* oxidases, like *T. thermophilus*, are positioned on different branches as well. According to this tree, Sulfolobales represent the closest neighbours to an ancestral 'ur-oxidase'.

Interestingly, all subunit-I sequences clustering on the branch with *D. ambivalens* oxidase have in common the deficiency of at least four amino acid positions forming the putative for proton pumping pathway [233], as reviewed in Ref. [161]. These are two asparagines and one aspartate in the helix II-III loop, and a glutamate adjacent to the HXH motif in helix VI. Therefore it has been assumed that all oxidases of this branch might not be actively translocating protons [161], which remains to be confirmed experimentally, however.

Similar conclusions can be drawn from the phylogenetic tree of Rieske [2Fe2S] proteins of Fig. 12b. This presentation includes the recent discovery of two separate Rieske FeS proteins in *Sulfolobus* [195,196]. According to this tree an ancestral iron-sulfur protein has to be assumed as a common root preceding the archaea-bacteria split. A gene duplication must have occurred within the archaea at approximately the same time as the major split into photosynthetic and respiratory bacteria. Notably, essentially identical trees were found for the evolution of *b*-type cytochromes including the two a^{587} *b*-type analogs of *Sulfolobus* [162]. With regard to structural association between *b*-type cytochromes and Rieske [2Fe2S] proteins the functional nucleus may be regarded also prior to the formation of more recent bc_1-complexes and to the split into the archaeal and bacterial domains. This assumption is tempting since such a device may have worked as a proton-translocating energy converter also under anaerobic conditions with oxidases using other terminal acceptors than molecular oxygen. An existing example is provided by b_6f complexes. At any rate, the involvement of iron-sulfur redox chemistry in early states of cellular life and even in prebiotic evolution has been impressively worked out [258–260].

10. Concluding remarks and open questions

Questions remaining at the end of an excursion through recent literature on bioenergetics of *Sulfolobus* could be: is there anything relevant to be added to our present knowledge on respiratory energy conservation in general, and, what are the future perspectives?

Actually, investigation of *Sulfolobus* has brought considerable insight into molecular structures and evolutionary relations of bioenergetic systems. In addition to that this hyperthermophilic organism provides a large number of interesting proteins and lipid components excellently suitable for studies on the molecular basis of extreme thermostability. Adenylate kinase and pyrophosphatase as described in chapter V are typical candidates. They have the advantage of being easily accessible to site-directed mutagenesis, crystallization, and also, because they are small, to dynamic NMR investigations. Since evolution obviously started with development of extreme thermophilic organisms it appears more reasonable to modify such hyperthermostable proteins, adapting them stepwise to lower temperatures, rather than going the opposite way in order to understand thermostability.

Regarding ATP-synthase the results from *Sulfolobus* were the first to show decisively that the principle structure of this machinery has been completely developed already at very early stages of

cellular evolution. The general structure of a catalytic headpiece connected by a stalk to a membrane residing ion channel has been strongly conserved giving rise to F-type and V-type ATPases. While their evolutionary relations appear conceivably solved, the functional coupling of archaeal ATPases to their proton translocating membrane part is totally open. No F_0-like complex has been isolated in intact form as yet from archaebacteria, nor could other components than an analog to the small DCCD binding polypeptide be identified. Also on the genetic level information is insufficient.

Unexpected and novel structural concepts were detected by molecular analysis of electron transport complexes, especially of the terminal oxidases of *Sulfolobus*. Both, SoxABCD and SoxM, are combining core structures of respiratory complexes III and IV. It is hardly possible to prove whether or not the genetic association of apocytochrome *b* with an aa_3 type oxidase as found in SoxABCD is an ancient invention. However, an attractive hyopthesis would be that it reflects an early evolutionary state from which a later development and division into two individual complexes started by aquisition of additional genes initiating the split into present forms of complexes III and IV. The genetic organization and morphological structure of the SoxM complex from *Sulfolobus* could resemble an intermediate state of such a development. Though presumably being expressed and assembled as a unique functional entity, the complex indeed integrates two substructures closely related to individual analogs of either complex III, or IV (Fig. 11). As a future approach the coordination of expression and assembly of its individual components might shine light on this issue. In summary, highly elaborate electron transport complexes have to be assumed already in very early organisms.

Evaluation of sequence homologies of subunit-I strongly favours the view that terminal oxidases have a monophyletic origin reaching back to ages before oxygenic photosynthesis. This holds equally for the Rieske FeS proteins. Surprisingly, two different membrane-associated respiratory Rieske proteins are expressed in *Sulfolobus*. This is a unique case within prokaryotes; the co-existence of two Rieske proteins is known only from photosynthetic eukaryotes containing two different endosymbiont-derived organelles, chloroplasts and mitochondria. One *Sulfolobus* Rieske protein is contained in the SoxM complex. Actually, the physiological association of the other (SoxL) with the electron transport system needs further investigation. Eventually additional constituents exist which so far escaped detection. Its capability to transfer electrons between cytochrome *c* and the SoxABCD complex fortunately could be used to demonstrate the ability for active proton pumping by this terminal oxidase. Though it is the first direct demonstration with a terminal oxidase from *Sulfolobus*, the question whether additional respiratory proton pumps are present also needs further experimentation. High H^+/O ratios observed with whole cells support this assumption. The SoxM complex would in fact be a likely candidate for efficient proton pumping, eventually including two energy transducing sites. Resolution of the branched electron transport system in this respect requires a separate functional reconstitution into liposomes of SoxM which has not yet been achieved. In addition, cellular or environmental parameters regulating electron flow through both branches are totally unknown.

Also for *Sulfolobus strain 7* (resp. *S. solfataricus*) information on the true path of electrons as well as the real structure of the proposed Q-oxidase complex are in a rather preliminary state [160,204]. At least the operation of a Q-cycle with a mono-heme *b*-cytochrome appears unlikely, because it would imply the interaction of only one heme-*b* centre with two different Q-redox sites of different redox potential.

A number of unsolved problems also becomes evident on the level of carbon metabolism of *Sulfolobus*. The fact that a non-phoshorylative pathway of sugar catabolism is operating calls for a solution how this organism generates glycogen from unphosphorylated intermediates or from amino acids. A simple reversal of the non-phosphorylating reaction sequence appears rather unlikely. It is a future task, therefore, to identify such pathways together with the respective enzymes, but also to investigate principles of their mutual regulation.

A common denominator for solution of many open questions summarized above is the necessity to search for a suitable genetic transformation and selection tool for *Sulfolobus*. For example, the differentiation between proton pumps in both respiratory branches could be envisaged if one branch could be genetically

disrupted. But nothing practical is available today. However, preliminary and promising research with stable plasmids is in progress [26,261] and might help to answer most of these questions in the near future. Nevertheless, *Sulfolobus* is the only extremely thermoacidophilic archaeon of which the bioenergetic system has been elucidated to a large extent.

11. Note added in proof

The archaebacterium *D. ambivalens* has recently been reclassified as *Acidianus ambivalens* [Fuchs, T., Huber, H., Burggraf, S. and Stetter, K.O. (1996) Syst. Appl. Microbiol. 19, 56–60].

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