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Review

Cytochromes of archaeal electron transfer chains

Mathias Lübben *

Lehrstuhl für Biophysik, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany

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Abbreviations: CoM-SH, 2-mercaptoethanesulfonic acid; CQ, CQH_2, caldariellaquinone, -ol; F_{420}, $F_{420}H_2$, ([*N*-L-lactyl-γ-L-glutamyl]-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate), oxidized and reduced form; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; HS-HTP, *N*-7-mercaptoheptanoylthreonine phosphate; CoM-S-S-HTP, heterodisulfide or 'mixed' disulfide of CoM-SH and HS-HTP; Q, QH_2, quinone, -ol; PAG(E), polyacrylamide gel (electrophoresis); TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

* Corresponding author. Fax: +49 234 7094238.

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1. Introduction

Since their discovery, the archaebacteria (= archaea) have attracted the attention of both molecular biologists and biochemists. By now, a number of primary structures from various structural ribonucleic acids and proteins of archaea are known by gene sequencing. The molecular phylogeneticists use these data for the derivation of evolutionary relationships of organisms down to the early stages of life. Trees have been constructed (Fig. 1), which separate the Eucaria and Bacteria from the so-called 'third urkingdom' of life [1,2] formed by the Archaea [3]. Alternative views exist [4,5], but a discussion of phylogeny goes beyond this text.

Archaea have interesting molecular characteristics, such as specific surface layer structures [6,7] and ether-linked lipids built from isoprenoid units [8,9]. Their molecular structures differ significantly from those found in eubacteria and eukaryotes, which has been extensively reviewed in [10,11].

The most fascinating feature archaea have in common is their preference for unusual living conditions. They survive in an environment which we, from our anthropomorphic viewpoint, consider to be impossibly hostile to supporting life [12]. On the other hand, they are not a homogeneous family of organisms (Fig. 1). Some representatives live at extremely high temperatures (thermophiles) or at high salinities (halophiles), whereas others perform unique metabolic pathways like methane formation, which requires strict anaerobiosis (methanogens). Archaea thrive in ecological niches, and the extreme habitats are less favourable for competitors, which then considerably reduces selective pressure. Therefore, the need for molecular adaptation by mutation is much less than compared with other microorganisms like enterobacteria. Archaea are therefore considered 'slow-clock' organisms with respect to their rates of evolutionary change. They should then resemble their ancestors more closely than eucarya and eubacteria do [2].

There is no doubt that life originated in an anaerobic hydrosphere [13,14]. Speculations have been made about the development and evolution of metabolic pathways from this environment. At the stage of a primeval inorganic world, energy could have been conserved by simple redox reactions [15], which is a prerequisite for autotrophic growth without light energy. Specifically, it has been pointed out that a small concentration of oxygen was locally present in the early atmosphere, due to photolysis of water in the ultraviolet light [16]. The first steps of aerobic metabolism could have evolved early, perhaps before multiphenotypical populations of pre-cells assembled into the ancestors of Eukarya, Bacteria and Archaea

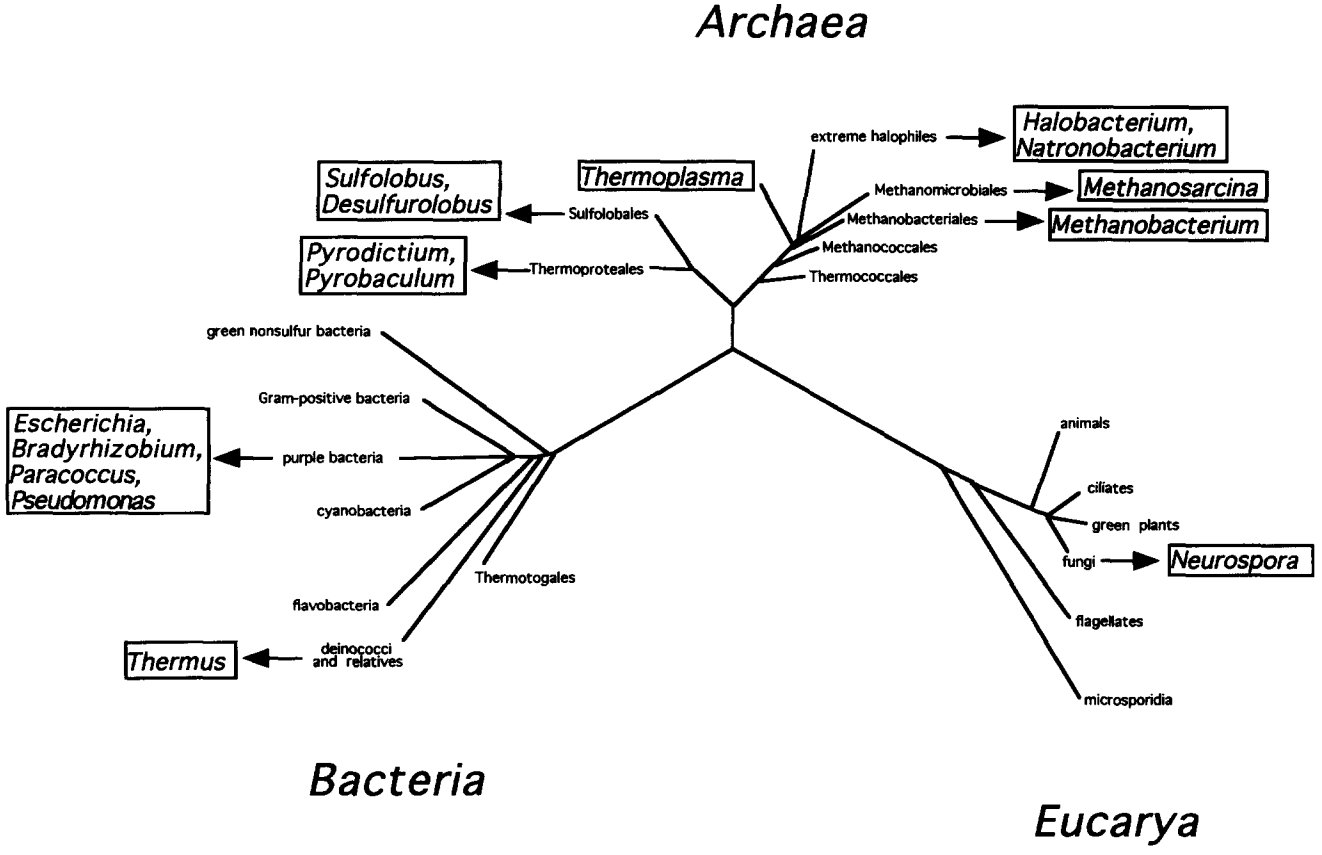

Fig. 1. Unrooted phylogenetic tree, taken from [2], modified after [197,17]. The branch lengths represent evolutionary distances, as calculated after alignment of 16 S rRNA sequences. The relationships of the microorganisms discussed here (boxed) are emphasized by assignments to the respective taxa.

[17]. This may have happened 2–3 billion years ago. Archaeological findings are witnesses of a much later period: one group reported the successful cultivation of halophilic archaea from rock salt that had been buried for more than 0.2 billion years. The survivors may be regarded as living fossiles [18]. Extant Archaea from extreme environments are descendants of ancient precursors. It is almost impossible to decide whether newly discovered molecular or physiological features are evolutionary remnants or just specific adaptations to their living conditions.

Archaea are also interesting because of their potential for biotechnological applications, which is being actively investigated [12,19,20]. However, many fundamental questions are unexplored. The capability of archaea to survive or even depend on the 'extreme' living conditions is closely linked to their mechanisms of energy conversion.

Many organisms yield energy by oxidoreduction reactions, which are carried out in membrane-bound electron transfer chains involving cytochromes. These are haem proteins which have various functions in the cell. The proteins exist in soluble and membrane-bound forms. The latter occur in the cytoplasmic membrane of eubacteria, in the inner membranes of mitochondria and in thylakoids of chloroplasts. In eubacteria and eukaryotic organelles, cytochromes contribute to electron transfer reactions in energy transduction, oxygen binding and sensing, and also in the key reactions of the anaerobic denitrification pathway.

Archaea also use the basic principles of energy conversion, but the catalysts are different from the well-known proteins of eubacteria and mitochondria. This review is intended to summarize current knowledge about the structure and function of electron transfer chains of archaea, in which cytochromes play a central role. Specific features of archaeal cytochromes will be discussed, while emphasizing similarities to eubacterial systems.

2. Anaerobic electron transfer reactions

2.1. Methanogens

2.1.1. Energy production by methanogenesis

The most fascinating mechanism of energy conversion discovered in archaea is the formation of methane from molecular hydrogen and from simple compounds, such as carbon dioxide, formate, methylamines and acetate. Methanogenesis is a strictly anaerobic metabolic process, during which the liberated free energy is used to drive ATP synthesis. Several reviews about the molecular aspects of methanogenesis have been published recently [11,21–24]. Enzymes required for methane production have either cytoplasmic or membrane location. The formation of transmembrane ion gradients in methanogens appears to be an anaerobic equivalent to the oxidative generation of electrochemical potential [25]. Some steps of the methanogenic reaction sequence are linked to the generation and consumption of sodium-motive force, reviewed in [26]. However, the proton seems to be the major coupling ion in methanogens.

During methane production a variety of recently discovered enzymatic reactions is carried out predominantly by cytoplasmic enzymes and involves a number of novel coenzymes, reviewed in [23]. The final step of methanogenesis consists of two partial reactions, (1) the reduction of the coenzyme M – bound methyl group (CH_3-S-CoM) by another soluble cofactor HS-HTP, resulting in the formation of an intermediate (CoM-S-S-HTP), the so-called 'heterodisulfide':

$$\mathrm{CoM-S-}\mathbf{CH_3} + \mathrm{HS-HTP} \rightarrow \mathrm{CoM-S-S-HTP} + \mathbf{CH_4}(\Delta G^{0\prime} = -45\mathrm{kJ/mol}) \quad (1)$$

and (2) or (3), the regeneration of the cofactor HS-HTP by reducing equivalents in the form of hydrogen in molecular (H_2) or coenzyme-bound ($F_{420}H_2$) form:

$$\mathrm{CoM-S-S-HTP} + \mathrm{H_2} \rightarrow \mathrm{CoM-SH} + \mathrm{HS-HTP}(\Delta G^{0\prime} = -40\mathrm{kJ/mol}) \quad (2)$$

and

$$\mathrm{CoM-S-S-HTP} + \mathrm{F_{420}H_2} \rightarrow \mathrm{CoM-SH} + \mathrm{HS-HTP} + \mathrm{F_{420}}(\Delta G^{0\prime} = -28\mathrm{kJ/mol}). \quad (3)$$

These partial redox reactions yield large free energies, which can be conserved in vivo in the form of proton gradients.

2.1.2. Proton translocation coupled to heterodisulfide reduction

In some methanogens, such as *Methanosarcina* and *Methanolobus* species the heterodisulfide reduction is coupled to transmembrane proton translocation [24,27,28]. The heterodisulfide reductase is an integral membrane protein that forms a functional complex with cytochrome *b* and with hydrogenase or F_{420} dehydrogenase. Electrons are taken up from the substrates H_2 or $F_{420}H_2$ and passed through the system, which releases protons on the outside as schematically outlined in Fig. 2 for the $F_{420}H_2$-dependent complex. The impermeant $F_{420}H_2$ acts from the inside (Fig. 2), whereas in the H_2-dependent complex the diffusible substrate H_2 could reach the hydrogenase from either side of the membrane (not shown in Fig. 2).

Cytochromes are directly involved in this process. In its membrane-bound and solubilized form the F_{420}-dehydrogenase reduces cytochrome *b* of *Ms. mazei* and *Ms. barkeri* [29,30]. The cytochromes of reduced cytoplasmic membranes could be rapidly oxidized by addition of CoM-S-S-HTP. Rotenon (a complex I inhibitor) and myxothiazol, antimycin and HQNO (inhibitors of the cytochrome

bc_1 complex) did not affect the reaction. The significance of the partial inhibition of cytochrome reduction observed with CO and azide is unclear [29].

Cytochromes of the *b*- and *c*-type have been identified only with the Methanosarcinaceae [31–34]. The cytochromes are exclusively membrane-bound in *Methanosarcina mazei* strain Gö1. At room temperature, α, β and Soret peaks at 556, 514 and 434 nm were present in redox difference spectra of cytoplasmic membranes, the broad α band were better resolved into 4 distinct signals

Table 1
Functional and structural properties of cytochromes isolated from aerobically grown archaea

Type [references]	Molecular size; subunit structure	Primary structure of the apoprotein	Cofactors	Spectral features; redox potential	function
Methanosarcina barkeri					
cytochrome *b* [36]	subunits: 46 and 23 kDa (PAGE)	unknown	0.6 mol cytochrome *b*, 20 mol non-haem iron, 20 mol S, 0.2 mol FAD per 69 kDa complex	ΔA_{max} = 558, 428 nm (red.-ox.)	heterodisulfide reductase
Halobacterium salinarium					
cytochrome *b*-558 [105]	subunits: 15.4 and 11.7 kDa (PAGE)	unknown	1 mol haem B/mol	A_{max} = 414, 526 nm (ox.); 425, 528, 558 nm (red.); E_m (pH 8.0) = −0.075 V	unknown, electron transmitter
cytochrome *b*-562 [105]	subunit: 25 kDa (PAGE)	unknown	1 mol haem B/mol	A_{max} = 417, 530 nm (ox.); 431, 531, 562 nm (red.); E_m (pH 8.0) = −0.340 V	unknown, electron transmitter
cytochrome *a* [115,116]	42 kDa (gel filtration) subunit: 40 kDa (PAGE)	coxI, homologous to subunit I of haem-copper oxidase	2 mol haem A-type/mol	A_{max} = 420, 598 nm (ox.); 441, 602 nm (red.); 430, 600 nm (red./CO); E_m (pH 8.0) = +0.310 V (n = 1.3)	terminal oxidase (electron donor unknown)
Natronobacterium pharaonis					
cytochrome *b/c* [88]	subunits: 18 and 14 kDa (PAGE)	unknown	haem B and C	A_{max} = 413 nm (ox.); 423, 525, 558, shoulder at 552 nm (red.); E_m = −0.112 and −0.007 V	unknown, electron transmitter
cytochrome ba_3 [88]	subunit: 40 kDa (PAGE)	unknown	Cu; haem B and A_S	A_{max} = 414, 440, 592 nm (ox.); 426, 440, 532, 560, 592 nm (red.); 426 (red./CO)	unknown, electron transmitter
Thermoplasma acidophilum					
cytochrome *b* [130,131]	subunit: 18 kDa (PAGE)	'ORF 2' of putative succinate dehydrogenase operon	at least 1 mol B-type haem per mol; 1.57 mol Cu/mol haem	ΔA_{max} = 562, 558, 553 nm (red. − ox.); E_m (pH 7.0) = −0.150 V and 0.075 V	cytochrome *b* of putative succinate dehydrogenase
Sulfolobus acidocaldarius					
cytochrome *b*-558/566 [146] [a]	65 kDa (PAGE)	unknown	1 haem B per mol	ΔA_{max} = 566, 558, 427 nm (red. − ox.); ΔA_{max} = 418 nm, ΔA_{min} = 428, 558 nm (red./CO − red.) E_m (pH 7.0–7.5) = 0.375 V	unknown
SoxABCD complex [85,95,150]	280 kDa (gel filtration); two subunits with 38 kDa (Sox B and Sox C), 27 kDa (SoxA), 5 kDa	SoxB; homologous to subunit I of haem-copper oxidase. SoxC; homologous to cytochrome *b* of bc_1 complex. SoxA; homologous to subunit II of haem-copper oxidase	4 mol haem A_S per mol; 1 mol Cu per mol haem	ΔA_{max} = 606, 586, 440 nm (red. − ox.); ΔA_{max} = 434, 595 nm, ΔA_{min} = 449 nm (red./CO − red.). E_m (pH 7.0–7.5) = 0.020 V, 0.100 V, 0.220 V and 0.370 V	terminal quinol oxidase complex
cytochrome aa_3 [85,94,151]	100 kDa (gel filtration); 38 kDa (PAGE)	SoxB; homologous to subunit I of haem-copper oxidase	2 mol haem A_S/per mol; 1 mol Cu per mol	ΔA_{max} = 441, 604 nm (red. − ox.); ΔA_{max} = 595, 434 nm, ΔA_{min} = 445 nm (red./CO − red.); E_m (pH 7.0–7.5) = 0.220 V and 0.370 V	catalytic subunit of quinol oxidase

Table 1 (continued).

Type [references]	Molecular size; subunit structure	Primary structure of the apoprotein	Cofactors	Spectral features; redox potential	function
SoxM complex [94,162] [b]	subunits: 45 kDa (SoxM); 38 kDa (apocytochrome *b*); 30 kDa Rieske 2Fe-2S	SoxM; homologous to subunit I + III of haem-copper oxidase. apocytochrome *b*; homologous to cytochrome *b* of bc_1 complex	haem A_S and B	ΔA_{max} = 445, 562, 592, 605 nm (red. − ox.); ΔA_{max} = 596, 433, 420 nm, ΔA_{min} = 447 nm (red./CO − red.)	terminal oxidase complex
***Sulfolobus* strain 7**					
cytochrome *a* complex [149]	150 kDa (gel filtration) subunits: 37, 23 and 14 kDa (PAGE)	unknown	A-type haems; ratio of 3.5 mol of haem A-type per mol of cytochrome a_3	ΔA_{max} = 441, 583, 603 nm (red. − ox.); ΔA_{max} = 431, 596 nm, ΔA_{min} = 448 nm (red./CO − red.)	terminal oxidase activity with heterologous cytochrome *c*
Desulfurolobus ambivalens					
cytochrome aa_3 complex [94,178]	subunits: 40, 27 and 20 kDa (PAGE)	unknown	haem A_S; ratio of 1 mol cytochrome *a* to 1 mol cytochrome a_3; 0.82 mol Cu/mol of cytochrome a_3	ΔA_{max} = 442, 603 nm (red. − ox.); ΔA_{max} = 428, 590 nm, ΔA_{min} = 442 nm (red./CO − red.)	terminal quinol oxidase complex

A_{max}, A_{min}, ΔA_{max}, ΔA_{min} = absorbance maxima and minima of electronic absorbance or redox difference spectra
ox. = oxidized, red. = reduced sample; red. − ox. = difference of reduced and oxidized sample; red./CO = reduced sample in the presence of carbon monoxide; red./CO − red. = difference of reduced sample in the presence of carbon monoxide and reduced
[a] Günter Schäfer, personal communication
[b] Jose Castresana and Matti Saraste, unpublished data

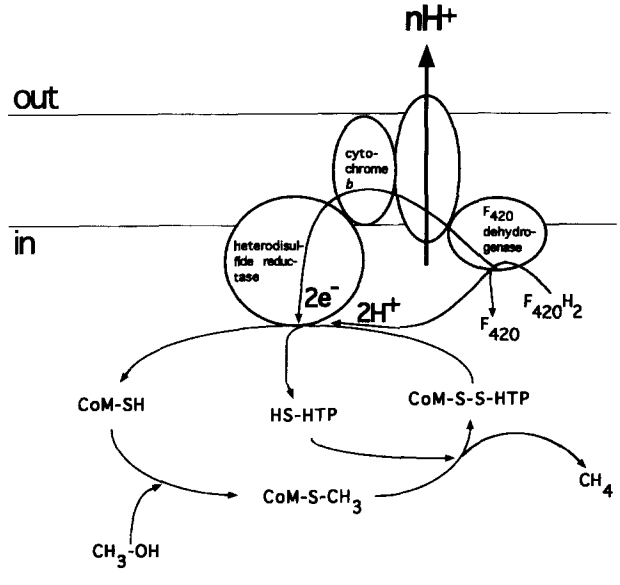

Fig. 2. Electron transfer of the heterodisulfide reductase complex of the anaerobe *Methanosarcina* strain Gö1. The scheme shows the entry of the substrate methanol (CH_3-OH) and the fueling of the proton pump with the hydrogen donor $F_{420}H_2$. In total, the transfer of electrons *via* cytochrome *b* and hydrogen to methanol leads to the release of methane. The free energy of this reaction is used to translocate protons across the cytoplasmic membrane. Activation of methanol by the binding of coenzyme M, and the liberation of methane in the cytoplasm are enzymatically catalyzed reactions. The symbols ...-SH and ...-S-S-... indicate the redox state of the involved coenzymes and coenzyme-substrate complexes. Another heterodisulfide reductase complex, in which molecular hydrogen operates as the donor of reducing equivalents (see text) is not shown for the sake of clarity. Modified after [24].

at lower temperature. Binding of carbon monoxide to cytochromes was detected [29]. CO binding had been observed previously for membrane-bound cytochrome b_{559} of *M. barkeri*, as deduced by a (CO reduced) − (reduced) difference spectrum with a maximum at 418 nm and minima at 433 and 559 nm [31]. Pyridine haemochrome analysis of the membrane-bound cytochromes revealed haems B and C. All the cytochrome *b* and *c* species have low redox midpoint potentials between −240 to −135 mV [29].

The heterodisulfide reductase from *Methanosarcina barkeri* is a multisubunit protein having nine different polypeptides with molecular masses ranging from 15–46 kDa, which co-purifies with hydrogenase (F_{420} not-reducing) [35]. The purified complex had absorbance features typical of *b*-type cytochromes (Table 1). The broad α peak measured at room temperature could be better resolved at 77 K into signals at 562, 558 and 548 nm. In addition to cytochrome *b*, the complex has non-haem iron, acid-labile sulfur, nickel and FAD as cofactors. The isolated *M. barkeri* enzyme uses molecular hydrogen as electron donor and as reductant of cytochrome *b* [35]. Recently, the preparation of a two subunit heterodisulfide reductase from *Ms. barkeri* strain Fusaro consisting of 46 and 23 kDa polypeptides was reported [36]. Haem binding was assigned to the 23 kDa subunit, which apparently corresponds to cytochrome *b*. The authors concluded that the 46 kDa polypeptide is the catalytical subunit of the heterodisulfide reductase.

The presence of *b*-type cytochromes is apparently not obligatory for the heterodisulfide reductase of

methanogens. No cytochromes could be detected in membranes [32] or in the purified complex of the hydrogenotrophically grown *Methanobacterium thermoautotrophicum* [37]. Moreover, after breakage of the cells, the heterodisulfide reductase of this organism is soluble. Nothing is known about the coupling of the reductase to proton translocation. It is possible that the enzyme of this organism operates with alternative electron carriers.

One of the haem binding proteins of *Ms. mazei*, having 27 kDa apparent molecular mass, was purified and characterized by amino acid sequencing. Its gene was identified (*vhoC*) in a transcriptional unit (*vhoGAC* operon) comprizing the large and small subunits of Ni/Fe hydrogenase [38]. Computer analysis of the putative cytochrome *b* predicts four hydrophobic regions, which could be membrane-spanning stretches. This pattern resembles the cytochrome *b* belonging to the HydC subunit of quinone-reactive Ni/Fe hydrogenase of the anaerobic eubacterium *Wolinella succinogenes* [39]. However, VhoC from *Ms. mazei* has no sequence homology to other proteins.

2.2. Other anaerobes

Non-methanogenic archaea that grow anaerobically have metabolic reactions coupled to electron transfer via cytochromes. Only a few of these redox reactions have been investigated in detail. One of these is a recently reported anaerobic electron transfer chain from *Pyrodictium brockii*, a hyperthermophilic anaerobe [40,41]. The archaeon grows by hydrogen-sulfur autotrophy, characterized by the reduction of elemental sulfur by hydrogen to produce H_2S and energy. Sulfide production and hydrogen uptake are sensitive to HQNO, which makes the involvement of a quinol very likely. The *Pyrodictium* quinone has not been identified, but after photochemical inactivation of the endogenous quinol pool the electron transfer reaction could be restored by mitochondrial ubiquinones with different isoprenoid side chains [42]. A *c*-type cytochrome became reduced in the presence of molecular hydrogen. The cytochrome had an α absorbance band at 553 nm, and it could be solubilized by Triton X-100. SDS PAGs of membrane extracts gave a haem-specific-stained band of 13–14 kDa. A specific role of the electron transfer reaction in energy transduction has yet to be demonstrated.

Membranes of the hyperthermophilic anaerobic sulfur reducer *Archaeoglobus fulgidus* [43] contain *b*- and *c*-type cytochromes, but their exact role in electron transport of that organism has not been attributed [44].

In the absence of oxygen, different archaea are capable to respire with nitrate as the terminal electron acceptor. They carry out certain partial reactions or even the entire pathway of denitrification, which is bound to anaerobiosis in eubacteria. Each of the individual electron transfer steps in the sequence

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

is catalyzed by metalloenzyme complexes in which cytochromes are either directly involved or closely linked (reviewed in [45,46]). Halobacterial species and *Pyrobaculum* are known to be facultative aerobes that can denitrify. None of the reported archaeal cytochromes has yet been explicitly demonstrated to play a specific role in the process of denitrification. Archaeal cytochromes, which are anaerobically expressed in the presence of nitrate, will therefore be described in the context of the aerobic electron transfer chains (see below).

3. Energy metabolism of aerobic archaea

The energy metabolism of aerobic archaea resembles that of eubacteria. Hetereotrophically grown archaea degrade glucose via modified Entner-Doudoroff pathways, in which metabolites are phosphorylated at different stages [11,47]. While Halobacteria carry out substrate level phosphorylation, the thermoacidophiles *Thermoplasma* and *Sulfolobus* seem to be unable to synthesize net ATP from these processes. The carbohydrate breakdown produces significant amounts of reducing equivalents. In this respect aerobic electron transfer chains have two important roles: (i) to oxidize the reduced coenzymes which maintains high metabolic fluxes and (ii) to convert redox energy into electrochemical potential and into ATP by membrane-bound catalysts. In fact, oxidative phosphorylation has been shown to exist in the archaeal kingdom [48–50].

Halobacteria were the first archaea for which chemiosmotic principles were demonstrated to be operative. They readily form oriented membrane vesicles, a prerequisite to study phosphorylation. Protonmotive force generated by artificially imposed H^+-gradients, membrane potential or by light led to ATP synthesis in appropriately 'stuffed' vesicles [51]. The photophosphorylation process involves the light-driven proton pump bacteriorhodopsin or the chloride pump halorhodopsin [51,52]. The activities of both have been extensively reviewed elsewhere [53–55]. The existence of this kind of catalyst sheds some light on the versatility of archaea in utilizing different sources for energy transduction. In the dark, during anaerobiosis, halobacteria ferment amino acids, like arginine [56]. In the presence of oxygen they have a respiratory electron transfer chain.

The thermoacidophiles investigated so far are more difficult to study, because they do not readily form closed membrane vesicles. Chemiosmotic phosphorylation by coordinated use of electron transfer proteins and ATP synthase could be demonstrated with intact cells of *Sulfolobus* [50]. Eubacterial as well as archaeal acidophiles have a cytoplasmic pH close to neutrality [57–60]. It has been shown for various acidophiles that respiration is necessary for maintaining the large proton gradients, that respiration-driven proton pumps exist, and that the cellular

ATP synthesis mainly relies on the proton as the coupling ion [61,62].

Archaea are highly specialized to their natural habitats. They have developed unusual variations of known processes and novel metabolic pathways. It is interesting to know how archaea integrate the separate catalysts in order to perform electron transport-driven phosphorylation. Recent studies have shown that archaeal electron transfer systems and their molecular components are constructed in a similar manner to eubacteria.

4. Structure of aerobic electron transfer chains

4.1. General composition

Electron transfer chains consist of a sequence of membrane-bound proteins which catalyze the electron transport from donors having low reduction potential (high energy level) to acceptors with high reduction potential (low energy level) (Fig. 3). At several stages of the electron transfer chain, redox energy is converted into an electrochemical proton gradient across the cytoplasmic membrane. Different substrate dehydrogenases forward the metabolite-abstracted electrons and protons to the quinol pool. Quinol is oxidized by haem proteins of the cytochrome *b*-, *c*- and *a*-type, and by the bc_1 and terminal oxidase complexes. Under aerobic conditions the preferred electron acceptor is molecular oxygen. During anaerobic growth, alternative respiratory chains exist which utilize other acceptors like nitrate (see above) and fumarate. Haem proteins have essential functions also in these anaerobic electron transfer chains [63,64] (for reviews, see [65,66]).

4.2. Terminal oxidases

Bacteria sometimes have respiratory chains branching at the level of the quinone pool. The synthesis of different respiratory pigments enables the microbe to respond to environmental factors. Fig. 3 illustrates an example of the variety of terminal oxidases synthesized by a eubacterium, e.g., *Paracoccus denitrificans* [67].

Two major classes of cytochrome oxidases are known, the cytochrome *bd*-type quinol oxidases and the haem-copper oxidases [68–72]. The latter comprises a superfamily [73,74] of quinol and cytochrome *c* oxidases, which are characterized by the binuclear (also named 'bimetallic') reaction centre consisting of one copper and one molecule of haem A, O or B oriented in close proximity. Molecular oxygen is reduced to water at this site [75]. A second molecule of haem A, O or B binds to another site. The haem-copper oxidases are named after the haem cofactors (lower-case letter) and after their ligand binding properties (index 3 after the ligand-binding haem), i.e., different combinations have been assigned as cytochromes aa_3, caa_3, cao_3, ba_3, bo_3, oo_3, bb_3 and cbb_3 in the literature (for reviews, see [72,73,76]; beside other nomenclatures, the one given above appears to be the least confusing). Several haem-copper oxidases have been demonstrated to be vectorial proton pumps. The catalytic subunits I of haem-copper oxidases retain similar primary structures, irrespective of the substrate oxidized or the nature of haem cofactor. The highly conserved ligand patterns of the

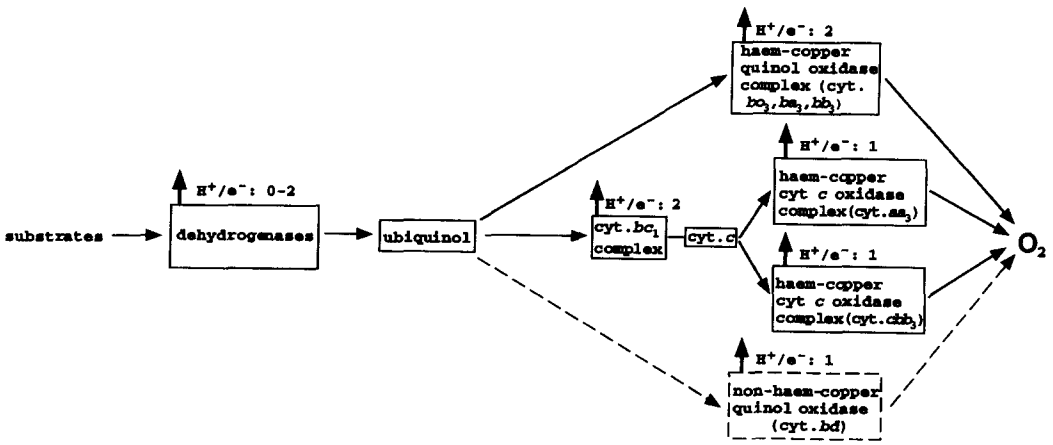

Fig. 3. The respiratory chain of aerobically grown *Paracoccus denitrificans*, as a paradigm of a branched electron transfer chain in an α purple bacterium. The dehydrogenases transfer electrons from substrates to the quinone acceptor pool, which is a branching point of electron transport to the various terminal oxidases [65,67,93,198–204]. Although it has been questioned whether *Paracoccus* has a cytochrome *bd* [67], it is included for completion and symbolized with dashed lines. Electron flow through the various branches involves specific complexes and has different energetic efficiencies. The H^+/e^- ratio of a proton translocator (i.e., the number of protons liberated per electron at the outside) is a measureable quantity, whose value is shown for each complex (arrows). Protons are transferred across the membrane by scalar and vectorial mechanisms; see text for further explanations (Section 5.6.4). The electrochemical work done by the complexes is represented by the number of charge equivalents translocated per electron transported (q/e^- ratio), $q/e^- = 1$ for the bc_1 complex and cytochrome *bd*, and $q/e^- = 2$ for the haem-copper-type cytochrome *c* and quinol oxidases. Therefore oxidation of quinol by combined activities of cytochrome bc_1 and cytochrome *c* oxidase ($q/e^- = H^+/e^- = 3$) is thermodynamically most efficient.

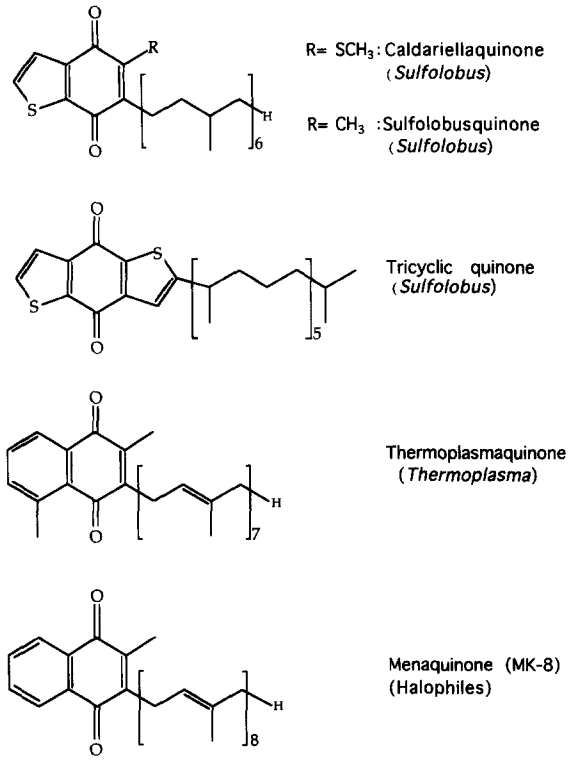

Fig. 4. Structures of archaeal quinones. *Thermoplasma* also has menaquinone (MK-7). Other archaea have menaquinones with partially (halophiles, *Thermoproteus*) or completely (*Archaeoglobus*, *Thermoproteus*, *Pyrobaculum*) saturated side chains [81]. In thermoplasmaquinone the positions 5 and 8 of the methyl group in the aromatic ring are spectroscopically equivalent and could not be clearly assigned [82].

apoprotein are diagnostic for this type of oxidase (Fig. 8A). The histidine ligands provide a unique geometry, into which the catalytic metal centres are embedded [77]. Some of the histidines have been suggested to be directly involved in the mechanism of proton translocation [78].

5. Electron transfer in aerobic archaea

5.1. Cofactors and coenzymes

5.1.1. Archaeal quinones

Quinones are lipid soluble, low-molecular-weight carriers of molecular hydrogen. They occur in superstoichiometric amounts compared to macromolecular respiratory catalysts. Besides the oxidized and the reduced forms, a half-reduced semiquinone form exists. Quinones are reduced by the substrate dehydrogenases. They become oxidized by the bc_1 complex, or directly by the action of the cytochrome *bd* or certain haem-copper oxidases (Fig. 3). Often these reactions are inhibited by the quinone analogue, HQNO.

The quinone composition of some archaea is known (Fig. 4) (for reviews and further references, see [79–81]). Halophiles have menaquinones with unsaturated isoprenoid chains, mostly MK-8. The menaquinone core occurs in some *Pyrobaculum* species as well, but some of the isoprenyl double bonds are reduced. The structure of the *Thermoplasma acidophilum* compound thermoplasmaquinone [82] is closely related to menaquinone (Fig. 4). *Sulfolobus acidocaldarius* has three different quinone species, which are characterized by sulfur atoms at the ring positions of caldariellaquinone [83], sulfolobusquinone and the so-called tricyclic quinone (Fig. 4). The quinone composition seems to vary upon the oxygen supply during growth. Caldariellaquinone was found to be the predominant species (more than 60% of total quinones) under all conditions tested [84]. It acts as a substrate of the isolated cytochrome aa_3 oxidase of *Sulfolobus* [85]. One group has made attempts to study the biosynthesis of caldariellaquinone, showing that its aromatic core is derived from tyrosine, as is the aromatic portion of ubiquinone in bacteria [86].

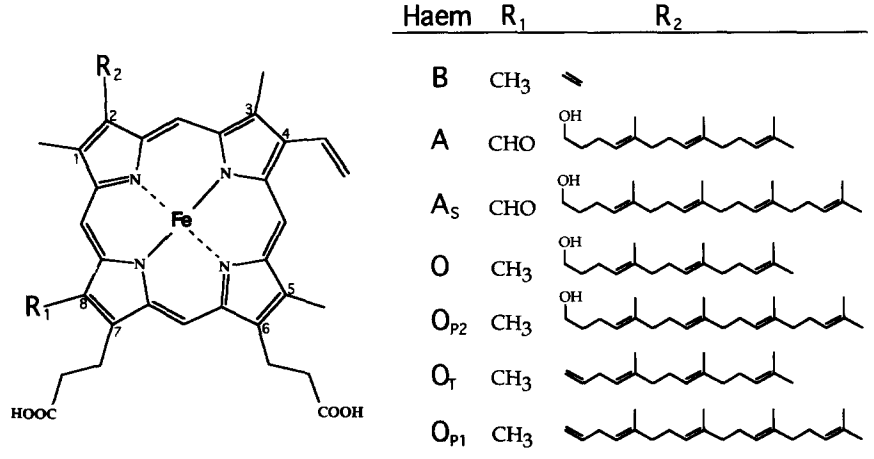

Fig. 5. Structure of archaeal, bacterial and eukaryotic organellar haems. The indices S, P1, P2 and T stand for *Sulfolobus*, *Pyrobaculum* and *Thermoplasma*, from which the haem variants have been isolated.

5.1.2. Archaeal haems

Haems are metal complexes of tetrapyrrol compounds that often form the functional heart of electron transfer proteins. In cytochromes of mitochondria and eubacteria, the cofactors are haem A, B, C, O or D. The nature of the haem molecule and its axial ligand environment determine the midpoint redox potential (E_m) of the cytochrome. Certain haem groups therefore are associated with particular electron carrier proteins. Archaea have haem B in various *b*-type cytochromes. Haem C was demonstrated in archaea by typical pyridine haemochromogen spectra obtained from membrane residues after removal of the extractable haems [29,87,88].

Haems A and O, which have long isoprenoid side-chains at position 2 (Fig. 5), are found in haem-copper oxidases. This kind of terminal oxidase species seemed to be obligatorily characterized by its prenylated haems, whose function is still matter of speculation [89]. Recently, in *Bradyrhizobium*, *Rhodobacter* and *Paracoccus* the FixN-type [90] ('cbb_3') novel haem-copper oxidases were discovered, that bind protohaem as a cofactor [67,91,92], but are fully active as vectorial proton pumps [93].

Oxidases of aerobically growing archaea (see below) have neither haem A nor O. Instead, four novel prenylated haems were isolated from various archaea (Fig. 5), which are named after their mother compounds haem A or O, and the microorganism from which the haem species was firstly isolated (*Sulfolobus*, *Thermoplasma* or *Pyrobaculum*). The novel haems A_S, O_T, O_{P1} and O_{P2} [94] are altered at their side-chains at position 2 (Fig. 5). Archaeal haems optically resemble haems A and O, because the structural modifications are at a site distant from the metal centre. In the case of haem A_S, this holds true for the isolated compound as well as for the apoprotein-bound state [94]. Haem A_S is unequivocally the cofactor of the two cytochrome oxidases isolated from *Sulfolobus acidocaldarius* [94,95] and of the halobacterial cytochrome ba_3 [88]. Table 2 shows the haem composition of different archaea. Prenylated haems of archaea could not be detected other organisms. An interesting exception is the eubacterium *Thermus thermophilus*, which has an archaeal haem composition. There is no haem A in cytoplasmic membranes, whereas haem A_S occurs in both of the isolated cytochrome oxidases caa_3 and ba_3 of *T. thermophilus* [94].

5.2. Halobacterium

5.2.1. General

Extreme halophiles are microorganisms requiring extreme salt concentrations of 250–300 g/l for survival [96]. They are capable of converting energy by the three different mechanisms, fermentation, photosynthesis and respiration (see above). Most of the published work about respiratory electron transport has been carried out with *Halobacterium halobium*, which has been recently renamed to *Halobacterium salinarium*. The structural integrity of halophilic biomolecules and a lot of biological activities tested in vitro require high salt concentrations. This includes the stability of halobacterial ribosomes [97] and of soluble proteins as well as of membrane proteins [98,99]. A concentration of 3 M NaCl or more is necessary for the measurement of O_2 uptake [87,100].

Different substrate dehydrogenases fuel the halobacterial respiratory chains. Earlier reports describe oxygen uptake by membranes, and the reduction of cytochromes by NADH, succinate and α-glycerophosphate [87,101,102]. Attempts to solubilize and purify succinate dehydrogenase were made [99]. A menadione reductase could be isolated, and its activity in membrane vesicles was used for the determination of the sidedness of the vesicles [103].

5.2.2. Cytochromes b and c

A number of *b*-type cytochromes were identified in *Halobacterium cutirubrum* and *salinarium* [87,102,104,105]. Their reducibility in the membrane-bound state was studied with different substrates. Some of the proteins were purified, and their molecular data are

Table 2
Haem composition [a] of membranes of archaea

Organism	Haem				Characteristics [b]
	A_S	O_T	O_{P1}	O_{P2}	
Sulfolobus acidocaldarius [c]	+	–	–	–	thermoacidophile, obligately aerobic
Desulfurolobus ambivalens	+	–	–	–	thermoacidophile
Halobacterium salinarium	+	–	–	–	halophile
Natronobacterium pharaonis [c,d]	+	–	–	–	haloalkaliphile
Thermoplasma acidophilum	–	+	–	–	thermoacidophile
Methanosarcina barkeri	–	–	–	–	methanogen, strictly anaerobic
Pyrobaculum aerophilum	+	–	+	+	thermophile

[a] Haem B (= protohaem IX) is present in all samples. Archaeal membranes have neither haem A nor haem O. Haems C and D were not analyzed, after [94].

[b] The indicated organisms are facultative aerobes, unless specified.

[c] Haem A_S is a cofactor of cytochrome oxidases of *Natronobacterium* and *Sulfolobus* [94]

[d] Haem analysis done with isolated cytochromes

listed in Table 1. At least four different cytochrome *b* species covering a wide range of redox potentials from −340 mV to +261 mV [102,104,105] are reported for *H. salinarium*. The cytochromes appear to be organized in a branched electron transfer chain. For instance, succinate addition to membranes reduces certain cytochromes *b* species, whereas others remain unaffected. The reaction is partially sensitive to the bc_1 complex inhibitor antimycin [104]. Although cytochrome *c* appears to be absent from *H. salinarium* [106], electronic absorption spectra of solubilized proteins show peak asymmetry exhibiting shoulders at 554 and 549 nm [102,104,105], which is reminiscent of *c*-type cytochromes. This peak was better resolved when spectra were taken at liquid nitrogen temperature. Signals corresponding to cytochrome *c* were also observed in the redox spectra of *H. cutirubrum* membranes [87], and the presence of haem C was demonstrated by pyridine haemochromogen. Fujiwara et al. were able to partially purify a soluble *c*- type cytochrome of *H. salinarium* [106].

At low concentrations, the quinol analogue HQNO was found to partially inhibit the oxidation of NADH, but not of succinate in membrane particles of *H. cutirubrum* [98]. This would indicate the involvement of quinol compounds in electron transfer, but the site of action could not be assigned precisely. Menaquinone was identified in Halobacteria (see above); there is no convincing evidence for the existence of a bc_1 complex in *H. salinarium* [105].

5.2.3. Cytochromes a

Pigments with an absorbance peak of 592 nm in the presence of CN^- were assigned as cytochrome a_1 in *H. cutirubrum* and *salinarium* [87,98,100,107]. Cytochrome a_1 is considered to be a haem A binding protein that reacts with molecular oxygen. The existence of cytochrome a_1 in halobacterial species was questioned by other authors, who assign the respective absorption band to a peroxidase complex [105,108]. Similar observations were made with the previously described 'cytochrome a_1' from *Bradyrhizobium japonicum*, which was identified later as a soluble catalase-peroxidase haemoprotein that binds haem B as a cofactor [109,110]. The so-called cytochrome a_1 from *Acetobacter aceti* was, on the other hand, identified as a ba_3-type haem-copper oxidase [111,112]. It should be noted that the halobacterial strains used and the growth conditions applied in these studies were considerably different. This may account for all the variations of the cytochrome composition, and especially for the expression of 'cytochrome a_1'.

Spectroscopic evidence for the existence of cytochrome *o* and cytochrome aa_3 oxidases has been presented for *H. cutirubrum* and *salinarium* [100,107]. The existence of distinct terminal oxidases in branched electron transfer chains of halophile membranes is further supported by the partial CN^- inhibition of oxidase activity with the artificial substrate TMPD [105].

At low ionic strength it is possible to demonstrate the oxidation of (horse heart) cytochrome *c* by *H. salinarium* membranes [106]. This activity decreases with higher salt concentrations, which is similar to findings with non-halophilic eubacteria [113,114] but in contrast to other enzyme reactions of halophiles. Cytochrome *c* and TMPD oxidase activity are lost completely after detergent solubilization of membranes [115]. The authors purified a carbon-monoxide-binding 40 kDa cytochrome *a*, which has no catalytic activity but which still can be partially reduced with TMPD (Table 1). The optical spectra were reminiscent of cytochrome aa_3. DNA sequencing of the corresponding gene identified the protein as a homologue of subunit I of the haem-copper oxidase superfamily [116] (Fig. 8A). Genetic constructs of chimeric proteins consisting of fragments from subunit I of halobacterial cytochrome aa_3 and *E. coli* cytochrome bo_3 expressed non-functional protein. Respiration could not be restored in mutants deficient of both *E. coli* terminal oxidase, indicating the importance of authentic intramolecular contacts for correct folding and catalytic activity [117].

The isolated cytochrome *a* probably binds haem A_S (Table 2), because no other A-type haem could be detected in cytoplasmic membranes. Surprisingly no copper was found in the protein, which may explain its lack of activity. At present, there is neither biochemical nor genetic evidence for the association fo cytochrome *a* with other subunits. The natural substrate of the halophile cytochrome aa_3 is unknown.

5.3. Natronobacterium pharaonis

Another interesting halophile being studied is the alkaliphilic *Natronobacterium pharaonis*. This archaeon generates a membrane potential by the light-driven Cl^--pump halorhodopsin [118]. In the dark, *N. pharaonis* is capable of conserving energy by oxidative phosphorylation although the pH difference between medium and cytoplasm is reversed [119]. This bioenergetic scenario is known from other alkaliphiles [120]. It has not yet been demonstrated, whether the proton is the coupling ion in *Natronobacterium*.

The membranes are rich in cytochromes *a*, *b* and *c*. Fractionation of cytochromes by ion exchangers yields a complex consisting of cytochromes *b* and *c*. The protein is composed of two polypeptides with apparent molecular masses of 18 and 14 kDa (Table 1), with the smaller of which presumed to be the cytochrome *c*, by haem-specific staining of SDS-PAGs [88].

The authors further described the preparation of an *a*-type cytochrome [88,119] (Table 1). The peaks in redox difference spectra as well as the characteristic band shifts after binding of carbon monoxide indicated the existence of cytochrome a_3. Pyridine haemochrome analysis demonstrated equimolar amounts of B-and A-type haems; the latter was identified as haem A_S (Table 2). The authors

regard the protein as a potential ba_3-type oxidase; activity data have not been reported. The preparation of cytochrome ba_3 consisted of a singular subunit with apparent molecular mass of 40 kDa. Sequencing and molecular characterization of the protein is in progress.

A high molecular weight *c*-type cytochrome is membrane-bound and may be a possible electron donor for terminal oxidases (M. Engelhard, personal communication). Another potential electron donor for quinol oxidases is menaquinone, which has been identified in *N. pharaonis* [121]. From this archaeon, a blue copper protein, named halocyanin, was purified, sequenced and physicochemically characterized [122,123]. The redox midpoint potential of +0.183 V (at pH 7.3) makes halocyanin also a possible candidate for the electron donor of terminal oxidases [124].

5.4. *Pyrobaculum aerophilum*

Recently, the facultative aerobe *Pyrobaculum* was isolated from marine habitats [125]. It lives at 100° C and can utilize organic and inorganic sources for aerobic and anaerobic respiration. The cytochrome expression of this archaeon differs considerably when the oxygen tension is varied [94]. *Pyrobaculum* is an interesting organism for future studies of molecular properties of respiratory proteins and of the regulation of cytochrome synthesis.

Different *b*-type cytochromes have been detected in cytoplasmic membranes by redox difference spectroscopy. In total, there are three different carbon monoxide binding species. In membranes from anaerobically and aerobically grown cells, signals similar to cytochrome bo_3 occur. In membranes from aerobically cultured cells there is an additional pigment with the CO-binding features of cytochrome a_3. The lack of an absorbance peak typical for cytochrome aa_3 (600–606 nm) may be interpreted as evidence for a ba_3-type cytochrome. These membranes had CN^--sensitive TMPD oxidase activity and showed oxygen uptake with the substrate menadiol [94]. The existence of several prenylated haems (A_S, O_{P1} and O_{P2}) (Table 2) supports the suggestion that different quinol oxidases of the ba_3- and bo_3-type may be present. Membrane-bound cytochromes *c* of *Pyrobaculum* were excluded by pyridine haemochromogen spectroscopy [94].

Anaerobic growth of *Pyrobaculum* uses nitrate as an electron acceptor while neither menadiol nor TMPD oxidase activity could be measured in its membranes [94]. The anaerobically expressed high-spin haem B-binding pigment is perhaps one of the membrane-bound cytochromes which are involved in denitrification [64,126].

5.5. *Thermoplasma acidophilum*

Early investigations reported *b*-, *c*- and *d*-type cytochromes in the cytoplasmic membranes of *Thermoplasma* [127–129]. This was confirmed in part by a later study, which presented evidence for the existence of cytochrome *b* and *d*. A species absorbing at 595 nm in redox spectra was tentatively identified as a_1 [130]. It is better assigned as b_{595} (see above), since neither haem A nor A_S is present under the applied growth conditions in this archaeon [94].

A cytochrome *b* [130] with apparent molecular mass of 18 kDa could be purified to homogeneity, Table 1. At first it was assumed to be a terminal oxidase, since the purified protein formed a carbon monoxide complex with absorption spectrum similar to cytochrome bo_3. Moreover, it had more than one mole of copper per mole of protein bound [130].

The gene encoding the cytochrome *b* was isolated via primers derived from the N-terminal amino acid sequence. The primary structure of the polypeptide is essentially unrelated to any of the known haem-copper or cytochrome *bd*-oxidases [131]. Instead, the gene (named ORF2) is placed between two adjacent open reading frames (named ORFs1 and 3) within a putative succinate dehydrogenase operon. ORF2 is predicted to be a hydrophobic polypeptide with 3 possible transmembrane regions. Histidine residues that could act as potential haem ligands are located within or next to the hydrophobic domains. The histidine array typical for haem-copper oxidases (Fig. 8A) is absent in *Thermoplasma* cytochrome *b*.

ORF1 of the operon is similar to a subunit of fumarate reductase. It has several cysteine-rich sequence patterns corresponding to proteins that bind iron-sulfur centres. Both succinate dehydrogenase and fumarate dehydrogenase complexes have been shown to contain *b*-type cytochromes in different organisms [63,132,133]. The authors favour the assignment as a succinate dehydrogenase because the protein is expressed during aerobic growth.

The pigments absorbing at 595 and 625 nm in redox difference spectra seem to derive from cytochrome *bd*. As yet, this cytochrome species has not been found in other archaea. Cytoplasmic membranes had oxidase activities of maximum rates with methylated naphthohydroquinones and these activities could be inhibited by HQNO. The enzyme was insensitive to CN^- at low concentrations [94,130] and to antimycin and myxothiazol. These properties are are characteristic of a *bd*-type quinol oxidase.

Evidence for the existence of cytochrome bo_3 comes from redox spectra of cytoplasmic membranes reacted with CO. Moreover, a *Thermoplasma*-specific prenylated haem (called haem O_T, see Table 2) could be isolated, which also suggests the presence of a haem-copper oxidase.

5.6. *Sulfolobus acidocaldarius*

5.6.1. General

The genus *Sulfolobus* belongs to the hyperthermophilic and acidophilic archaea [134–136]. It has a wide distribution and different species and strains have been isolated from hot and acidic biotopes (geothermal areas, uranium mines) of different places in the world, like Yellowstone

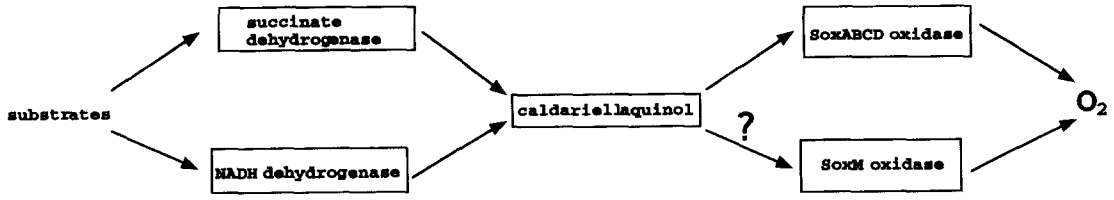

Fig. 6. Scheme of electron transfer chain in *Sulfolobus*. Additional substrate dehydrogenases may exist. Natural quinols other than caldariellaquinol, like Sulfolobusquinonol and tricyclic quinol (see Fig. 4 for structures), could also be substrates of SoxABCD. Quinol oxidation of SoxM complex was not be demonstrated unequivocally, therefore alternative electron donors could be considered (see text).

National Park, Iceland, Beppu lake district and Germany [137]. It requires low pH (1–3.5) and high temperatures (70–95° C) for optimum growth. In some cases it is difficult to compare data from different laboratories on this organism. This is due to the use of different species and strains of *Sulfolobus* under the same assignment, and has caused a remarkable confusion which is discussed in [138,139]. Most of the published data relevant to bioenergetics were obtained from studies of the type strain *S. acidocaldarius* DSM 639 and those are predominantly discussed in this section. *S. acidocaldarius* DSM 639 is an obligate aerobe that only grows heterotrophically. Special findings obtained with *Sulfolobus* strain 7, which is presumed to be '*S. acidocaldarius*' will be emphasized in a separate chapter.

In the first studies of the bioenergetics of *Sulfolobus* the relation between oxygen uptake and formation of a transmembrane proton gradient was established [140]. Respiration of intact cells is fully maintained with endogenous substrates, which probably originate from storage products. Broken cells most effectively oxidize the added substrates NADH and succinate. Initially, the electron transfer chain of heterotrophically grown *Sulfolobus acidocaldarius* was studied extensively with whole cells or with membrane particles, and then the respiratory chain was dissected into purified complexes. In the meantime, biochemical, molecular biological and biophysical studies provided many of the data. The scheme in Fig. 6 depicts the sequence of components in the electron transfer chain of *Sulfolobus*.

NADH and succinate dehydrogenase [141–143] were

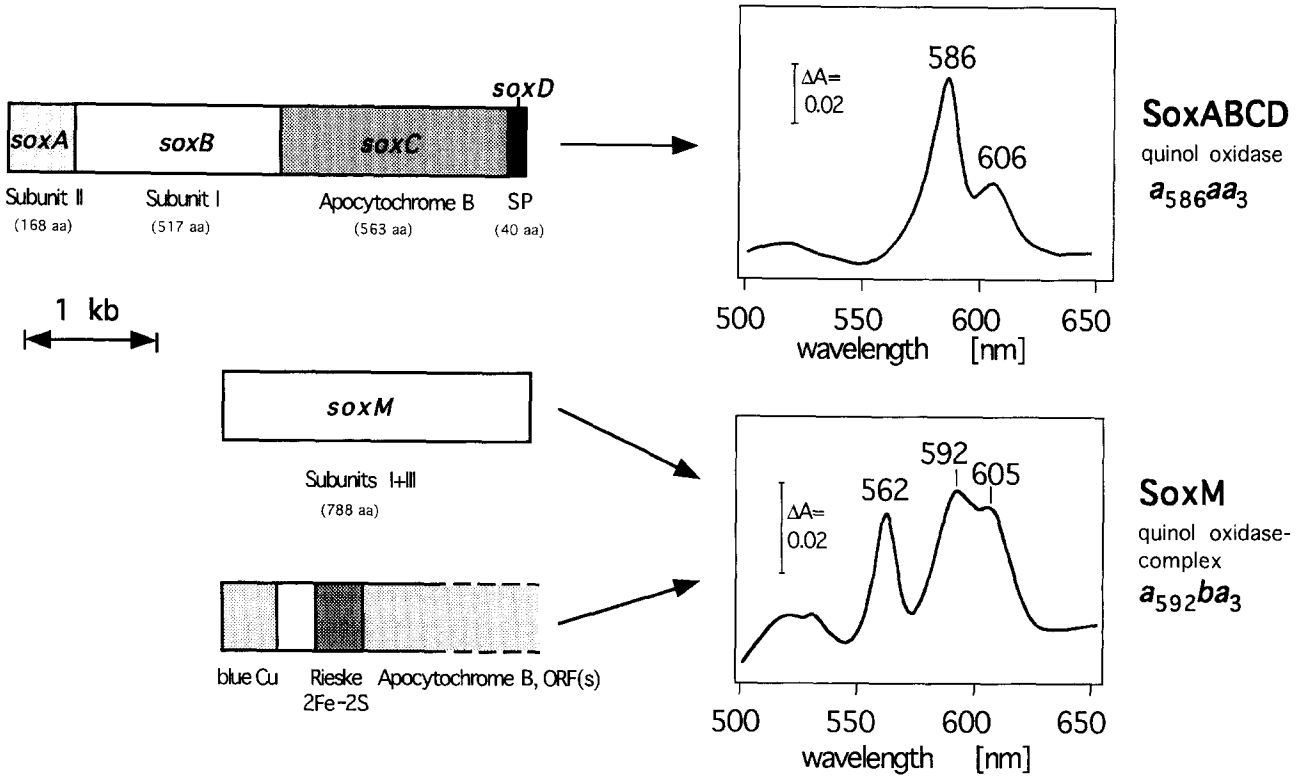

Fig. 7. Gene organization and structural models of terminal oxidase complexes of *Sulfolobus*. The gene products encoded in the *soxABCD* and *soxM* operons combine to form the SoxABCD and the SoxM oxidases. At least one other transcriptional unit providing further components is partially characterized (Jose Castresana and Matti Saraste, unpublished data) and it is schematically drawn to the bottom. The SoxM complex has an apocytochrome *b*, which is nonidentical to SoxC and an EPR-detectable Rieske 2Fe-2S centre, both of which are present in bacterial and mitochondrial bc_1 complexes. It is not known whether these *Sulfolobus* oxidases are vectorial proton pumps.

isolated, and three quinol species (see above) were identified. Besides several cytochromes *b*, there are at least three different polypeptides that bind the A-type haem A_S, which constitute the terminal oxidases SoxABCD and SoxM complex.

5.6.2. Cytochromes b

Sulfolobus expresses *b*-type cytochromes having absorbance maxima at 558, 562 and 566 nm, as seen in room temperature redox difference spectra [140,144,145]. The α absorbance peaks become sharper and slightly blue-shifted at low temperatures, but no additional signals could be resolved [146]. One of these *b*-type cytochromes, further denoted as $b_{558/566}$, was further studied (Table 1). The pigment has a double α absorbance band of 566 and 558 nm at room temperature, which shifts to 562 and 553 nm at 77 K [146]. Polypeptides of 18–20, 30 and 67 kDa have been copurified with cytochrome $b_{558/566}$.

Some properties of cytochrome $b_{558/566}$ were reminiscent of haem-copper oxidase: (i) the content of tightly bound Cu and (ii) the formation of a complex of CO with a high spin haem B which spectroscopically resembles cytochrome bo_3 [146]. However, without added solubilizers, the expected CO-binding signal of cytoplasmic membrane particles expressing the cytochrome $b_{558/566}$ was absent. Partial unfolding of the protein by detergents could therefore explain an artificial formation of ligand-binding high-spin haem B [147]. The purified protein had no catalytic activity with quinols or TMPD [146]. Recent data suggest that cytochrome $b_{558/566}$ is a 65 kDa polypeptide, to which one haem is bound (Günter Schäfer, personal communication). It has a redox midpoint potential of +0.375 V [148].

The synthesis of cytochrome $b_{558/566}$ depends on the oxygen tension in the growth medium: At high oxygen supply the pigment can not be detected [148]. At limiting concentration of oxygen the synthesis of cytochrome $b_{558/566}$ is induced to absorbance levels comparable with the *a*-type cytochromes. The functional role of this cytochrome remains unclear.

Another cytochrome *b* absorbing at 562 nm appears to be always present in the membranes. It is discussed below in the context of cytochrome *a*.

5.6.3. a-type cytochromes

The most abundant haemoproteins in *Sulfolobus* are the *a*-type cytochromes. They are represented by the two absorbance peaks at 586 and 606 nm of cytoplasmic

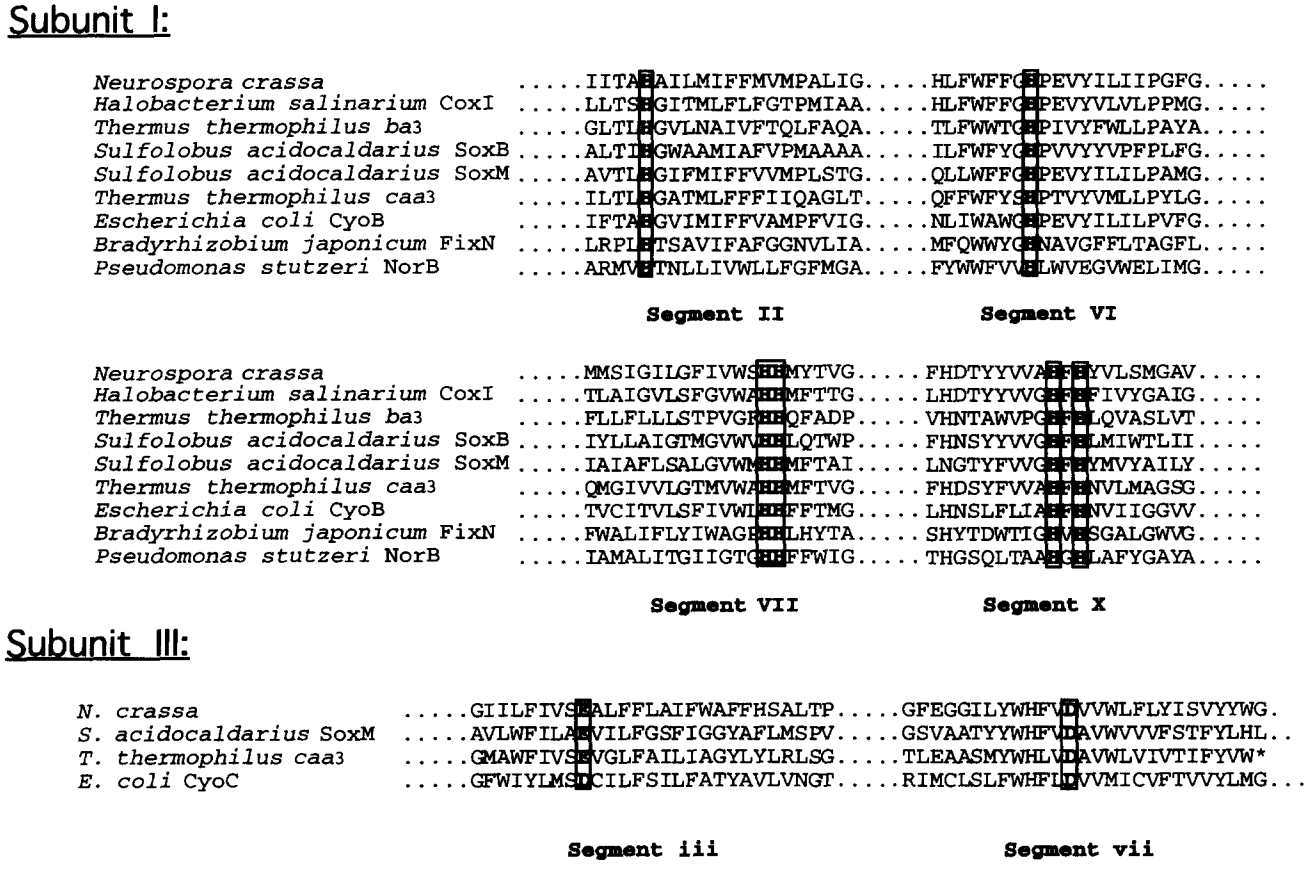

Fig. 8. Alignment of predicted transmembrane sequence fragments containing the haem-copper oxidase signatures. Sequence data were obtained from the EMBL database. Subunit I: For the *E. coli* cytochrome bo_3 the histidines (boxed) were demonstrated to be the ligands of copper and of haem irons. The polypeptides belong to haem-copper oxidases, which use cytochrome *c* or quinol as substrates; except NorB, the Subunit I-homologue from NO reductase [64]. The known archaeal sequences are from *Sulfolobus* and *Halobacterium*. Subunit III: The carboxylic acids in segments iii and vii (boxed) are highly conserved. Note nature's menace in the segment iii of SoxM ("...EVIL...") to scientists investigating archaeal cytochromes.

membranes [140,144]. A complex consisting of the pigments was purified [149,150], Table 1. Both cytochromes contain haem A_S as a prosthetic group and belong to the SoxABCD oxidase complex, which is encoded by the *sox* operon [150], shown in Fig. 7. The purified complex consists of four to five nonidentical polypeptides [95]. Four bands are visible on SDS PAGs, corresponding to the subunits SoxB and C (both 38 kDa), SoxA (27 kDa), SoxD and another polypeptide (both 5 kDa). The apparent molecular masses determined electrophoretically differ considerably from the calculated values [150], presumably due to their high content of hydrophobic amino acids. All subunits have been identified by sequencing of N-termini and/or internal fragments, whereas the SoxA, B and C subunits have also been identified immunologically with peptide-specific antisera. Despite the lack of cytochrome *c* in *Sulfolobus* [140], membranes exhibit cyanide-sensitive oxidase activity with added cytochrome *c* (from horse heart) [140,141,144]. The SoxABCD complex described in [95] is completely inactive with horse heart cytochrome *c* and has high turnover numbers only with quinols and TMPD used as substrates [95]. One part of the SoxABCD oxidase has been spectroscopically identified as cytochrome aa_3, as deduced by its absorbance maximum at 606 nm in redox difference spectra [85,150,151], and by the characteristic spectrum of its carbon monoxide complex. The spectroscopically determined ratio of haem A_S to cytochrome aa_3 is 4:1, which suggests the presence of three low-spin haems and one ligand binding high-spin haem per complex [150].

The cytochrome aa_3 portion of the SoxABCD complex was identified as SoxB, a homologue of subunit I of haem-copper oxidase [150], see Fig. 7. This polypeptide binds the two haem redox centres corresponding to cytochrome *a* and cytochrome a_3 parts. Two residual haem A_S molecules have to be accomodated by another part of the complex. The apoprotein of cytochrome a_{586} is most probably SoxC, which has potential haem binding sites, and is structurally related to cytochrome *b* of the mitochondrial complex III (or bc_1 complex, see also Fig. 3) [150]. In contrast to the bis-histidyl ligation of the haem irons found with bacterial and organellar cytochromes *b*, there is spectroscopic evidence (from magnetic circular dichroism and electron paramagnetic resonance techniques) for the contribution also of methionine to haem A_S binding of SoxC [152]. Interestingly, SoxC has an extended C-terminal sequence, which is unrelated to other proteins and may comprise 3–4 additional predicted transmembrane regions. SoxD (or 'SP') is a small hydrophobic peptide, without homology to other proteins. SoxA corresponds to subunit II of haem-copper oxidase [150], which is assumed to provide the substrate binding part of the protein. It is an amphipathic polypeptide with a single predicted N-terminal transmembrane span and a hydrophilic C-terminus. The latter domain lacks the characteristic residues shown to be ligands of Cu_A, and in this respect SoxA resembles subunit II of eubacterial quinol oxidases.

The Sox oxidase is encoded by the *sox* operon, which comprises the structural *soxABCD* genes [150]. Nothing is known about the genetic location of the other small polypeptide. Gene products similar to CtaA and CtaB, which have a role in haem A biosynthesis [153] and are located adjacent to the caa_3-type terminal oxidase operons of bacilli [154,155] have not yet been isolated.

The pigment absorbing at 606 nm could be copurified with the SoxB component as a single polypeptide by using a highly disruptive detergent [85]. This preparation had caldariellaquinol and TMPD oxidase activity and is apparently free of associated SoxC and SoxA [148]. SoxB itself can therefore be considered as a minimum form of the oxidase. The catalytic subunit contains the metal-binding ligand signatures (Fig. 8A). The binuclear haem A_S-Cu_B reaction centre of the isolated SoxB preparation and the

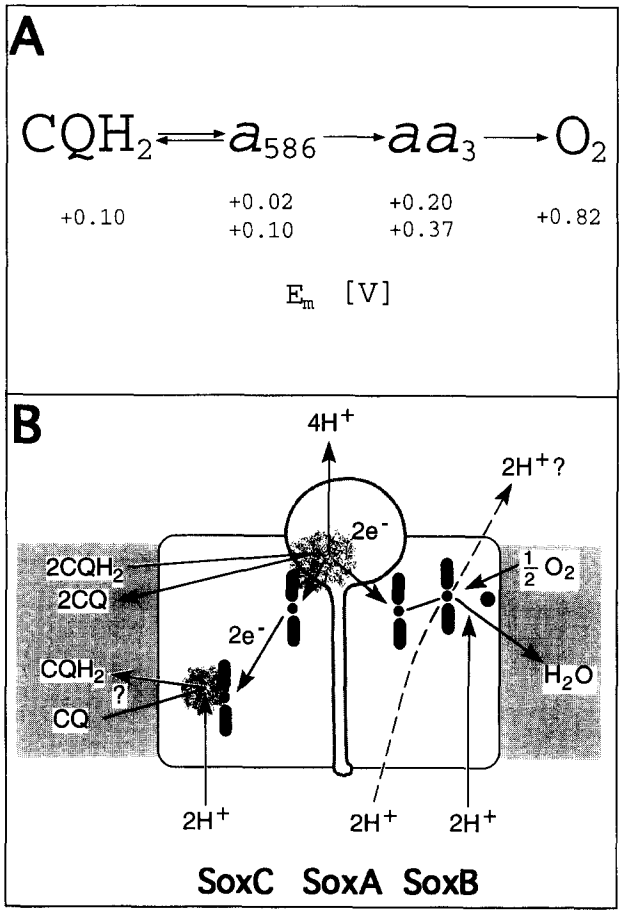

Fig. 9. (A) Putative electron flow in SoxABCD oxidase. SoxC and SoxB are the apoproteins forming the pigments $a_{586\ and\ aa3}$. The redox midpoint potentials (E_m) were spectroscopically determined from the purified components, with data taken from [85,148]. Operation of the Q-cycle [161,205] similar to bc_1 complexes requires partial reversibility of electron transfer between the quinol and the cytochrome *b* homologue. (B) Model of the function of SoxABCD oxidase working as a miniaturized cytochrome $bc_1 + aa_3$ "supercomplex" (see explanations in the text). Modified after [150].

haem-apoprotein interaction were probed with biophysical techniques [85,156–158]. Redox midpoint potentials (E_m) determined with optical and electron paramagnetic resonance methods of +0.20 to +0.21 V and +0.37 V were consistent [85,156].

Redox potential determinations obtained with partially purified cytochrome a_{586} (corresponding to SoxC see above) indicated two redox centres with +0.02 V and +0.10 V [148]. Combined with the half reduction potential of caldariellaquinol (E_m = +0.1 V) [85], one can sort the redox potential data determined from the isolated pigments in an electron transfer scheme shown in Fig. 9A.

Why is SoxABCD more complicated than a singular subunit quinol oxidase? The assembled multi-subunit complex probably operates more efficiently than SoxB alone. An association with the cytochrome *b* portion of bc_1 complexes does not occur in eubacterial quinol oxidases. In an attempt to explain the binding of SoxB to the cytochrome *b* homologue SoxC, a model of the SoxABCD complex was proposed, in which the cytochrome *b* and cytochrome aa_3 homologues of SoxABCD represent the catalytic cores of complexes III and IV (Fig. 9B). These cytochromes may functionally cooperate in the SoxABCD complex as has been described for a multisubunit respiratory supercomplex ($bc_1 + aa_3$) found in some eubacteria [159,160]. It has been suggested that the *Sulfolobus* complex links electron transfer between the Q-cycle [161] and terminal oxidase. In the putative reaction scheme shown in Fig. 9B, one half of the electrons from CQH_2 pass the SoxC portion in a Q-cycle like manner, whereas the other half directly enters the terminal oxidase part provided by SoxB [150]. If the whole process is coupled to proton pumping (see below), the extra redox loop introduced by SoxC would increase the free energy yield of quinol oxidation. SoxABCD could thus do the work of two electron transfer complexes by using a minimum number of subunits and may thus be regarded as an economy version of one half of the respiratory chain.

SoxM is another terminal oxidase that copurifies with a pigment absorbing at 562 nm (further denoted as cytochrome b_{562}) [162]. The *soxM* gene encodes a fusion of subunits I and III of a haem-copper oxidase similar to cytochrome caa_3 of *Thermus thermophilus* [163] and to artificial constructs of the *E. coli* bo_3 oxidase [164]. SoxM has 19 stretches of hydrophobic amino acids predicted to be transmembrane segments (Fig. 10A, B). It has the typical sequence signatures in subunit I of haem-copper oxidases, the invariant histidines in segments II, VI, VII and X (Fig. 8, Fig. 10B). The highly conserved acidic residues [72] in the subunit III part of the fusion are also present. SDS-PAGE resolves a polypeptide with an apparent molecular mass of 45 kDa. It is not known whether posttranslational cleavage into pieces corresponding to subunit I and III occurs. SoxM has been prepared in a complex (designated as SoxM complex) with a protein immunologically related to SoxC (see below) and an iron-

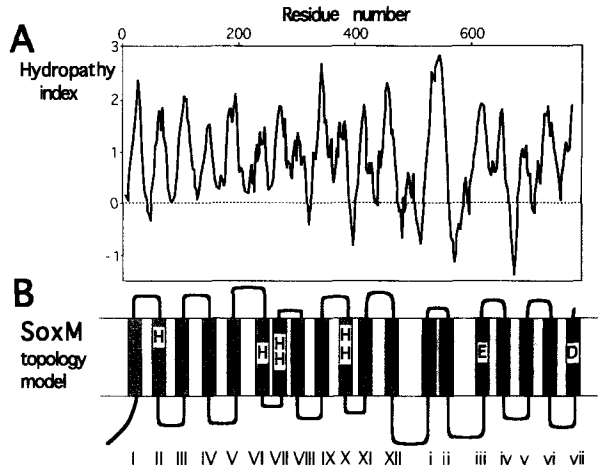

Fig. 10. The SoxM fusion protein of Subunits I and III of haem-copper oxidase. (A) calculated hydrophobicity from sequence data [206]. (B) Topological model based on the transmembrane organization of the homologous *E. coli* cytochrome bo_3 [207]. The signatures of haem-copper oxidases located within very hydrophobic amino acid sequences are marked, i.e., the histidines in the N-terminal Subunit I portion and the conserved carboxylic acids in the in the C-terminal Subunit III portion. Predicted transmembrane regions are drawn in grey (Subunit I, segments I–XII) and black (Subunit III, segments i–vii).

sulfur protein [165], both of which are homologues of cytochrome *b* and Rieske 2Fe-2S protein of complex III in mitochondrial and eubacterial respiratory chains [162].

The membrane-bound 2Fe-2S cluster of *Sulfolobus* has a strongly pH-dependent redox potential of +0.4 V [166]. Added horse heart cytochrome *c* reduces the Rieske protein in the presence of the oxidase inhibitor cyanide [167]. Thus it is possible that the Rieske iron-sulfur centre provides the electron entry site responsible for the observed oxidation of horse heart cytochrome *c* by cytoplasmic membranes (see above). Due to its complex formation with the Rieske protein, SoxM can be suggested to receive the electrons and to act as terminal oxidase with this substrate. However, the cytochrome *c* oxidase activity of the SoxM preparation [162] was found to be negligible under the applied test conditions (M. L. and Matti Saraste). Recently, the gene encoding the 30 kDa Rieske iron-sulfur protein was cloned and identified by its N-terminal amino acid sequence. Located adjacently to it are open reading frames, which encode an apocytochrome *b* and another subunit of haem-copper oxidase (Fig. 7). Next to this cluster in the reverse orientation there is a stretch of DNA which codes for a homologue of blue copper proteins (Jose Castresana and Matti Saraste, unpublished observations). Intriguingly, another Rieske-type 2Fe-2S protein, being nonidentical with the one mentioned above, was discovered from the same strain of *Sulfolobus* (Günter Schäfer, personal communication). It is not known whether these gene products take part of the SoxM oxidase complex.

Spectroscopically, SoxM consists of cytochrome b_{562}, as it has a low-spin haem B. Further peaks at 592 and 605 nm are related to the haem A_S group. The absorbance

maximum at 592 nm was formerly assumed to originate from the SoxC protein, because the 38 kDa band of the SoxM complex cross-reacted with antisera raised against the N-terminal peptide of SoxC [162]. However, protein sequencing identified it as the apocytochrome *b* which is encoded within the Rieske gene cluster (Fig. 7) (Jose Castresana and Matti Saraste, unpublished observations).

The apoprotein of cytochrome aa_3, SoxB, is absent from the complex [162], so that the signal at 605 nm appears to be a different cytochrome *a* (Fig. 7). SoxM oxidizes TMPD and is inhibited by azide and cyanide. The carbon monoxide binding difference spectrum has the features of cytochrome a_3, exhibiting maxima at 433 and 596 nm and a minimum at 447 nm. Small contributions of a ligand-binding high-spin haem B could be due to partial denaturation or to facultative binding of haems B and A_S in the reaction centre. Since the ratio of haem A_S to B is roughly 3:1, it is possible that the apocytochrome *b*, like SoxC, binds two haems A. SoxM can have a haem B at the low-spin site, whereas the high-spin site is occupied with haem A_S. The use of different haem species at the two binding sites of cytochrome oxidases is known for eubacterial and archaeal oxidases (see [94,168], and references therein). Alternative binding of different haems to the same site of the apoprotein ('haem promiscuity') was already reported for some eubacteria [169–171].

5.6.4. Proton translocation

The capability of translocating protons across the membrane is a typical feature of haem-copper oxidases [172] (Fig. 3). Protonmotive force can be generated by scalar and vectorial processes (Fig. 3). By the scalar mechanism, substrate oxidation and water formation take place at different sides of the membrane, and quinol oxidation liberates the so-called 'scalar' protons on the outside, while protons are consumed at the inside by synthesis of water (for example, see Fig. 9B, straight arrows). The other mechanism involves the translocation of 'vectorial' protons by active transport in a carrier-like manner (Fig. 9B, dashed arrow). Mitochondrial and eubacterial haem-copper oxidases pump two vectorial protons per oxygen atom reduced, i.e., 1 H^+/e^-.

Acidophiles like *Sulfolobus* certainly have operative proton translocators, since growing cultures continuously create cytoplasmic space with a higher pH than the medium. In principle, generation of a proton gradient by a scalar mechanism would be sufficient to fulfil this task, provided that the oxidase activity is high enough. A higher efficiency of energy conversion could be obtained if protons would also be actively transported across the membrane. SoxABCD and SoxM complex are so different from other haem-copper oxidases, that *a priori* vectorial proton pumping is questionable unless proven explicitly.

Using tetraether lipids prepared from *Sulfolobus* [173] the isolated SoxB preparation was successfully reconstituted into lipid vesicles [174]. A majority of the oxidase particles seemed to be incorporated in the same orientation as in the cytoplasmic membrane. A significant net ΔpH (inside alkaline) and membrane potential (inside negative) built up after addition of the artificial substrate TMPD in presence of the reductant ascorbic acid. Incorporation into liposomes of a preparation consisting of both cytochrome aa_3 *plus* cytochrome a_{586} (SoxABCD oxidase complex, see above) did not generate a protonmotive force higher than the SoxB preparation alone. It is not clear from these data whether the isolated SoxB functions as a vectorial H^+ pump. [174]. The reduced form of TMPD freely permeates the membrane and delivers substrate to the inversely incorporated oxidase molecules, which then leads to proton translocation in the opposite direction. A proton/oxygen stoichiometry can not be determined, unless the oxidase has a unique orientation in the liposome membrane.

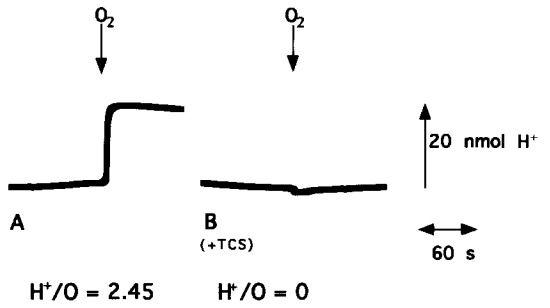

Fig. 11. Demonstration of vectorial proton translocation of *Sulfolobus* cells by oxygen-pulse method. (A) cells (3 mg of protein) were suspended in 0.1 mM K^+-Mes, 0.1 mM EDTA, 50 mM K_2SO_4 (pH 5.8–6.0). 15 nmol of valinomycin per mg of cell protein was added. The suspension (total volume = 3.5 ml) was kept at 40° C in a closed water-jacketed reaction vessel and was made anaerobic by argon bubbling in the presence of 15 mM ascorbic acid and 30 μM TMPD. Oxygen pulses (3.8 nmol O_2) were generated by rapid injection of 20 μl of 50 mM K_2SO_4. (B) as in (A), but with 8 nmol of the protonophore TCS per mg of protein present.

These complications can be avoided by carrying out the so-called oxygen pulse experiments (see Fig. 11) in which intact *Sulfolobus* cells provide a 100% 'right-side-out' orientation. Conditions can be adjusted so that oxygen uptake rates with endogenous substrates are negligible compared to those with the substrate/mediator pair ascorbate/TMPD. The artificial electron donors fuel the terminal oxidases, since their redox midpoint potentials are in the appropriate range. In fact, the reaction releases up to 3 protons per oxygen atom into the medium, a single scalar proton from the oxidation of ascorbate to dehydroascorbate ($H^+/O = 1$), *plus* two additionally translocated, vectorial protons ($H^+/O = 2$). The experimental ratios are between $H^+/O = 2$ to 3 (Fig. 11), which means that *Sulfolobus* cells have at least one oxidase that can pump protons vectorially. Since both SoxABCD and SoxM oxidases are expressed at comparable levels [162], the identity of the proton pump is not clear. Earlier experiments with intact *Sulfolobus* cells showed a total number of up to 12 protons per oxygen with endogenous substrate [138,175], which

would indicate the presence of several coupling sites in the cytoplasmic membrane.

5.7. Thermoacidophiles related to S. acidocaldarius

Sulfolobus strain 7 is an isolate from the Beppu lake district (Japan). It probably represents another species or it may even belong to another genus than the type strain of *S. acidocaldarius*, because the cell wall glycoproteins of both organisms are dramatically different [176,177]. From strain 7, Wakagi et al. reported a cytochrome *a* preparation (Table 1), which had maxima at 583 and 603 nm in redox difference spectra [149]. Spectroscopically, the complex closely resembles the SoxABCD complex. Apparently the isolated protein retained horse heart cytochrome *c* activity, which could be observed in intact membranes of strain 7 as well as in *S. acidocaldarius* DSM 639. However, high concentrations of detergents were necessary for this reaction from *S. acidocaldarius* strain 7. It has yet to be clarified whether this effect is due to activation of the enzyme or of its exogenously added substrate, cytochrome *c*. The purified protein has subunits with apparent molecular masses of 37, 23 and 14 kDa [149]. The authors claimed that in addition to cytochrome *c* oxidase, a distinct caldariellaquinol oxidase exists in membranes of this strain.

A cytochrome aa_3-type caldariellaquinol oxidase was purified from *Desulfurolobus ambivalens*, a facultative aerobe closely related to *Sulfolobus* [178]. Qualitatively this organism has a cytochrome pattern similar to *S. acidocaldarius*. However, in redox difference spectra of membranes from *D. ambivalens* the peak of cytochrome aa_3 at 603 nm was much more intense than the cytochrome *b* signal at 562 nm and the band at 587 nm, which is reminiscent of SoxC. The stoichiometric relations of the aa_3 and the *a*-587 portion, as derived for SoxABCD oxidase of *Sulfolobus*, did not seem to apply for *Desulfurolobus*. The gene encoding Subunit II was already cloned, confirming its affiliation to the haem-copper oxidase family (Günter Schäfer, personal communication).

The three-subunit complex (Table 1) contains approximately 1 Cu per aa_3 and it has three polypeptides with molecular masses of 40, 27 and 20 kDa.

6. Archaeal complexes: novel inventions or variations of a theme?

The heterodisulfide reductase is an example of a proton pump working in a highly specialized environment. No eubacterial system operates in a similar fashion. On the other hand, it shows that the concept of coupling electron transfer to ion movements is very generally utilized. Thus, energy conserving reactions involving cytochromes are not restricted to chains in which oxygen is the terminal electron acceptor. The principle of anaerobic electron transfer coupled to ion transport may function also in other, as yet unidentified systems in archaea and bacteria.

The above compilation of data describes a number of electron transfer chain catalysts of aerobically grown archaea, which have a similar architecture to that for eubacteria or mitochondria. In these cases it would be expected that they also have analogous functions, i.e., formation of protonmotive force and the process of oxidative phosphorylation.

In some cases the individual components combine to form novel complexes. For instance, this occurs in the SoxABCD oxidase of *Sulfolobus*, where subunits homologous to complex III (apocytochrome *b*) and IV (subunit I and II of haem-copper oxidase) together build a novel unit (Figs. 7, 9B). The SoxM-oxidase is another formation, in which the components apocytochrome *b* and the Rieske protein occur in complex III and the SoxM is a fusion of subunits I plus III of haem-copper oxidase (Figs. 7, 10B). The SoxM complex may also integrate other gene products expressed from the third transcriptional unit (Fig. 7).

The halobacterial systems are not yet resolved enough to draw further conclusions. In the electron transfer systems of *Sulfolobus*, individual components apparently have been mixed in a combination different from bacterial and mitochondrial complexes. Archaeal complexes seem to be derived from a series of evolutionary events happening in restricted habitats. Subunits or catalytical cores from a basic arsenal could have been shuffled to create entities with novel properties. The process may have led to several differently shaped complexes in bacteria and archaea, due to the different environmental conditions, and the fine-tuning of molecular adaptation.

Another point of interest is the nature and the position of electron donors in the archaeal respiratory complexes. The electron-rich substrate for *Sulfolobus* oxidases, at least for SoxABCD, appears to be caldariellaquinol. One may further ask how ferredoxin [179] interacts with the respiratory chain of this organism? The role of small blue copper proteins in archaea [122,123] remains to be elucidated. For example, halocyanin could well be a substrate of the terminal oxidase of *Natronobacterium pharaonis*. The product of the recently discovered gene encoding a blue copper protein in *Sulfolobus* might be a similar case. The cytochrome oxidase of the eubacterium *Thiobacillus ferrooxidans* uses rusticyanin, an endogenous blue copper protein occurring in the periplasmic space [180]. Although soluble cytochrome *c* does not occur normally in aerobic archaea, membranes of *Sulfolobus acidocaldarius* and of *Halobacterium salinarium* show in vitro cyanide-sensitive horse-heart cytochrome *c* oxidation. This feature remains to be elucidated.

7. Evolution of respiration

Energy conversion by light or redox reactions can be considered as a prerequisite for developement of the au-

totrophic growth mode. Redox reactions between simple inorganic compounds like H_2S and pyrite ($= FeS_2$) could have provided the driving force for a primitive metabolism that developed in an ancient anaerobic era [15,181,182].

In earlier hypotheses, the development of respiration was dated to the geological period, when oxygen concentration in the atmosphere began to increase. This assumption was based mainly on a logical idea that respiratory reactions are meaningful only when the terminal electron acceptor O_2 exists in sufficient amounts [183–185]. The independent rise of aerobic metabolism in several evolutionary lines (being defined by the phylogenetic trees based on 16 S rRNA comparison [2]) was postulated in order to explain the fact that extant aerobic organisms do not form a monophyletic clade [2,184,186].

Analysis of primary structures of respiratory catalysts is an alternative approach to study their phylogenetic relationships. One could attempt to reconstruct the evolution of respiration when the molecular history of an appropriate enzyme is known. Haem-copper quinol and cytochrome *c* oxidases are functionally diverse and structurally closely related, and they form a superfamily [187], which is suited for investigation of phylogenetic relationships. The amino acid sequences of the catalytic subunit I of both oxidases are highly conserved, especially in the segments forming the metal-binding signatures (Fig. 8A). From sequence alignments comprising these fragments alone, a phylogenetic tree has been computed [188]. Most probably, an early gene duplication has led to the generation of two homologous polypeptides of Subunit I in the eubacterium *Thermus* and in the archaeon *Sulfolobus* (Fig. 12). The two types of cytochrome oxidase belong to different branches of the tree, suggesting that the enzyme had already existed in the common precursor cell before archaea and bacteria diverged into different domains.

In a recent study [189], the distance analysis was based on comparisons of complete sequences of subunits I and II.

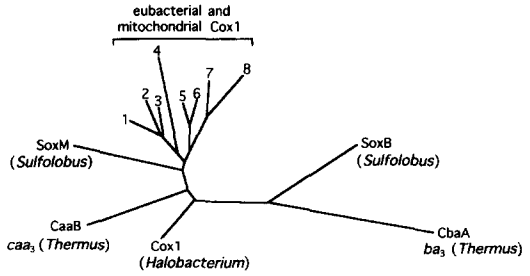

Fig. 12. Relationship of haem-copper oxidases. The unrooted tree was constructed after alignment of partial amino acid sequences, which form the metal-binding regions of Subunit I (Histidine signatures) and computation of sequence distances. The figure was taken from [188] and modified to underline the grouping of the oxidases of *Thermus* and *Sulfolobus*. Original sequence data were extracted from the EMBL database. (1) *Neurospora crassa* Cox1, (2) *Paracoccus denitrificans* CtaD, (3) *Bradyrhizobium japonicum* CoxA, (4) *Bradyrhizobium japonicum* CyoB, (5) Thermophilic bacterium PS3 CaaB, (6) *Bacillus subtilis* CtaD, (7) *Bacillus subtilis* QoxB, (8) *Escherichia coli* CyoB.

Dendrograms calculated mainly from sequence data of subunit I data further corroborate the fact that the oxidase was already present in the ancestor of archaea and eubacteria. Photosystem II, which is the catalyst responsible for photosynthetic oxygen production, developed after the archaea-bacteria split exclusively in bacteria and plastids. Thus, cytochrome oxidase appears to be older than its substrate, molecular oxygen [189].

The search after the uroxidase [189] led to the finding of FixN, which is the subunit I of a cytochrome oxidase in nitrogen-fixing bacteria [90]. FixN is distantly related to the haem-copper oxidase family (Fig. 8A). In its natural environment the enzyme has to function under almost anaerobiosis, i.e., an oxygen concentration in the nanomolar range [190]. Low oxygen tension may also have prevailed during early steps of life. Thus it is plausible that aerobic metabolism could operate within the confined environments of a low concentration of bulk oxygen.

New light on ancestral stages of cytochrome oxidase was shed upon by the exciting discovery of an even more distant relation between NorB [64] and subunit I of the haem-copper oxidase superfamily [76,191]. NorB is the catalytic subunit of nitric oxide reductase, an enzyme involved in the strictly anaerobic process of denitrification. Like subunit I, NorB is an integral membrane protein with 12 putative transmembrane regions [64], it has the histidine-rich sequence patterns typical for oxidases in the corresponding regions (Fig. 8A). The existence of a binuclear high-spin haem iron-copper centre has been proposed from the amino acid sequence [76]. High-spin and low-spin haems have been identified spectroscopically in NorB [126], whereas Cu_B and its possible integration into a binuclear centre remains to be demonstrated.

Another link between respiratory and denitrification proteins is the similarity of sequence patterns for Cu_A binding ligands in subunit II of oxidase and the subunit of nitrous oxide reductase [191]. It appears possible that a common ancestral protein used the same ligand geometry for carrying out electron transfer on different substrates and that a specialization for acceptors developed at a later stage. The versatile transition metal copper is embedded in a limited number of different ligand environments, in which it contributes to distinct electron transfer steps. Notably copper is present as a cofactor in almost all terminal oxidases of respiration and in oxidoreductases of denitrification [66,192], which suggests a common theme in the construction of these catalysts.

8. Conclusions and perspective

Archaea have respiratory catalysts, which are homologous to those of eubacteria and mitochondria. This could already be predicted from the numerous reports on the isolation and physicochemical characterization of the respiratory proteins. In some cases the relationships have been

objectively proven by comparison of the primary structure, which yields a quantitative measure of phylogenetic distance. This approach has been extensively applied to archaeal haem-copper oxidases. Eubacteria have complex electron transfer chains and they express multiple terminal oxidases (Fig. 3). Branched respiratory chains exist also in archaea; however, little is known about 'alternative' oxidases. For example, of the archaea tested, only *Thermoplasma* shows features of cytochrome *bd* oxidase. This remains to be studied in more detail.

The FixN oxidase was detected in N-fixing eubacteria, and enzymes with similar properties were isolated also from *Rhodobacter* species [91,92] and *Paracoccus* [67]. These purple non-sulfur bacteria have enormous metabolic flexibility and can adapt to diverse environmental conditions, e.g., they grow at a wide range of oxygen tensions. Certain archaea thrive also under microaerobic conditions that require the expression of high oxygen-affinity oxidases, e.g., *Pyrobaculum* or some halophiles. These archaea could well have cytochrome oxidases related to FixN. The same might be expected for nitric oxide and nitrous oxide reductase, the enzymes of denitrification distantly related to oxidase [191]. Archaeal homologues of these reductases are not known. However, the process of denitrification is reported to operate in halobacteria as well as in the thermophile *Pyrobaculum* [125,192–196]. In the latter organism the occurrence of nitric oxide and other intermediates of the reaction sequence was proven [125]. Thus, it can be assumed that the enzymes nitric oxide and nitrous oxide reductase are expressed in *Pyrobaculum*. If one wants to understand the phylogenetic relationships of respiratory enzymes and the evolution of respiration itself, it is necessary to search for the homologous proteins also in archaea.

Initially it was postulated that archaeal cytochromes are catalysts with simpler structure than their eubacterial or eukaryotic counterparts, but that they function identically. If this were true, archaeal representatives of this class of proteins would be ideal subjects for functional and structural studies. In regard to the archaeal electron transfer proteins it is difficult to state whether we deal with more simple or complex systems. For instance, the SoxABCD oxidase combines the core structures of two respiratory complexes in the minimum number of four subunits, whereas the simplest comparable eubacterial system requires seven. SoxABCD condenses one branch of the respiratory chain into a single complex and presumably is also functional as such. Although SoxABCD has a simpler composition, the oxidase as a whole is more difficult to study. However, further dissection of the SoxABCD oxidase yielded a single polypeptide carrying out quinol oxidase function. The polypeptide is a good candidate for further structural investigations. Similar relations may be also be discovered with other catalysts and in this respect, further screening of archaeal proteins, especially those involved in energy transduction, is a promising task. In addition to the possibility of finding representatives which can be easier studied, further questions of the evolution of respiratory systems may be addressed.

9. Notes added in proof (received 2 February 1995)

(1) A recent paper reports on the reduction kinetics of membranes from *Sulfolobus* [208]. The data suggest that 'cytochrome a_{587}' is a redox carrier which equilbrates in two different redox pools. This is consistent with the presence of two nonidentical apocytochrome *b*-haem AS complexes which belong either to SoxABCD or to SoxM oxidase.

(2) Another publication describes the biochemical characterization of a 32 kDa Rieske-type protein from *Sulfolobus* [209]. The N-terminal amino acid sequence of this purified protein differs from the one reported from the 30 kDa component of the SoxM complex preparation in [162]. The latter polypeptide has been identified as Rieske-type protein by gene sequencing (Jose Castresana et al., unpublished data). It is possible that two Rieske-type proteins exist in *Sulfolobus*.

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