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Review

Multiple Rieske proteins in prokaryotes: Where and why?

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Abstract

Many microbial genomes have been sequenced in the recent years. Multiple genes encoding Rieske iron-sulfur proteins, which are subunits of cytochrome *bc*-type complexes or oxygenases, have been detected in many pro- and eukaryotic genomes. The diversity of substrates, co-substrates and reactions offers obvious explanations for the diversity of the low potential Rieske proteins associated with oxygenases, but the physiological significance of the multiple genes encoding high potential Rieske proteins associated with the cytochrome bc-type complexes remains elusive. For some organisms, investigations into the function of the later group of genes have been initiated. Here, we summarize recent finding on the characteristics and physiological functions of multiple high potential Rieske proteins in prokaryotes. We suggest that the existence of multiple high potential Rieske proteins in prokaryotes could be one way of allowing an organism to adapt their electron transfer chains to changing environmental conditions.

Keywords: Cyanobacteria; Sulfolobus; Gene family; Cytochrome bc_1; Cytochrome b_6f

1. Introduction

Since the first discovery of the Rieske iron-sulfur protein [1], the Rieske proteins have been recognized to be a large protein family containing functionally diverse groups. The first group, the "true", or perhaps more appropriately "high potential", Rieske proteins are present in many photosynthetic or respiratory electron transfer chains of all three kingdoms of life. The second group consists of the so-called Rieske-type, or low potential Rieske proteins, which appear to be equally widely distributed [2]. Both groups host the same cofactor, an [2Fe–2S] iron–sulfur cluster bound to the protein via two cystein-sulfur atoms and two nitrogen atoms from the imidazole ring of histidine residues and display a number of distinctive properties compared to [2Fe–2S] clusters with a pure sulfur ligation. This centre displays a rhombic EPR spectrum of the reduced form with a g_y in the range from 1.89 to 1.91 compared to 1.92 to 1.94, redox potentials that are significantly (between 100 mV and 800 mV) more positive than in the case of a pure sulfur ligandation, and one to two protonation equilibria are associated with the redox reaction [3,4]. Previously, the later effect was believed to be restricted to the "high potential" Rieske proteins. However, recent results published in Zu et al. [5] suggest that the redox potentials of all Rieske-[2Fe–2S]-clusters display the same principle pH dependence, but very different pK_{a1}-values [5], which range from 4 to 8 for high potential Rieske proteins [6] and is 9.8 for the low potential Rieske ferredoxin BphF [5], the only member of this group analyzed in detail.

The majority of currently identified Rieske proteins can be assigned to one of these two groups. However, some Rieske-like proteins cannot be clearly classified, indicating the possibility of a third group of Rieske-like proteins. The first characterized members of this new group are the Rieske subunits of the arsenit oxidases [7–9]. An initial phylogenetic analysis [8] suggested that these proteins form a distinct group beside the bacterial and archaeal high potential Rieske proteins (see also Fig. 3).

The function of Rieske proteins is electron transfer. Low potential Rieske proteins may occur as individual proteins, like the Rieske ferredoxins, or as domains of hydroxylases, or oxygenases. Their physiological electron donors are other iron–sulfur-clusters and cytochromes. The electron acceptor is

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either the low potential Rieske iron–sulfur-cluster of a hydroxylase or oxygenase, or oxygen bound to active site of these enzymes [2]. To date, the majority of the characterized high potential Rieske proteins are essential subunits of the cytochrome bc-type complexes. Different types of these complexes can be distinguished based on the nature of the high potential cytochrome (c_1, f, or other) and the di-heme low potential cytochrome (cytochrome b vs. cytochrome b_6 plus subunit IV). However, they all share the same basic minimal structure shown in Fig. 1 using the beef heart mitochondrial complex as a model. The electron donors of these complexes are typically quinols, and electron acceptors are high potential redox proteins, like cytochromes, blue copper proteins [2] or high potential iron–sulfur proteins [10].

The Rieske subunits of the arsenit oxidases are firmly attached to the large subunit of the enzyme that harbors the active site with the molybdenum cofactor and the [3Fe–4S] iron–sulfur cluster. The electrons are transferred from the active site via the [3Fe–4S] cluster to the Rieske cluster. Oxidants for the Rieske cluster are periplasmatic cytochromes, or copper proteins, which in turn are suggested to be re-oxidized by the photosynthetic reaction center [8] or the terminal oxidases of non-photosynthetic organisms.

The principle electron transfer reactions of a cytochrome bc-type complex are outlined in Fig. 1. A quinol is bound to the quinol oxidation site Q_o and transfers the first electron along

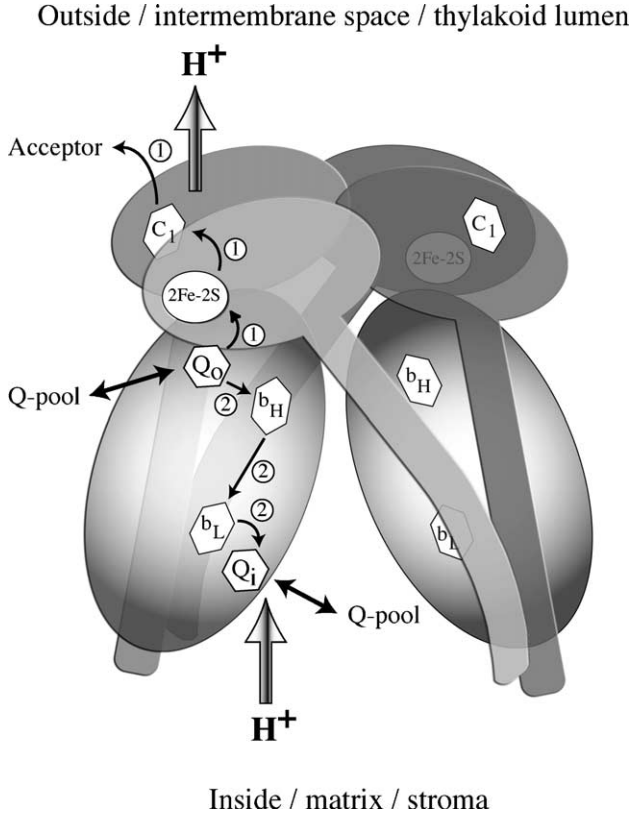

Fig. 1. Schematic view of the redox cofactor bearing subunits and electron transfer reactions of a cytochrome bc_1 complex. The numbered arrows indicate the path of the first, respectively second electron. The coordinates of the beef heart mitochondrial complex (1PPJ.pdb) were used as template to draw the scheme.

with a proton to the Rieske protein. The globular domain of now reduced, protonated Rieske protein reorientates to a position close to the heme group of the high potential cytochrome and transfers the electron to this redox center. The second electron from the quinol is transferred through the heme groups b_H and b_L to the quinone at the quinone reduction site Q_i. At this point, it should be noted that the details of the reactions at the Q_o side are still a matter of debate. The quinone generated at the Q_o side is then exchanged with a quinol from the pool and the reaction starts again. Since the protons generated at the Q_o site are released to one side of the membrane (the outside of the cell or the corresponding compartment in the case of organelles) and the protons needed for protonation at the Q_i site are taken up from the opposite side of the membrane, this results in the generation of a proton gradient across the membrane [11].

All Rieske proteins contain several conserved sequence motifs [2]. The most strictly conserved residues are the iron–sulfur-cluster-binding motifs Box I [**C**x**H**xGC] and Box II [**C**xC**H**x(S,A,G)x(Y,F)]. The iron–sulfur-cluster is coordinated by the first cysteine and histidine of each motif, whereas the second cysteine is involved in the formation of a disulfide bond. The presence of this disulfide bond, or at least of the second conserved cysteines, is one of the most important differences between a high potential and the low potential Rieske proteins. This bond, or at least the conserved cysteines, is present in all known high potential Rieske proteins, but is not typical for low potential Rieske proteins. Currently, the Rieske-subunits of the arsenit oxydases from *Alcaligenes*, *Chloroflexus* and *Cenibacterium* are the only examples of Rieske proteins not associated with a cytochrome bc-type complex, but containing this disulfide bond [8].

As a consequence of their different functional contexts, high and low potential Rieske proteins display different midpoint potentials. They are in the range of +100 to +570 mV for the high potential and in the range from −150 to +5 mV for the low potential Rieske proteins. An intermediate midpoint potential in the range of +60 to +100 mV was reported for the Rieske subunits of the arsenit oxidases [8].

Based on their properties, the Rieske subunits of the arsenit oxidases appear to represent a link between the high and low potential Rieske proteins. The tight and rigid binding of the Rieske subunit to the large subunit of the arsenit oxidases and its function as an electron transfer center are reminiscent of the low potential Rieske centers of the oxygenases and hydroxylases. Whereas, the relatively high midpoint potential, the membrane anchor and presence of the disulfide bond adjacent to the iron–sulfur cluster resemble the Rieske subunits of the cytochrome bc-type complexes.

The general structure of a high-potential Rieske protein is shown in Fig. 2A for the protein from the bovine mitochondrial cytochrome bc_1 complex. The protein consists of a single transmembrane helix that is linked by a flexile hinge region to the iron–sulfur-cluster bearing globular domain. The globular domain consists of 3β-sheets and a single α-helix and can be further divided into a large domain and a smaller rubredoxin-

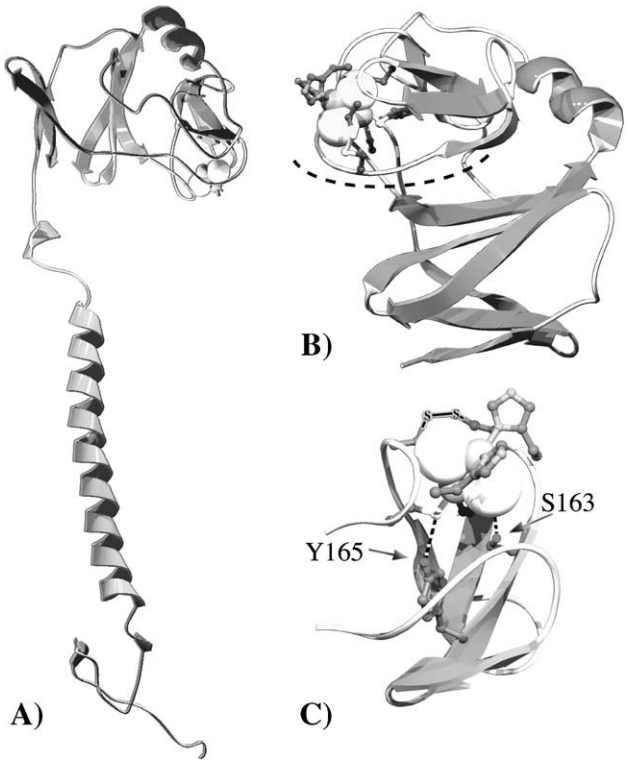

Fig. 2. (A) Overview of the structure of the Rieske protein from the beef heart mitochondrial cytochrome bc1 complex (from the coordinates of 1PPJ.pdb). (B) Close up of the globular domain. Dashed line indicates the boundary between the two sub-domains. (C) Enlarged view of the iron–sulfur cluster binding sub-domain showing the disulfide bond and the two hydrogen bonds discussed in the text.

like iron–sulfur-cluster binding sub-domain build of 4β-strands [12] (Fig. 2B).

The disulfide bond in the immediate vicinity of the iron–sulfur-cluster is shown in Fig. 2C. Currently, the literature is unclear about the function of this structural element. First, the removal of this disulfide bond from the yeast protein by site-directed mutagenesis led to a protein with altered structure for the iron–sulfur-cluster judged by the changes of the EPR-spectrum and a significant drop of the midpoint potential by 115 mV [13]. In contrast, only a moderate decrease of the midpoint potential (~40 mV) and a slight shift of the EPR signals was observed upon chemical reduction of the disulfide bond of the *Thermus thermophilus* Rieske protein [14].

Side-directed mutagenesis [15,16] as well as crystallographic [17] studies have indicated that the number of hydrogen bonds to the iron–sulfur-cluster may be the most important determining factor for the redox potential. Two of these hydrogen bonds play a prominent role for the adjustment of the midpoint potential of the iron–sulfur-cluster in accordance with the quinone-substrate [18] and the pH of the environment of the protein [6]. The first and most important of these hydrogen bonds is formed between the hydroxyl group of serine 163 of the bovine Rieske protein (corresponding to S183 in yeast and S157 in *Paracoccus*) and the sulfide ion S-1 of the [2Fe–2S]-cluster (Fig. 2C) [15,16]. This hydrogen bond is frequently absent in Rieske proteins from menaquinone (E_m=−74 mV) [19] containing organisms. The midpoint potentials of these Rieske proteins are approximately in the range from +100 to +200 mV at neutral pH [6,18]. Rieske proteins from organisms containing quinones with higher midpoint potentials (ubiquinone [20], plastoquinone [21] and caldariella quinone [22]) contain both hydrogen bonds and display significantly higher midpoint potentials in the range from about +250 to +573 mV [6,18]. However, these simplified rules have to be applied with caution since the midpoint potential of the quinones and the Rieske proteins are pH and temperature dependent [6,23]. The importance of these factors can be demonstrated with the example of the alcalophile *Bacillus alcalophilus* which accommodates a Rieske protein with a midpoint potential of +150 mV at pH 7.0 even though this organism uses ubiquinone [6,24].

2. Multiple Rieske proteins in eukaryotes

In several of the completely sequenced genomes from eukaryotes multiple Rieske genes can be found. Organisms that are most likely to contain more than one Rieske iron–sulfur protein are higher plants and green algae. In higher plants, the Rieske protein subunits of the chloroplast cytochrome b_6f complex [25–28] and of the mitochondrial cytochrome bc_1 complex [29,30] are encoded in the nucleus. In several cases, both genes exist as single chromosomal copies, though each gene is encoded on the two parental chromosomes. Two Rieske proteins with 98% sequence identity were found in the tobacco chloroplast b_6f complex [26] and it was suggested that these isoforms originate from the two alleles located on the corresponding chromosomes. In tobacco and maize, the Rieske subunits of the mitochondrial bc_1 complexes are encoded by a small family of genes located on the same chromosome [26].

It was shown that all four members of this family are functionally expressed in tobacco [29] and the encoded mature proteins share about 99% sequence identity. The expression of some of these genes is differently regulated in an organ or tissue specific manner. The existence of this gene family could allow the plant to respond to various developmental or environmental stimuli [29]. All currently identified Rieske proteins of tobacco are nuclear encoded.

Following their synthesis in the cytosol the plastidic and the mitochondrial proteins are sorted to their destinations via N-terminal signal sequences that mediate the transfer of polypeptides across the chloroplast or mitochondrial membranes. This highly efficient sorting system prevents incorporation of mitochondrial Rieske proteins into the chloroplasts cytochrome b_6f complex or vise versa. Even though the structural and electrochemical properties of the mitochondrial and plastidic high potential Rieske proteins are relatively similar, it is not clear yet if the proteins are interchangeable.

3. Multiple Rieske proteins in prokaryotes

Several microbial genomes were found to contain more than one gene encoding a Rieske protein. The exact functions of the

encoded proteins are for the most part not yet understood. However, some recent data indicate different physiological functions of multiple Rieske proteins in prokaryotes. In the following section we will discuss species for which information on the role of multiple Rieske proteins are available.

3.1. Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans is an acidophilic bacterium, which can grow in the presence of Fe^{2+} or of reducing sulfur compounds. D. Lemesle-Meunier and coworkers mainly characterized the electron transfer reactions in this organism. It was suggested that two different cytochrome bc_1 complexes operating in opposite direction exist in this organism [31].

The first cytochrome bc_1 complex of *Acidithiobacillus* has been characterized in detail [32]. It contains a cytochrome b, a cytochrome c_1, and a Rieske subunit like all classical cytochrome bc_1 complexes. Two c-type cytochromes are present in membranes of *Acidithiobacillus* with midpoint potentials of +150 mV and +485 mV, respectively. Currently it is not clear which of these potentials can be attributed to the c-type cytochrome associated with the cytochrome bc complex. The midpoint potential of the Rieske centre was measured to be +490 mV, about 150 mV higher than in typical Rieske iron–sulfur centers from cytochrome bc_1 complexes.

Based on the thermodynamic properties of the redox centers it was suggested that the *Acidithiobacillus* complex functions in reverse direction under physiological conditions transferring electrons from a soluble cytochrome to ubiquinone [31,33]. In addition to the gene cluster encoding the subunits of this characterized cytochrome bc complex, the genome of *Acidithiobacillus* contains a second gene cluster also encoding the typical subunits cytochrome b, cytochrome c, and the Rieske protein of a cytochrome bc complex [31]. To our knowledge, the electrochemical characteristics of this second cytochrome bc complex are not yet described. However, it was suggested from functional studies that the second cytochrome bc complex acts in the forward direction, like “classical” cytochrome bc complexes [34]. The sequences of the two Rieske proteins show no major differences (compare Fig. 3). Both genes code for proteins of about the same size and have a similar midpoint potential as deduced from the sequence. A similar situation, namely two different enzymes each preferentially catalyzing one direction of a reversible reaction, can be found in the *E. coli* electron transfer chain. Depending on the growth conditions, *E. coli* cells preferentially express either a succinate dehydrogenase or a fumarate reductase [35].

3.2. Rhodospirillum rubrum and Rhodopseudomonas palustris

Two genes coding for Rieske iron–sulfur proteins have been identified in the genomes of each of these organisms: *rrub02003033* and *rrub02001746* in *Rhodospirillum rubrum* and *rpa1192 (petA2)* and *rpa1016 (petA1)* in *Rhodopseudomonas palustris*. *rrub02003033* is part of a putative operon structure consisting of *rrub02003033*, *rrub02003034* (cyto-

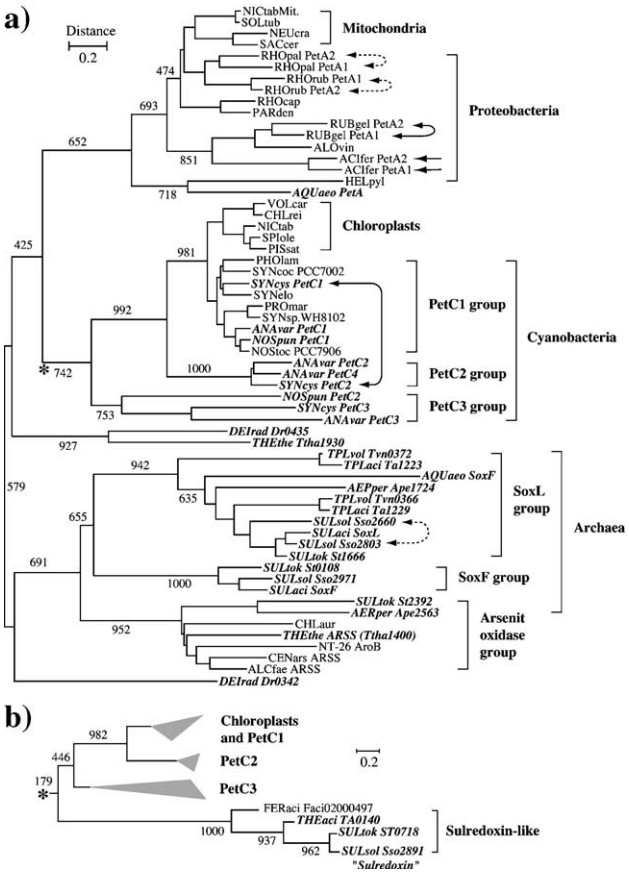

Fig. 3. (a) Unrooted phylogenetic tree of the Rieske proteins. Proteins that have been demonstrated to be able to substitute for each other in a cytochrome bc, or bf complex are indicated by arrows connected with a solid line. Arrows connected with a dashed line indicate proteins that are expected to be able to substitute for each other based on their similarity and the branching pattern. The petA proteins from the two bc complexes of *Acidithiobacillus* are marked with not connected arrows. For clarity, the bootstrap values (out of 1000 trails) are indicated for the main branching points only. (b) Simplified sub-tree demonstrating the possible placement of the Sulredoxin-like sequences. An asterisk indicates the position of the sub-tree within the main tree. Organisms: SACcer, bakers yeast; NEUcra, *Neurospora crassa*, SOLtub, potato; NICtab, tobacco, ACIfer, *Acidithiobacillus ferrooxidans;* DEIrad, *Deniococcus radiodurans*; RHOrub, *Rhodospirillum rubrum*; RHOpal, *Rhodopseudomonas palustris;* PARden, *Paracoccus denitrificans*; RUBgel, *Rubrivivax gelatinosus*; RHOcap, *Rhodobacter capsulatus*; ALOvin, *Allochromatium vinosum*; HELpyl, *Helicobacter pylori*; AQUaeo, *Aquifex aeolicus*; VOLcar, *Volvox carteri*; CHLrei, *Chlamydomonas reinhardtii*; SPIole, spinach; PISsat, pea; NOStoc PCC7906; PROmar, *Prochlorococcus marinus*; NOSpun, *Nostoc punctiforme*; SYNcys, *Synechocystis* PCC 6803; SYNcoc 7002, *Synechococcus* PCC7002; SYNelo, *Thermosynechococcus elongatus*; SYNsp. WH8102, *Synechococcus* sp. WH 8102; ANAvar, *Anabena* sp. *PCC 7120*; PHOlam, *Phormidium laminosum*; THEthe, *Thermus thermophilus*; SULaci, *Sulfolobus acidocaldarius*; AERper, *Aeropyrum pern ix;* SULsol, *Sulfolobus solfataricus* P2; SULtok, *Sulfolobus tokodaii*; TPLaci, *Thermoplasma acidophilum*; TPLvol, *Thermoplasma volcanium*; CENars, *Cenibacterium arsenoxidans*; CHLaur, *Chloroflexus aurantiacus*; ALCfae, *Alcaligenes faecalis*; NT-26, arsenite-oxidising bacterium NT-26 (Rhizobiaceae). The alignment and phylogenetic tree were calculated using the program ClustalX (version 1.83) [81]. The following parameters were used: Pairwise alignments: Identity matrix; gap opening penalty: 10, gap extension penalty: 0.1. Multiple alignments: BLOSUM series, gap opening penalty: 15, gap extension penalty: 0.3, threshold for delaying divergent sequences: 30%. Protein gap parameters: residue-specific penalties: on, hydrophilic penalties: on, hydrophilic residues: GPSNDQEKR, gap separation distance: 8, end gap separation: off. The correction for multiple substitutions was applied. The criteria outlined in [2] were used to evaluate the quality of the alignment and the reliability of the phylogenetic tree.

chrome b) and *rrub02003035* (cytochrome c_1), coding for the three subunits of a cytochrome bc_1 complex.

The same is true for *rpa1192*. However, *rpa1193* codes for a fusion protein consisting of an N-terminal cytochrome b domain and a C-terminal cytochrome c_1 domain.

The proteins encoded by the second Rieske gene in both organisms display approximately the same degree of similarity/dissimilarity to *rrub02003033*, or *rpa1192*, respectively, as the two *Acidithiobacillus* Rieske proteins among themselves. However, they are not constituents of additional cytochrome bc complex operons. Based on sequence similarity, it appears plausible that the proteins encoded by *rrub02001746* and *rpa1016* can functionally replace Rrub02003033 or Rpa1192, respectively, in the cytochrome bc complexes as it has been demonstrated for PetC2 in *Synechocystis* sp. PCC 6803 and for PetA2 in *Rubrivivax gelatinosus* (see below). Mutagenesis studies, like the ones reported in *Rhodobacter sphaeroides* [36], or in *Rhodobacter capsulatus* [37], could answer this question but to our knowledge no comparable studies have been accomplishes in *Rhodospirillum rubrum* or *Rhodopseudomonas palustris*.

3.3. Rubrivivax gelatinosus

The situation in *Rubrivivax gelatinosus* is very similar to that described for *Rhodospirillum rubrum* and *Rhodopseudomonas palustris*. The *Rubrivivax* genome contains two genes encoding Rieske iron–sulfur proteins. One of them *petA1* is part of a typical *petABC* operon, whereas the other one, *petA2*, is not [38]. The inactivation of one of these genes does not abolish the ability of the organism for photosynthetic growth, whereas the simultaneous inactivation of both *petA* genes or of *petA1* and *petB* resulted in a photosynthesis deficient phenotype [38]. Thus, it is clear that even with one *petABC* operon the proteins encoded by both *petA1* or *petA2* can substitute for each other to form a fully functional bc_1 complex [38].

Based on their phylogenetic analysis, Ouchane and coworkers concluded that the gene duplications leading to the existence of the two functional *petA* genes in *Rubrivivax gelatinosus*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* occurred independently within each lineage. Thus, the duplication of the *petA* genes in these organisms has to be a relatively recent event, in contrast to the gene duplications leading to the origin of the cyanobacterial *petC1*, *petC2* and *petC3* genes [38], and the archaeal *soxL* and *soxF* genes (Fig. 3). Since the situations found in *Rubrivivax gelatinosus*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* appear to be very similar with respect to the close similarities and the genomic context of the two *petA* genes of each of the organisms, it is very likely that the PetA proteins of *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* could also substitute for each other within the cytochrome bc_1 complexes of their respective organisms.

Besides the two discussed PetA proteins found in *Rubrivivax gelatinosus*, an additional third Rieske gene was identified (*petA3*) and the encoded protein shows similarities to PetA1 and PetA2. Because of a deletion in the *petA3* gene, PetA3 lacks the first iron–sulfur cluster binding box and, if the gene is expressed, the protein would be unable to bind the iron–sulfur-cluster. Therefore, *petA3* does most likely not code for a functional Rieske protein and it is unlikely that the protein is assembled into the bc_1 complex of *Rubrivivax gelatinosus* [38].

3.4. Aquifex aeolicus

The genome of the hyperthermophilic bacterium *Aquifex aeolicus* was completely sequenced in 1998 [39]. Analysis of the sequence information revealed that *Aquifex* contains two genes encoding for Rieske iron–sulfur proteins. The *petA* gene is part of the *petABC* operon, which additionally codes for the cytochrome b (*pet*B) and the cytochrome c_1 subunit (*petC*) of the cytochrome bc_1 complex. *petA* encodes a 181 aa long protein with a calculated molecular mass of 19.4 kDa. In contrast, the *Aquifex soxF* gene encodes a larger protein (238 aa) with a molecular mass of 26.9 kDa. The *Aquifex soxF* gene is located on a different locus of the chromosome and is not clustered with other cytochrome bc_1 complex genes. Corresponding to the presence of two Rieske proteins, two electrochemically distinct EPR spectra were observed in the membrane fractions of *Aquifex*. The midpoint potentials were determined to be +210 mV for PetA and +95 mV for SoxF [40]. In agreement with the observed low midpoint potential, one of two residues known to influence the midpoint potential is exchanged (Tyr to Phe, Fig. 2) in the SoxF protein. Purification and characterization of the cytochrome bc_1 complex from this organism has shown that only the *petA* gene product is functionally associated with the cytochrome bc_1 complex, and the authors concluded that the cytochrome bc_1 complex of *Aquifex* contains exclusively PetA [40]. Since the *Aquifex* SoxF protein was also identified in membrane preparations, it is conceivable that the protein is involved in other membrane bound electron transfer reactions. A hypothetical function of the *Aquifex* SoxF protein can be derived from the genomic context of the gene. It is located downstream of *dhsU* which encodes a flavocytochrome *c* sulfide dehydrogenase and upstream of *fccB* encoding the flavoprotein subunit of a sulfide dehydrogenase ([28], accession No.: NC_000918]. The sulfide dehydrogenase from *Pyrococcus furiosus* was found to consist of an α-subunit (SudA) containing a FAD cofactor and a β-subunit (SudB) which carries a [2Fe–2S] cluster with a redox potential (+80 mV) and an EPR spectrum [41] very similar to that reported for the *Aquifex* SoxF protein. Even though the FeS-cluster in SudB is coordinated by $Asp(Cys)_3$ ligands instead of the $(His)_2(Cys)_2$ coordination of the Rieske proteins, the very similar redox potential suggests that the *Aquifex* SoxF protein might be functionally equivalent to *Pyrococcus* SudB and thus is a subunit of a sulfide dehydrogenase.

The position of the *Aquifex* SoxF protein in the phylogenetic tree shown in Fig. 3 remains unclear. Under a wide range of parameters, it reproducibly segregates with the SoxL group of the archaeal Rieske proteins. Thus, the gene encoding this protein may have been acquired by horizontal gene transfer.

Transfer of the gene from *Thermoplasma*-like organisms containing a menaquinone derivate would also explain the low redox potential of the *Aquifex* SoxF protein.

3.5. Deinococcus radiodurans

Two Rieske protein-encoding genes have been identified in the genome of *Deinococcus radiodurans*. Together with one of the *Thermus thermophilus* proteins they form the deepest branch of the bacterial/eukaryotic Rieske proteins (Fig. 3). Both proteins display a relatively low degree of similarity to each other (Fig. 3). DEIrad_2 (Dr0435) is encoded upstream of a gene encoding a b type cytochrome (*dr0436*), suggesting that the two encoded proteins build the core of a cytochrome bc-like complex. The second Rieske protein encoding gene (*dr0342*) is located upstream of a gene (*dr0343*) encoding a protein of 340 amino acids, which is predicted to contain two transmembrane helices and a c-terminal cytochrome c domain. Thus Dr0343 remotely resembles the *Bacillus* c-type cytochrome encoded by the *qrcC* gene [42], though it shows no significant sequence similarity to this protein besides the cytochrome c domain. The genes located downstream of *dr0343* are predicted to code for proteins involved in cytochrome c biosynthesis (compare *Thermus thermophilus*). The sequence comparison and the phylogenetic analysis of these proteins (Fig. 3) reveal no obvious clues about the function of the Dr0342 protein as it does not segregate with any of the well-defined groups.

Although clear predictions of the functions of the two *Deinococcus* Rieske proteins is currently difficult, it appears safe to assume that at least the Dr0435 protein is a subunit of a cytochrome bc complex.

3.6. Thermus thermophilus

Most of the details discussed for *Deinococcus* can be applied to *Thermus thermophilus*. This organism contains two genes coding for Rieske-like proteins. One of the Rieske protein encoding genes (*ttha1930*) is located upstream of a gene coding for a b-type cytochrome (*ttha1931*). Both proteins show highest similarities to the proteins encoded by the *Deinococcus* genes *dr0435* and *dr0436* and have been demonstrated to be subunits of a cytochrome bc complex [43]. The second Rieske protein of *Thermus thermophilus* is encoded by *ttha1400*. This protein clearly segregates together with the proteins from arsenit oxidase group in Fig. 3. *ttha1401* located downstream of *ttha1400*, which encodes a membrane protein containing a cytochrome c domain, followed by genes encoding cytochrome c biosynthesis related proteins. The protein most similar to the Ttha1401 protein in the databases is Dr0343 from *Deinococcus*. Considering this, it is temping to assume that the Rieske proteins Dr0342 and Ttha1400 are performing the same or very similar functions even though they do not cluster together in the phylogenetic tree shown in Fig. 3. The position of Ttha1400 in the phylogenetic tree suggests it functions as a subunit of an arsenit oxidase.

However, such a conclusion without any experimental verification has to be drawn with caution.

4. Multiple Rieske proteins in cyanobacteria

Possible functions of multiple Rieske proteins are comprehensively studied in cyanobacteria. In some cyanobacteria, like *Thermosynechococcus elongates* and *Gloeobacter violaceus*, only one *petC* gene can be found encoding for a high-potential Rieske protein [44–46]. This single gene copy is organized in an operon with the *petA* gene, which encodes the cytochrome f subunit of the cyanobacteria cytochrome b_6f complex [47,48]. In contrast, several cyanobacteria have been shown to contain several isoforms of Rieske proteins, which are encoded by three or more *petC* genes. In Fig. 4, a sequence alignment of the four Rieske proteins from the mesophilic cyanobacterium *Anabaena* sp. PCC 7120 is shown. Two of the additional PetC sequences, PetC2 and PetC4, show about 70% sequence identity to the main Rieske protein PetC1, whereas PetC3 is only about 20% identical to PetC1. While PetC1, 2 and 4 have a similar molecular weight of about 19 kDa, the calculated mass of PetC3 is only about 14 kDa. In the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 three genes can be found, which encode for typical Rieske iron–sulfur proteins

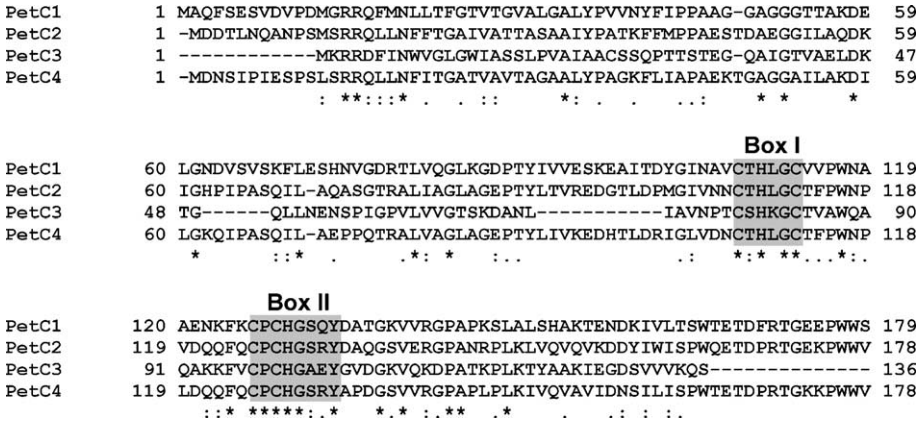

Fig. 4. Sequence alignment of the four PetC Sequences from *Anabaena* sp PCC 7120. Residues of the two boxes involved in binding of the iron–sulfur cluster are shaded.

[49] and the gene products show a high degree of similarity to the PetC1-3 proteins from *Anabaena*.

So why do some cyanobacteria need more than one Rieske protein? This is a hard question to answer but studies with *Synechocystis* have shown that all three genes are functionally expressed [49] and transcripts of the four *Anabaena* genes have also been identified [50]. These observations indicate that the encoded Proteins have some physiological function in vivo. Based on functional analysis, it was concluded that the *petC1* gene for the cyanobacterium *Synechococcus* PCC 7002 encodes for the essential Rieske subunit of the cytochrome b_6f complex, whereas *petC2* and *petC3* are silent in the function of the complex [51]. In *Synechocystis*, it was shown by deletional analysis that the high potential isoform PetC2 can partly substitute for the main PetC1 protein, although PetC2 is not as efficient as PetC1 in electron transfer [52]. While PetC1 and PetC2 share a high degree of sequence similarity, some critical residues in the flexible hinge region are replaced in the PetC2 protein. This glycine-rich region connects the transmembrane domain with the soluble domain and is critical for electron transfer. It was suggested that the replacement of amino acids in the PetC2 hinge region could be responsible for the lowered activity of the cytochrome b_6f complex in the *Synechocystis* Δ*petC1* strain [52]. While these results indicate that PetC2 can be a subunit of the cyanobacterial cytochrome b_6f complex, it is possible that the protein mainly fulfills a different function in vivo. In *Synechocystis* as well as in *Anabaena* the open reading frame *slr1185* (*petC2*) is located in a gene cluster together with two other genes (*slr1184* and *ssr1966* in *Synechocystis*). *ssr1966* encodes a small protein, which has two predicted transmembrane helices. The protein shows no significant homology to any protein of known function. The open reading frame *slr1184* encodes a 164 amino acid long transmembrane protein with one predicted N-terminal transmembrane helix. In addition, the protein contains a rhodanese-like domain. Although catalytic or noncatalytic rhodanese-like domains are present in many proteins, a general function and substrate specificity of rhodanese-like domains has not yet been established definitively, and the role of the noncatalytic rhodanese homology domains has been so far elusive. Proposed functions of rhodanese-like domains include cyanide detoxification, maintenance of the sulphate pool and thiamine biosynthesis (for a recent review see [53]). Interestingly, it was also suggested that these domains function in the formation of prosthetic groups for iron–sulfur proteins [54]. It is possible that after gene duplication the major in vivo function of *petC2* has changed and that PetC2 may now function together with Ssr1966 and Slr1184 (in *Synechocystis*). Analysis of the function of these proteins could help to understand the physiological role of PetC2 proteins in cyanobacteria.

In contrast to PetC2, PetC3 cannot complement for the deletion of *petC1* in *Synechocystis*. In spite of this, the PetC3 protein was co-isolated with the cytochrome b_6f complex of *Synechocystis*, suggesting that this protein is somehow involved in electron transfer through the cytochrome b_6f complex [49]. This leads to the question of whether different populations of cytochrome b_6f complexes with different functions exist in vivo in *Synechocystis* and what the physiological function of such multiple complexes could be.

As pointed out, the amino acid sequences of PetC3 from *Synechocystis*, as well as from *Anabaena*, show many differences when compared to the main PetC1 protein. The protein has a smaller molecular mass due to shortened N- and C-termini and two deletions in the middle of the proteins. Although these modifications alter the structural properties of the protein, the amino acids forming the iron–sulfur cluster binding domain are conserved. In general, Rieske proteins from various sources do not show a high degree of sequence identity but the overall three dimensional structure seems to be conserved. This is especially true for the cluster binding domain of these proteins [55,56]. Interestingly, the C-terminus of all PetC3 proteins is shortened compared to other family members. Several structural features of chloroplast/cyanobacterial Rieske proteins appear to have no direct counterparts in cytochrome bc_1 complex Rieske proteins [56,57]. These distinctive features include a C-terminal extension of cytochrome b_6f complex Rieske proteins compared to cytochrome bc_1 complex proteins. In this respect, the PetC3 proteins are more like cytochrome bc_1 complex Rieske proteins as they do not contain this C-terminal extension. This extension is neither necessary for transport of the protein into the thylakoid lumen nor for assembly of it into the cytochrome b_6f complex, and it was suggested that this extension is may be important for the interaction of the Rieske protein with cytochrome f [58]. In contrast to this, the structure of the cytochrome b_6f complex has been solved and it was shown that the C-terminal extension of cytochrome b_6f complex Rieske proteins is not in a close contact to any domain of cytochrome f [59,60], thus it appears that the function of this extension remains elusive and the absence of this extension in the PetC3 proteins cannot give any hints on the function of this protein in vivo.

It was very recently shown that in *Synechocystis petC3* encodes for a protein with a significantly lowered midpoint potential. While the midpoint potentials of PetC1 and PetC2 were determined to be approximately +300 mV, PetC3 has a potential of only +135 mV [49]. These differences can be explained by sequence analysis of the proteins. First it was shown by side-directed mutagenesis that the residues Ser163 and Tyr165 (high lightened in Fig. 2C) are involved in the formation of a hydrogen-bond network around the iron–sulfur cluster and substitution of these residues led to a lowered midpoint potential [16]. For the yeast *Saccharomyces cerevisiae* Rieske protein, the midpoint potential was lowered by 130 mV after the substitution of Ser183 by Ala and lowered by 68 mV after the substitution of Tyr185 by Phe (see above). Most cyanobacterial PetC3 proteins have the equivalent Ser183 residue substituted with another amino acid, which results in a considerably lowered midpoint potential such as that measured for PetC3 from *Synechocystis*. An exception to this is the PetC3 protein from *Nostoc punctiforem* which has a Box II sequence typical for a high potential Rieske protein and none of the two crucial amino acids is substituted. Nevertheless, in

the "typical" Box I sequence (CTH**L**GC), the highly conserved Leu residue is replaced by Ala in this protein. In *Rhodobacter capsulatus* replacement of this conserved Leu residue with Ala lowered the midpoint potential of the iron–sulfur cluster by about 50 mV [61]. In addition, in all cyanobacterial PetC3 sequences the residue, which is localized in between the two "crucial" Box II residues, is a Glu, and it has been suggested that this residue also contributes to lowering the PetC3 midpoint potential [62]. Taken together, this suggests that the *Nostoc punctiforme* PetC3 protein is also likely to have a low midpoint potential. But now we face the problem that plastoquinone cannot be oxidized efficiently at physiological pH if the midpoint potential of all PetC3 proteins is only about +150 mV. This would result in a loss of function of the cytochrome b_6f complex (Fig. 5). Nevertheless, since PetC3 was co-isolated with the cytochrome b_6f complex from *Synechocystis* PCC 6803, this subunit seems to fulfill some function in vivo. While in *Bacillus alclophilus* a Rieske protein with a midpoint potential of + 150 mV at pH 7.0 can be found and the organism uses ubiquinone, low midpoint potentials of +150 mV or less are typically found in bacteria which use menaquinone instead of ubi- or plastoquinone [6,63]. It is possible that PetC3 could be a subunit of a cytochrome b_6f complex which oxidizes menaquinol instead of plastoquinol. The gene products involved in naphthoquinol biosynthesis have all been identified in *Synechocystis* [64], but nothing is so far known about a pool of free naphtoquinol in cyanobacterial membranes. Clearly, two molecules of naphthoquinol serve as electron acceptors A1 in photosystem I complexes of higher plants, as well as in cyanobacteria [65–69] and both quinones have been located in the photosystem I structure of the cyanobacterium *Thermosynechococcus elongatus* [70]. While

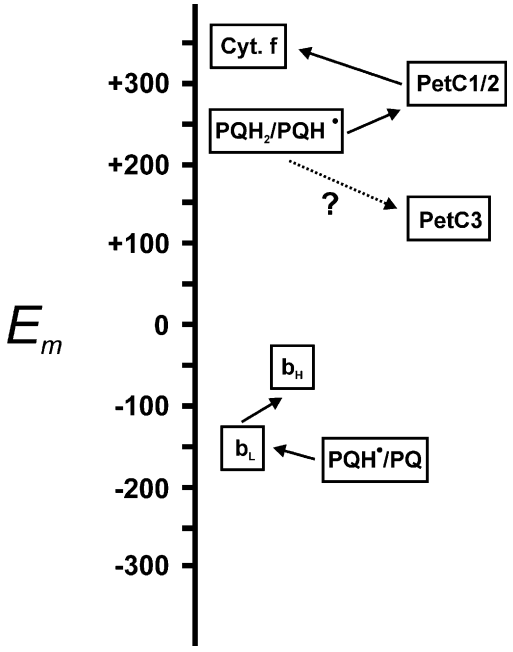

Fig. 5. Thermodynamic profile of the electron transfer reactions within the cyanobacterial cytochrome b_6f complex. While electron transfer from PQH_2 to PetC1 or PetC2, respectively, is thermodynamically possible, the transfer of an electron from PQH2 to PetC3 is not favored.

in higher plant thylakoids all phylloquinone was found to be bound to photosystem I reaction centres, the naphtoquinone/photosystem I ratio in membranes from the cyanobacterium *Anabaena* sp. PCC 7120 was found to be about 3 (instead of 2) [69]. This may indicates a pool of free naphtoquinone in cyanobacteria. Nevertheless, the physiological function of PetC3 is not clear yet and it is also possible that the PetC3 protein is not part of a cytochrome b_6f complex but fulfills a completely different function in vivo. This argument is strengthened by the observation that the protein is localized very distant from the clade of cytochrome b_6f complex Rieske proteins in phylogenetic trees [38] (Fig. 3).

In contrast to *Synechocystis*, other cyanobacteria like *Anabaena* contain an additional fourth PetC protein. Although the electrochemical properties of PetC4 are not yet known, it is highly likely that PetC4 is a high potential Rieske protein (see Fig. 4). Arnold et al. showed that the expression of *petC4* depends on nitrogen availability and a Δ*petC4* strain was not able to grow on medium without fixed nitrogen [62]. These observations suggest that PetC4 has a specific role in nitrogen fixing cyanobacteria and it could be a redox sensor for coordinating the change of electron pathways in heterocysts or could be necessary for accelerated respiration. In other nitrogen fixing cyanobacteria no *petC4* gene can be found and the function of PetC4 in heterocysts is probably confined to *Anabaena* PCC 7120.

The exact functions of the multiple Rieske proteins in cyanobacteria are not yet completely understood. In cyanobacteria, multiple electron transfer pathways are connected and the cytochrome b_6f complex has a central role in this electron transfer network [71]. It is intriguing to speculate that exchange of the cytochrome b_6f complex Rieske subunit is used to regulate electron transfer between individual complexes.

5. Multiple Rieske proteins in Archaea

Genes encoding Rieske proteins have been detected in some but not all currently sequenced archaeal genomes [6,72]. Some of these proteins have been isolated and characterized either from their native source or recombinant from *E. coli*. Information about functions of these proteins and other subunits of archaeal cytochrome bc homologous complexes is still limited.

Two different Rieske proteins have been characterized from *Sulfolobus acidocaldarius* (DSM 639) [73,74]. Both proteins have been purified from *Sulfolobus* membranes [73,74] and characterized. SoxF has been purified as part of a terminal oxidase supercomplex representing a fusion between a cytochrome bc complex and a cytochrome c oxidase [73,75]. Furthermore, the soluble domain of SoxF has been expressed in *E. coli* and the structure was solved [76].

Genomic evidence indicates that SoxL together with the other proteins encoded by the *cbsAB-soxLN-odsN* gene cluster, namely the di-heme cytochrome b homologue SoxN and the unusual high potential b-type cytochrome CbsA, are also constituents of a cytochrome bc homologous complex [72]. However, the functional placement of this hypothetical

complex within the *Sulfolobus* respiratory chain remains unclear. The same is true for the functions of the non-cofactor bearing proteins CbsB and OdsN. Homologous gene clusters have been identified in *Sulfolobus solfataricus* (*sso2801*, *sso2802*, *sso2803* encoding the Rieske protein, *sso2805*, and *sso11196*) and *Sulfolobus tokodaii* (*st1664*, *st1665*, *st1666* encoding the Rieske protein, and *st1667*). The genome of *Sulfolobus tokodaii* contains two more genes encoding Rieske proteins (*st0108* and *st2392*). *st0108* is located upstream of a gene encoding a b-type cytochrome (*st0109*). However, these genes are not part of a gene cluster resembling the *soxM–soxE–soxFGHI* region in the genome of *Sulfolobus acidocaldarius or Sulfolobus solfataricus* (*sso2973* to *sso2968*). Nevertheless, *st0108* and *st0109* display the highest similarities to the *soxFG* genes of *Sulfolobus acidocaldarius* (Fig. 3). Hence, we propose the denotations *soxF* and *soxG* for the open reading frames *st0108* and *st0109* from *Sulfolobus tokodaii*. There are currently no data available concerning the function of St0108 and St0109. However, the pronounced sequence similarities to the *Sulfolobus acidocaldarius soxFG* gene products suggests that St0108 and St0109 are the core of a cytochrome bc homologous complex.

Sequence comparison shows that St2392 is rather distantly related to St0108 and St1666. Instead it appears to be the archaeal homologue to the Rieske subunits of the arsenit oxidases [8] (Fig. 3). Two more genes encoding Rieske proteins are present in the *Sulfolobus solfataricus* genome. *sso2660* (*soxL-1*) shows highest sequence similarities to other SoxL proteins. It is immediately flanked by genes encoding a protein similar to a proline permease (downstream) and a potential transposase (upstream, on the complement strand). Located further upstream is the *soxABC* operon encoding one of the terminal oxidases of the respiratory chain. Thus, judged by sequence similarity and the genomic context, the Sso2660 protein may be part of the *Sulfolobus solfataricus* respiratory chain. The pronounced similarity between Sso2803 and Sso2660 even suggests that the later protein may able to substitute for Sso2803 within the SoxLN complex as it has been demonstrated for the PetA proteins from *Rubrivivax* (see above).

The fourth Rieske protein of *Sulfolobus solfataricus* is encoded by *sso2891*. The protein was named "Sulredoxin" and has been isolated from the cytosolic fraction of *Sulfolobus* sp. strain 7 and characterized by Iwasaki and coworkers [77–79]. "Sulredoxin" is a soluble protein with a midpoint potential of +155 mV at pH 6.5. Two protonization equilibria with $pK_{(ox)}$ values of 6.23 and 8.57 were found to be associated with the redox reaction. Thus, it displays the redox properties of a Rieske protein associated with a respiratory or photosynthetic electron transfer chain. However, the observation that the potentials of SoxF and SoxL, which were proven to belong to the *Sulfolobus* respiratory chain, are 300 to 400 mV more positive [6] argues against a function of "Sulredoxin" as a subunit of a cytochrome bc homologous complex within the respiratory chain of *Sulfolobus solfataricus*. A data base search reveals 3 open reading frames potentially encoding proteins with a high similarity to "Sulredoxin": *ta0140* from *Thermoplasma acidophilum*, *st0719* from *Sulfolobus tokodaii* and *faci02000497* from *Ferroplasma acidarmanus*. In the data base, the Faci02000497 protein has been classified as "Ferredoxin subunit of nitrite reductases and ring-hydroxylating dioxygenases". However, the open reading frames up- and downstream of all of these four "Sulredoxin-like" genes provide no clues about the function of the proteins. In agreement with the observed relatively low redox potential the serine and tyrosine residues within the Box II sequence are exchanged with non-hydroxylated amino acids (see Fig. 2C). The attempt to include these sequences into the phylogenetic analysis shown in Fig. 3 resulted in a severe "destabilization" of the tree as indicated by a drop of the bootstrap values. Under a wide range of tested alignment parameters, the "Sulredoxin-like" group segregated together with the chloroplast/cyanobacterial sequences and often forms the deepest twig of that branch (Fig. 3b). Although this positioning has to be considered with utmost caution, the observation that these sequences are clustering so faraway from the sequences of the archaeal Rieske proteins with known or predictable functions suggests that this line has developed prior to the separation of archaea and bacteria and may represent a new subfamily of Rieske proteins.

Two genes encoding Rieske proteins have been identifies in the genome of aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. The protein encoded by *ape1724* shows 39% similarity to the *Sulfolobus acidocaldarius* SoxL protein. It is located upstream of *ape1725*, encoding a cytochrome b homologe and *ape1727*, which shows similarity to the *Sulfolobus acidocaldarius odsN* gene [72]. Hence, the Ape 1724 Rieske protein appears to be part of an archaeal cytochrome bc-homologous complex. The second Rieske protein encoded in the *Aeropyrum pernix* genome, Ape2563, appears to be a subunit of an arsenite oxidase [8] (Fig. 3).

Two gene pairs encoding a Rieske protein and a b-type cytochrome have been identified in each of the genomes of *Thermoplasma acidophilum* and *Thermoplasma volcanium*. In both organisms the proteins encoded by one of these gene pairs displays a higher similarity to the SoxLN proteins of *Sulfolobus*, whereas the proteins encoded by genes of the second pair approximately equal similarity to the SoxLN and SoxFG proteins from *Sulfolobus* [72] (Fig. 3).

The physiological significance of the multiple archaeal Rieske proteins remains unclear. Only a few of the proteins have been isolated, or expressed in *E. coli* and characterized in detail. In the case of *Sulfolobus*, the expression of both genes has been demonstrated by purification of the encoded proteins [74,75]. Even though it is tempting to speculate about a differential expression of the *soxL* and *soxF* genes depending on the growth conditions, no such data have yet been published. Nevertheless, the analysis shown in Fig. 3 clearly suggests that the gene duplication events that lead to the evolution of the different groups of the archaeal Rieske proteins occurred prior to the separation of the different *Sulfolobus* species (*soxF* vs. *soxL*) and perhaps even before the archaea–bacteria split (*soxF* and *soxL* vs. the "Sulredoxin-like" group).

The sequence analysis performed by Lebrun and coworkers [8], as well as results presented in Fig. 3, clearly indicate the existence of archaeal homologues of the bacterial arsenit oxidases. The clustering of archaeal and bacterial sequences in this arsenit oxidase group suggests that this subfamily of the Rieske proteins already existed prior to the split between the archaea and the bacteria (see also [80]).

It should be noted that in addition to the proteins and genes discussed in this section many archaeal genomes also contain genes encoding low potential Rieske proteins as judged by the absence of the conserved cysteine residues forming the disulfide bond in the vicinity of the iron sulfur cluster.

6. Concluding remarks

Gene families encoding for Rieske iron–sulfur proteins seems to be more common in bacteria and archaea when compared to eukaryotes. There is emerging evidence to suggest that Rieske protein homologues participate in far more reactions than respiratory or photosynthetic electron transfer. The exact function of many of the proteins identified as open reading frames in the genomes is currently under investigation. Initial results of the investigations were presented in the recent years. It is to be expected that more organisms with multiple Rieske protein genes will be identified with completion of more microbial genome projects. It is becoming clear that the Rieske proteins are as functionally versatile as many other redox proteins. Perhaps, a decade ago, it was still reasonable to ask whether an organism contained a Rieske protein, presently it appears almost more surprising that there are organisms like *Methanococcus jannaschii* that appear to have no Rieske proteins at all.

Bacteria seem to have evolved multiple mechanisms to adapt to changing environmental conditions and one main conclusion which can be drawn from the available data is that the use of multiple Rieske proteins in electron transfer reactions seems to allow the organism to adapt more easily to changing environmental conditions as it was suggested for the mitochondrial Rieske protein family as well as for microbial Rieske proteins. Future work will have to be done to elucidate the exact physiological roles of multiple Rieske proteins in prokaryotes.

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