Two different respiratory Rieske proteins are expressed in the extreme thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*: cloning and sequencing of their genes

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Abstract **We have isolated two genes encoding Rieske iron sulfur proteins from the genomic DNA of the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* (DSM 639). One of the genes, named *soxL*, codes for the previously isolated novel Rieske-I protein [1]. The second gene (*soxF*) [2] codes for the Rieske-II protein associated with the second terminal oxidase of *Sulfolobus* [3]. Both proteins exhibit only 24% identical residues. The Rieske-I protein shows a number of unusual features. (i) The distance between the two cluster binding sites is significantly larger than in all known proteins. (ii) An unexpected Pro → Asp exchange in one of the cluster binding sites. (iii) It shows some resemblance to the mitochondrial and plastidic Rieske proteins insofar as the *soxL* gene codes for a pre-sequence which is no longer present in the mature Rieske-I protein. Both proteins cluster together on a separate branch of the phylogenetic tree. To our knowledge this is the first proven case of two significantly different Rieske proteins in a prokaryote.**

Key words: Rieske; Archaea; Iron-sulfur protein; *soxL*; *soxF*; Sequence

1. Introduction

Rieske iron sulfur proteins are membrane associated compounds of the respiratory and photosynthetic electron transport chains of prokaryotic and eukaryotic organisms. They are known as subunits of the cytochrome bc_1 and b_6f complexes and contain a single [2Fe-2S] cluster with typical EPR features and unusually high, pH dependent midpoint potentials. A distinct, but closely related group are the soluble Rieske-type proteins associated with the bacterial oxygenases [4]. The characteristic EPR features and the significantly higher midpoint potentials of both the Rieske and the Rieske-type proteins compared to the [2Fe-2S] ferredoxins are attributed to the presence of two histidyl ligands in the coordination sphere of one of the iron ions [5–8]. Rieske and Rieske-type proteins can be differentiated by their sub-cellular localization and their midpoint potentials, which are in the range from +105 to +350 mV [9,10] and are pH dependent in the case of the Rieske proteins, whereas the midpoint potentials of the Rieske-type proteins are lower (−150 to +5 mV) [11–13] and pH independent. On a molecular level both types of proteins can be distinguished by the presence of three short stretches of amino acids which are strictly conserved in the Rieske proteins, but to a much lesser extent in the Rieske-type proteins [4,14].

Recently we reported the first isolation of a typical Rieske iron sulfur protein (Rieske-I) from an archaeal source, the membranes of the extreme thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* [1]. The purified protein contains one [2Fe-2S] cluster as determined by the iron content and EPR spin quantification and has an apparent molecular mass of 32 kDa. It displays a rhombic EPR spectrum with $g_{xyz} = 1.768$, 1.895, 2.035 and an average g-value of 1.902 indicating a nitrogen ligand-containing [2Fe-2S] cluster. The isolated protein shows ubiquinol cytochrome *c* reductase activity [1] as was reported for the isolated Rieske protein from beef heart mitochondria [15]. However, the presence of a typical bc_1 or b_6f complex in *Sulfolobus* is excluded by the complete absence of *c*-type cytochromes.

Surprisingly, EPR studies on *Sulfolobus* membranes revealed an abundance of 1.7–2.0 Rieske clusters per aa_3 terminal oxidase [1]. In fact, an EPR spectrum typical for a Rieske iron sulfur protein was found to be associated with an 29 kDa protein (Rieske-II) copurifying with the alternate oxidase of *Sulfolobus*, the SoxM complex [3].

Here we report the identification and sequencing of two different genes coding for Rieske iron sulfur proteins from *Sulfolobus* (Rieske-I, gene termed *soxL* and Rieske-II, gene termed *soxF* [2]). While the SoxF gene is located within a gene cluster coding for the subunits of the SoxM oxidase as discovered independently by others [2], the location of the SoxL gene is not associated with either one of the terminal oxidase gene clusters [2,16].

2. Materials and methods

The *S. acidocaldarius* (DSM 639) cells used in this study were grown at the 'Gesellschaft für Biotechnologische Forschung mbH', Braunschweig, Germany, essentially as described in [17].

Genomic DNA was prepared according to [18]. Clones containing an 1 kb *Eco*RI fragment and an 1.7 kb *Bam*HI/*Hin*dIII fragment in the plasmid pBluescript II KS$^-$ (Stratagene, Heidelberg, Germany) were isolated from genomic libraries of *S. acidocaldarius* DNA in *E. coli* XL2- Blue (Stratagene).

The *Eco*RI library was screened with an oligonucleotide probe, ACW GTI ACW GTI GCI ACH GGI TTY CCW AA, derived from amino acid positions 8–17 of the isolated Rieske-I protein [1]. The probe was 3′-end labelled with digoxigenin-11-ddUTP (Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

The *Bam*HI/*Hin*dIII library was screened with a 386 bp internally digoxigenin-labelled [19] PCR product derived from the *soxF* gene. This PCR product was obtained by chance with the primers GTN ACN GTN GCN ACN GGN TTY CCN AA derived from the N-terminal amino acid sequence of the *Sulfolobus* Rieske-I protein and TYR TAN TGN SWN CCR TGR CAN GGR CA derived from the consensus sequence of the C-terminally located cluster binding site of the known Rieske proteins.

Southern and colony hybridization were performed as described in

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[19,20]. The detection was chemiluminescent using CSPD (Boehringer) for Southern blots, or colorimetric using the NBT/BCIP protocol (Boehringer) [19] in the case of the colony hybridizations.

Cycle sequencing was carried out using the SequiTherm thermostable DNA polymerase (Epicenter Technologies, Madison, WI, USA) following the protocol described in [21]. Electrophoresis and blotting was performed using a GATC 1500 direct blotting electrophoresis apparatus (GATC, Constance, Germany). The gel concentration, electrophoretic conditions and colorimetric detection of the sequence blot was done following the manufacturer's instructions.

Sequencing of the 16S RNA genes was carried out by the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (Braunschweig, Germany).

EPR redox titrations were performed as outlined in [22].

Sequence alignments and the construction of phylogenetic trees were performed with the Clustal w1.5 programm. Refinement of the alignments was done manually.

Secondary structure analysis was done using the TMAP [23] and the PredictProtein [24] programs via Internet at the EMBL, Heidelberg, Germany.

3. Results and discussion

We have isolated two different fragments from the genomic DNA of *Sulfolobus acidocaldarius*, both containing genes encoding Rieske iron sulfur proteins. A 16S rRNA analysis was performed in order to ensure that the culture from which the DNA used in this study was isolated was free of contaminations [25] and was identical to the *S. acidocaldarius* type strain DSM 639 (data not shown).

The gene located within the 1 kb *Eco*RI fragment (*soxL*) codes for a novel Rieske protein (Fig. 1), whereas the 1.7 *Bam*HI/*Hin*dIII fragment contains the *soxF* gene and is identical to the corresponding region of the 4.6 kb fragment described by Castresana et al. [2]. The amino acids 13–29, 31 and 32 of the SoxL protein are identical with the N-terminal amino acid sequence determined for the previously purified Rieske-I protein of *S. acidocaldarius* [1], proving that this protein is encoded by the *soxL* gene. The discrepancy at position 30, which was assumed to be a leucine, is most likely the result of a protein sequencing error.

A possible transcription start site [(A/T)($T_{/C}$)($G_{/A}$)] [26] is located immediately upstream of the start codon (Fig. 1). Sequences matching the consensus for box A [(T/C)TTA(T/A)A] [26] or for the ribosomal binding site [(A/G)GGTG] [16] are not present. The absence of box A may indicate that the *soxL* gene is part of a larger operon. However, there are also a number of examples of archaeal promoters which do not contain the box A consensus sequence [26]. A pyrimidine-rich stretch of 19 nucleotides containing the sequence TTTTTAT is located downstream of the stop codon and may act as a transcription termination signal [2,27].

Surprisingly the *soxL* gene encodes a 12 amino acids long N-terminal extension which is not present in the mature Rieske-I protein isolated from membranes [1]. In this respect it resembles the eukaryotic Rieske proteins which initially contain targeting sequences that are removed in the process of membrane insertion [28–30]. It appears reasonable to assume that the pre-sequence of the Rieske-I protein is also involved in the membrane targeting of the protein, especially since it shows similarities to the hydrophobic domain of the eukaryotic signal peptides [31].

In contrast, the Rieske-II protein contains no cleavable presequence [2] but shows a remarkable similarity in the N-terminal region to other prokaryotic Rieske proteins and even to the OmpA leader sequence of *E. coli* (Fig. 2). This suggests that the N-terminal part of the Rieske-II protein contains the signals for membrane targeting and/or insertion as was suggested in the case of the *Rhodobacter sphaeroides* Rieske protein [32].

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          TGG GTG GCG TAG CAG AGA GGA GGA GGA AGT TTC TGA AGT CAC TGA TAT TTG GGA
                    9          18          27          36          45          54

Met Ala  Ala Ala  Ala Val Val Gly Ile Ile Pro Gly Val Ser Val Leu Val Pro Pro Thr
ATG GCT GCA GCT GCA GTT GTA GGA ATT ATA CCT GGA GTC AGT GTT CTA GTA CCA CCT ACA
          63          72          81          90          99          108

Val Thr Val Ala Thr Gly Phe Pro Lys Ser Leu Leu Ile Asp Ser Ser Gly Asn Pro Val
GTT ACT GTT GCA ACT GGA TTT CCA AAG TCA CTA TTA ATA GAT TCC TCA GGA AAT CCG GTA
          123         132         141         150         159         168

Lys Ala Ser Ser Leu Pro Val Asn Ser Pro Tyr Ile Met Val Phe Glu Tyr Pro Met Thr
AAG GCT TCG TCT TTA CCA GTA AAT AGT CCC TAT ATC ATG GTC TTT GAA TAC CCA ATG ACT
          183         192         201         210         219         228

Gly Glu Pro Asn Phe Leu Ile Asn Leu Gly Asp Ser Ser Gly Arg Pro Val Glu Ile Asn
GGA GAG CCT AAT TTC TTA ATA AAC TTA GGC GAT TCG AGT GGG AGA CCA GTT GAG ATC AAC
          243         252         261         270         279         288

Pro Thr Lys Val Val Ile Pro Gln Thr Gly Lys Thr Tyr Asp Phe Pro Gly Gly Val Gly
CCT ACC AAG GTA GTA ATA CCT CAA ACG GGG AAA ACC TAT GAC TTC CCT GGA GGA GTA GGT
          303         312         321         330         339         348

Pro Asn Lys Ser Ile Val Ala Tyr Ser Ala Ile Cys Gln His Leu Gly Cys Thr Pro Pro
CCT AAT AAG TCA ATT GTC GCT TAT TCA GCC ATT TGT CAA CAT CTG GGT TGT ACA CCG CCG
          363         372         381         390         399         408

Tyr Ile His Phe Tyr Pro Pro Asn Tyr Val Asn Ser Gly Gln Leu Thr Ala Ser Glu Pro
TAT ATT CAC TTT TAT CCT CCC AAT TAT GTC AAT TCA GGT CAG CTA ACA GCT TCA GAG CCA
          423         432         441         450         459         468

Asp Gln Leu Thr Ala Ala Ala Leu Leu Ala Ala Arg Gln Ala Asn Val Pro Ala Leu Ile
GAC CAA CTG ACA GCA GCT GCT TTA TTA GCA GCG AGA CAA GCA AAT GTA CCA GCT TTG ATA
          483         492         501         510         519         528

His Cys Asp Cys His Gly Ser Thr Tyr Asp Pro Tyr His Gly Ala Ser Val Leu Thr Gly
CAT TGT GAC TGC CAT GGG TCA ACT TAT GAT CCT TAC CAC GGT GCA TCT GTA TTA ACT GGA
          543         552         561         570         579         588

Pro Thr Val Arg Pro Leu Pro Ala Val Ile Leu Glu Trp Asp Ser Ser Thr Asp Tyr Leu
CCC ACA GTA AGA CCT TTA CCT GCC GTT ATA CTC GAA TGG GAC TCC TCA ACA GAT TAC TTA
          603         612         621         630         639         648

Tyr Ala Ile Gly Ser Val Gly Val Ala Val Tyr Pro Asn Gly Ser Asn Gly Ile Pro Ser
TAT GCC ATA GGA TCT GTT GGT GTA GCA GTT TAT CCA AAT GGC AGT AAT GGG ATA CCT TCT
          663         672         681         690         699         708

Gln Asp Pro Thr Glu Asp Leu Ser Ser Ser Phe Gly Thr Ser Val Gly Glu Lys Ile Thr
CAA GAT CCA ACT GAA GAT CTA AGC TCA AGT TTT GGC ACC TCT GTG GGA GAG AAG ATA ACA
          723         732         741         750         759         768

Val Ser Gln Thr Glu Asn Pro Phe Ser Ser
GTG TCT CAA ACA GAA AAT CCC TTC TCC TCA TAA GTC CAA CGC TTT ATT TTT ATA TTT TAG
          783         792         801         810         819         828

ATT ATG GAA
          843
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Fig. 1. Nucleotide sequence of the *soxL* gene. The DNA sequence has been deposited in the EMBL data bank under accession number X97067. The potential transcription start and termination sites are shown underlined. The amino acids of the pre-sequence are printed in italics. The N-terminal amino acids determined by protein sequencing are shown in bold letters. The underlined amino acids indicate a potential transmembrane helix of the precursor protein.

Both *Sulfolobus* Rieske proteins contain the typical iron sulfur cluster binding motifs that are strictly conserved in all Rieske and also to some extent Rieske-type proteins (Fig. 3). The pattern of conservation CxHLGC for the first and CPCHGSxY (x = any amino acid) for the second site is typical of the membrane bound Rieske proteins of the photosynthetic and respiratory electron transfer chains rather than of the soluble Rieske-type proteins of the bacterial oxygenases. A remarkable and unexpected exception is the proline → aspartate exchange at position 143 of the Rieske-I protein. However, this exchange does not alter the EPR characteristics of the Rieske-I protein which are almost indistinguishable from those of other Rieske proteins [1,33]. In addition EPR redox titration of the purified Rieske-I revealed a midpoint potential of +270 ± 10 mV (at pH 7.5) (data not shown) which is similar to the value of +320 mV determined for the sum of both Rieske clusters in *Sulfolobus* membranes [22]. Since the existence of two clearly different Rieske centers in the membranes of *S. acidocaldarius* is now firmly established, the results of previous studies performed on membranes [22,33] need to be reconsidered in order to identify the contributions of the in-

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                                    ++ Hydrophobic stretch
Ecoli OmpA                    MKKT--------------AIAIAVALAGFATVAQA ... ... ...
Sulac Rieske-II  1          MDRRTFLRLYLLV-------GAAIAVA-----PVIK---PALDYVGYFYS
Synechococ       1    MTQLSGSSDVPDLGRRQFL--NLLWV-----GTAAGTAL-GGLYPVIKYFIPP-SSGGAGGG
Nostoc           1    MAQFSESADVPDMGRRQFM--NLLTFGTV--TGVALGAL----YPVVKYFIPP-ASGGAGGG
Parde            1    MSHADEHAGDHGATRRDFLYYATAGAGTVA-AGAAA--WTLVNQMNPSADVQALASIQVDVS
Rhosh            1        MSHAEDNAGTRRDFLYHATAATGVVV-TGAA--VWPLINQMNASADVKAMSSIFVDVS
Rhoca            1        MSHAEDNAGTRRDFLYHA-AATGVVV-TGAA--VWPLINQMNASADVKAMASIFVDVS
Rhoru            1  MAEAEHTASTPGGESSRRDFLIYGTTAVGAV---GVALAVWPFIDFMNPAADTLALASTEVDVS
Synechocys      12  -IMTQISGSPDVPDLGRRQFM--NLLTFGTIT--GVAAGAL----YPAVKYLIPP-SSGGSGGG
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Fig. 2. Alignment of the N-terminal sequences of the *Sulfolobus* Rieske-II protein with the sequences of other bacterial Rieske proteins and the ompA leader sequence. The conserved positive charges and the hydrophobic region are indicated. Abbreviations: *Sulac*, *Sulfolobus acidocaldarius*; *Synecococ*, *Synechococcus*; *Parde*, *Paracoccus denitrificans*; *Rhosh*, *Rhodobacter sphaeroides*; *Rhoca*, *Rhodobacter capsulatus*; *Rhoru*, *Rhodospirillum rubrum*; *Senechocys*, *Senechocystis*.

dividual proteins to the observed effects. EPR studies concerning this problem are in progress.

One major difference to other Rieske proteins is the enlarged distance between the cluster binding sites of the *Sulfolobus* proteins. While it is 12–18 residues for other proteins it is 24 amino acids in the case of the Rieske-II protein and even 44 residues in the case of the Rieske-I protein. At present there is no obvious reason for this enlarged distance. It may reflect either an adaptation to extreme temperatures or an altered function.

The overall similarity of the *Sulfolobus* Rieske proteins to the proteins of other organisms is rather low (7–22% identical residues). The similarity between both *Sulfolobus* proteins is only slightly higher (24%). This is also reflected in the phylogenetic tree constructed from the protein sequences (Fig. 4). With the exception of the position of the *Chlorobium* sequence, the tree for the Rieske proteins is comparable to that derived from the 16S rRNA sequences [34], assuming an endosymbiotic origin of the eukaryotic proteins. Both *Sulfolobus* proteins cluster together on a separate branch. Therefore a horizontal gene transfer for one or both genes for the *Sulfolobus* proteins from bacteria or eukaryotes appears unlikely. It is also striking that the evolutionary distance between these two proteins is almost as large as between the mitochondrial and the plastidic Rieske proteins.

The functional assignment of the *Sulfolobus* Rieske proteins is still incomplete. Protein chemical [3] and genetic [2] evidence suggests that the Rieske-II protein is a component of the SoxM terminal oxidase complex, apparently combining the essential components of a cytochrome *bc* complex, in this case more properly addressed as a cytochrome *b*Rieske complex, and of a terminal oxidase. However, there is no protein chemical or genetic evidence [16] for an association of the Rieske-I with the other quinol-oxidase (SoxABCD) of *Sulfolobus*, especially since the SoxABCD oxidase can be easily isolated in a highly active form without any trace of Rieske protein (Gleißner et al., in preparation). Given the low sequence similarity of both proteins it is also very unlikely that they can functionally replace each other, i.e. that the SoxM oxidase can form a functional complex with either one of the *Sulfolobus* Rieske proteins. Since the Rieske-I protein is clearly able to interact with quinols and cytochromes as demonstrated by its ubiquinol-cytochrome *c*-reductase activity [1], it may function within a so far unrecognized reaction sequence of the *Sulfolobus* respiratory chain.

To our knowledge *Sulfolobus* is the first proven case of two significantly different Rieske proteins occurring in a prokaryotic organism. The diversity between these two proteins is dramatically higher than of the iso-forms of Rieske proteins previously reported from chloroplasts [35], which could be easily explained by the existence of two slightly different alleles.

Our results suggest that the existence of both *Sulfolobus* Rieske proteins is the result of a gene duplication event that took place shortly after the bacteria/archaea separation. A long, independent evolution of both proteins would explain

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                          SGQLTASEPDQLTAAALLAARQANV
                                              *
Sulac Rieske-I    AICQHLGCTPPYIHFYPPNYVN     PALIHCDCH-GSTYDPYHGASVLTGPTVR ┐
Sulac Rieske-II   DVCVHLGCQLPAQVIVSSESDPGLYAKGADLHCPCH-GSIYALKDGGVVVSGPAPR │
Parde             GVCTHLGC-VPIGDGAGD---------FGGWFCPCH-GSHYDT--SGRIRRGPAPQ │
Rhosh             GVCTHLGC-VPMGDKSGD---------FGGWFCPCH-GSHYDS--AGRIRKGPAPR │
Sacchcer          GICTHLGC-VPIGEA-GD---------FGGWFCPCH-GSHYDI--SGRIRKGPAPL │ Rieske
Bosta             GVCTHLGC-VPIANA-GD---------FGGYYCPCH-GSHYDA--SGRIRKGPAPL │ proteins
Soltu mito.       GVCTHLGC-IPLPNA-GD---------FGGWFCPCH-GSHYDI--SGRIRKGPAPY │
Synechococ        AICTHLGCVVPWNTA-----------ENKFMCPCH-GSQYDE--TGKVVRGPAPL │
Spiol clp.        AVCTHLGCVVPFNAA-----------ENKFICPCH-GSQYNN--QGRVVRGPAPL │
Chlorolim         AVCTHLGCLVNWVDA-----------DNQYFCPCH-GAKYKL--TGII-SGPQPL │
Bacsub            PICKHLGCTVNWNSDPKN---------PNKFFCPCHYGLYEKD--GTNVPGTPPLA ┘
Pseupu TODB       DTCTH-GDWALSDGY-LD---------GDIVECTLHFGKFCVR--TGKVKALPACK ┐ Rieske-type
Pseupu BNZP3      NQCRHRGMRICRADA-GN---------AKAFTCSYH-GWAYDT--AGNLVNVPYEA ┘ proteins
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Fig. 3. Alignment of the iron sulfur cluster binding regions of representative Rieske and Rieske-type proteins. The asterisk indicates the Pro→Asp exchange at position 143 of the *Sulfolobus* Rieske-I protein. Abbreviations: *Sacchcer*, *Saccharomyces cerevisiae*; *Bosta*, *Bos taurus*; *Soltu*, *Solanum tuberosum*; *Spiol*, *Spinacia oleracea*; *Chlorolim*, *Chlorobium limnicola*; *Bacsub*, *Bacillus subtilis*; *Pseupu*, *Pseudomonas putida*; mito., mitochondria; clp., chloroplasts; TODB, ferredoxin component of the toluene dioxygenase; BNZP3, benzene dioxygenase; all others as indicated for Fig. 2.

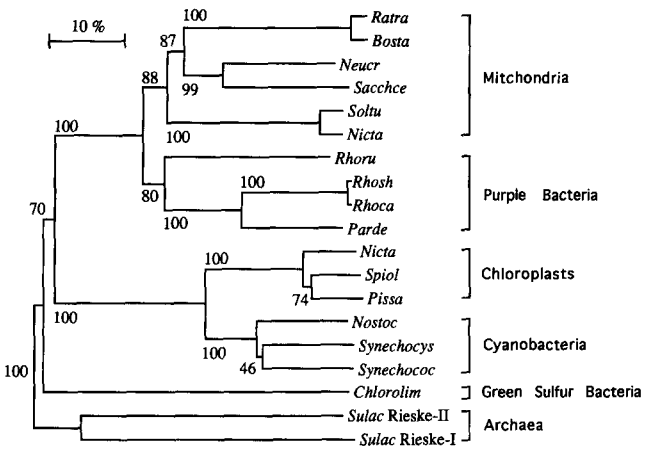

Fig. 4. Phylogenetic tree of the Rieske iron sulfur proteins. Abbreviations: *Ratra, Rattus rattus*; *Neucr, Neurospora crassa*; *Nicta, Nicotiana tabacum*; *Pissa, Pisum sativum*; all others as indicated in Figs. 2 and 3. The numbers indicate the bootstrap confidence levels.

their low similarity as well as the apparently different signal structures for membrane targeting.

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