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A Novel Rieske Iron-Sulfur Protein from the Hyperthermophilic Crenarchaeon *Pyrobaculum aerophilum*: Sequencing of the Gene, Expression in *E. coli* and Characterization of the Protein

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The crenarchaeon *Pyrobaculum aerophilum* is with an optimal growth temperature of 100 °C one of the most thermophilic organisms known to possess an aerobic respiratory chain. The analysis of DNA sequences from the *Pyrobaculum* genome project lead to the identification of an open reading frame potentially coding for a Rieske iron-sulfur protein. The complete gene (named *parR*) was cloned and sequenced. The deduced amino acid sequence displays unusual amino acid exchanges and a so far unknown sequence insertion. The N-terminus shows similarities to bacterial signal sequences. Several forms of the gene were expressed in *E. coli* in order to verify the classification as a Rieske protein and to facilitate biophysical studies. Soluble, thermo-stable proteins with correctly inserted iron-sulfur clusters were expressed from two versions of the gene. The Δ1-23 truncated holo-protein is redox active. It displays the typical spectroscopic properties of a Rieske protein. The redox potential was determined to be +215 mV at pH 6.5 and is pH dependent above pH 7.5 revealing the influence of two protonation equilibria with pKa values of 8.1 and 9.8. Phylogenetic analysis demonstrates that the parR protein clusters together with the two other available archaeal Rieske sequences from *Sulfolobus* on a separate branch of the phylogenetic tree apart from the proteins from thermophilic bacteria like *Aquifex* and *Thermus*.

KEY WORDS: *Pyrobaculum aerophilum*, Archaea, hyperthermophile, Rieske protein, sequence, expression, redox potential, pH-dependence.

INTRODUCTION

Rieske iron-sulfur proteins are essential components of the cytochrome bc_1 and b_6f complexes and as such almost ubiquitously distributed among the bacteria and eucarya. Like the plant type ferredoxins they harbour a [2Fe-2S] cluster as a prosthetic group. However, unlike in these proteins one of the iron ions is liganded by the nitrogens of two conserved histidins in addition to the cysteins and the sulfide ions. As a consequence the Rieske proteins display distinctive spectroscopic properties as well as unusually high redox potentials in the range of +100 to +400 mV (Schmidt *et al.*, 1994, Riedel *et al.*, 1993, Trumpower 1981), whereas the potentials of the ferredoxins can be as low as −420 mV (Mason and Cammack 1992). Intermediate redox potentials in the range from −150 to +5 mV are reported for the Rieske-type proteins (Mason and Cammack 1992). These components of many bacterial hydroxylases and oxygenases share the histidyl ligandation and the spectroscopic properties

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of the Rieske proteins, even though they display only minimal sequence similarities (Mason and Cammack 1992).

The structures of the spinach chloroplast (Carrell *et al.*, 1997) and the bovine heart mitochondrial Rieske proteins (Iwata *et al.*, 1996), as well as site directed mutagenesis experiments (Schroter *et al.*, 1998, Denke *et al.*, 1998) identified two critical amino acids: Ser-163 and Tyr-165 in the bovine heart protein, the homologous residues Ser-130 and Tyr-132 of the chloroplast protein, Ser-157 and Tyr-159 in the Rieske protein of *Paracoccus denitrificans* (Schroter *et al.*, 1998) as well as Ser-183 and Tyr-185 of the yeast protein (Denke *et al.*, 1998), which contribute to the difference of the mid-point potentials between the Rieske and the Rieske-type proteins. The OH-groups of the serine residues are hydrogen bonded to one of the sulfide ions of the FeS cluster, whereas the OH-groups of the tyrosine residues are contributing hydrogen bonds to one of the cystein sulfurs liganding the cluster.

Two groups can be distinguished among the Rieske proteins studied in detail so far: The first group involves the proteins from the ubi-, plasto- and caldariella quinone containing organisms in which the serine as well as the tyrosine residues are conserved. These proteins display mid-point potentials in the range of +280 to +375 mV (Trumpower, 1981, Schmidt *et al.*, 1997). The second group includes the proteins from the menaquinone containing organisms. In these proteins the serine is replaced by a non hydroxylated residue, mostly glycine or alanine. The mid-point potentials of these proteins are in the range of +105 to +160 mV (Riedel *et al.*, 1993, Trumpower, 1981).

As of today only sparse data are available concerning the occurrence, the function and the properties of archaeal Rieske proteins. Two membrane bound proteins have been described from the hyper thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* (DSM 639) (Schmidt *et al.*, 1996, Schmidt *et al.*, 1995, Castresana *et al.*, 1995, Lubben *et al.*, 1994). One of these (soxF) seems to be a subunit of a terminal oxidase super complex together with a cytochrome homologous to cytochrome *b* or -b_6 (Castresana *et al.*, 1995). The functional context of the other *Sulfolobus* Rieske protein (soxL) has not been elucidated so far. It displays the least similarities to all other members of this family (Schmidt *et al.*, 1996). In addition, a soluble Rieske protein of unknown function has been purified and characterized from *Sulfolobus sp*. strain 7 (Iwasaki *et al.*, 1996, Iwasaki *et al.*, 1996).

Here we report the identification and sequencing of a gene coding for a Rieske iron-sulfur protein from the hyper thermophilic crenarchaeon *Pyrobaculum aerophilum* (Volkl *et al.*, 1993) as well as the partial characterization of the recombinant protein expressed in *E. coli*. *P. aerophilum* is a facultative micro aerophilic organism thriving on a variety of different carbon sources. Under anaerobic conditions nitrate is utilized as terminal electron acceptor of the respiratory chain (Volkl *et al.*, 1993). With an optimal growth temperature of 100 °C *P. aerophilum* is one of the most thermophilic organisms known to contain an aerobic respiratory chain. Hence, and due to the phylogenetic location of *Pyrobaculum* on the lowest branch, at the base of the crenarchaeota (Burggraf *et al.*, 1997) this organism deserves special interest with respect to the evolution of the components of aerobic respiration chains.

MATERIALS AND METHODS

DNA Techniques

630 bp of DNA were amplified from the fosmid SPAF 3G8 (Fitz-Gibbon *et al.* 1997) in a PCR reaction using the primer parRext (5′ ACA AAC ACC TAC AAG TCC CTA TAT) and parRBam-02 (5′ AGA GCG CTT GTG AGG ATC CGG GAT TT) and a proofreading polymerase. The product was purified by agarose gel electrophoresis, phosphorylated by treatment with T4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany), ligated into the vector pBS II SK^+ (Stratagene, Heidelberg, Germany) which was digested with EcoRV and dephosphorylated with calf intestinal phosphatase (Boehringer Mannnheim, Germany) (plasmid named pBS/parR) and transformed into E. coli XL2 Blue (Stratagene, Heidelberg, Germany) as described in (Schmidt *et al.*, 1997). Three of the clones were sequenced as described in (Schmidt *et al.*, 1996) in order to identify potential PCR artefacts.

A total of five different plasmids for the expression of the parR gene in E. coli were constructed: For the expression of the full length of the protein as well as a Δ1-23 truncated form the coding region was amplified from the plasmid pBS/parR using the primer parR-Bam-02 in combination with either parRExp-02 (5′ TAT GGT GGA TGA GAA CAG GAG GAA C) or parRExp-03 (TAT GTT GGC AAC GCC GCT TGT GG). The DNA was purified and phosphorylated as above and ligated into the plasmid pet 11a (Stratagene,

Heidelberg, Germany) which was prepared for the ligation by digestion with NdeI followed by a treatment with Klenow DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) to generate blunt ends and dephosphorylation as described above. The ligated DNA was transformed into *E. coli* XL2 Blue, colonies were selected, plasmid DNA isolated and the orientation of the inserts checked by digestion with BamHI. Plasmids with correctly oriented inserts were finally transformed into the expression strain *E. coli* BL21DE3 (Stratagene, Heidelberg, Germany). The expression plasmid for a Δ1-84 truncated protein was constructed starting from the same PCR reactions. However, the naturally occurring NcoI site at the position of Met85 in combination with the BamHI site introduced with the primer parRBam-02 were used for an oriented ligation into the vector pet 15b (Stratagene, Heidelberg, Germany). Expression plasmids for the Δ2-42 and Δ2-53 truncated proteins were constructed by PCR mutagenesis as described in (Schmidt et al. 1997) using the primer ANTI3a (5′ ATG TAT ATC TCC TTA AAG TTA AAC) in combination with the primer parRK42M (5′ AAT GCC CGA GCC CTC TGG CGC AA) or parRI53M (5′ GAT GTG TAA AGA CGT TGA CAG CT).

Overproduction of the parR Protein

The conditions for the expression of the recombinant proteins in *E. coli* were as given in (Schmidt *et al.*, 1997) with the following modifications: The temperature was kept at 37°C during the whole experiment, L-cystein was omitted from the medium for the over night culture and the ampicillin concentration was raised to 200 μg/ml. The cells were harvested and analysed for the expression of the parR proteins as described in (Schmidt *et al.*, 1997).

Purification of the Recombinant Protein

The parRΔ1-23 protein was purified from the *E. coli* cytosol by heat precipitation (10 min at 80°C) followed by a 15 min incubation on ice and a centrifugation step to remove the precipitated *E. coli* proteins.

EPR Spectroscopy and Redox Titrations

EPR redox titration were performed and EPR spectra recorded as described in (Anemuller *et al.*, 1994).

Miscellaneous Techniques

Phylogenetic analysis was performed and alignments were calculated using the program ClustalW1.7 (Thompson *et al.*, 1994). Secondary structure analysis was performed using the PHDsec program at the EMBL (Heidelberg, Germany) via internet (Rost and Sander 1993, Rost and Sander 1994). Homology modelling was done using the Swiss-Model server (Guex and Peitsch 1997) via internet. All other techniques were performed as described in (Schmidt *et al.*, 1995).

RESULTS

An amino acid sequence displaying similarities to the sequences of known Rieske proteins was detected upon translation of a DNA sequence from the fosmid SPAF 3G8 from a fosmid library of the entire *Pyrobaculum aerophilum* genome (Fitz-Gibbon *et al.*, 1997). A DNA fragment of 630 bp was amplified from this fosmid and cloned into the vector pBS II SK^+. Three clones were sequenced in order to identify possible PCR artefacts. An open reading frame of 561 bp was detected within the three identical sequences. Since the deduced protein sequence displayed pronounced similarities to the sequences of known archaeal and bacterial Rieske proteins (Schmidt *et al.*, 1996, Castresana *et al.*, 1995) the gene was named *parR* (***P****yrobaculum* ***a****erophilum* ***r***espiratory chain ***R***ieske protein). The sequence was deposited under the accession number AF049911 in the "GenBank" data base.

The N-terminus of the parR protein displays similarities to the proposed membrane targeting signal sequences of other Rieske proteins (Schmidt *et al.*, 1996, Schmidt *et al.*, 1997, van Doren *et al.*, 1993) (Figure 1a). The residues 8 to 34 display a strong hydrophobicity. The calculated transfer energy for the residues 8 to 22 is larger than the threshold of 86 [kJ mol^{-1}] (Engelman *et al.*, 1986) suggesting that this part of the protein is buried in the hydrophobic core of the membrane. The residues 30 to 60 display weak similarities to the flexible regions of the spinach chloroplast Rieske protein (Carrell *et al.*, 1997) and the bovine heart Rieske protein (Iwata *et al.*, 1998, Zhang *et al.*, 1998) connecting the membrane anchors with the main bodies of these proteins (Figure 1b).

The central part of the protein contains the well conserved motifs of the iron-sulfur cluster binding sites and the Pro-loop (Iwata *et al.*, 1996). However, a tyro-

Fig. 1. Comparison of the parR protein sequence with selected Rieske iron-sulfur protein sequences. a) Comparison of the N-terminal region with the N-termini of bacterial and archaeal proteins. Conserved residues and conservative replacements are shaded in grey. The conserved positive charges are marked by (+). Abbreviations: PYRaer, *Pyrobaculum aerophilum*; SULaci, *Sulfolobus acidocaldarius*; PARden, *Paracoccus denitrificans*; RHOsph, *Rhodobacter sphaeroides*; RHOcap, *Rhodobacter capsulatus*; RHOrub, *Rhodospirillum rubrum*. b) Comparison with the flexibe regions of the spinach chloroplast (SPIole clp.) and the bovine heart (BOStau mit.) Rieske proteins. Ile74, the pivot point of the bovine heart protein is marked by an asterix. c) Comparison of the central parts of the proteins containing the iron-sulfur cluster binding sites and the Pro-loop. Identical residues are shaded in grey. The Y->F exchange in the C-terminal cluster binding site of the parR protein is marked by an arrow. The extra cysteines between the c-terminal cluster binding motif and the Pro-loop of the Pyrobaculum sequence are shown bold. ⟨-⟩ marks the omission of 23 amino acids from the soxL sequence to simplify the figure. Abbreviations: AQUaeo, *Aquifex aeolicus*; THEthe, *Thermus thermophilus*; BACsub, *Bacillus subtilis*; CHLlim, *Chlorobium limnicola*; NICtab, *Nicotiana tabacum*, CHRvin, *Chromatium vinosum*; SACcer, *Saccharomyces cerivisiae*; clp., chloroplast; mit., mitochondria.

sine residue conserved in almost all known Rieske proteins (Tyr185 in the yeast protein (Denke *et al.*, 1998), Tyr159 in the *Paracoccus* protein (Schroter *et al.*, 1998)) is replaced by a phenylalanine (Phe157) and a so far unknown insertion of 12 amino acids is present between the C-terminal cluster binding motif and the Pro-loop (Figure 1c).

Since the relatively low growth yield of *Pyrobaculum* (Volkl *et al.*, 1993) impedes the isolation of sufficient amounts of the authentic protein, five clones for the expression of the *parR* gene in *E. coli* were constructed in order to verify the classification of the protein as a Rieske iron-sulfur protein and to facilitate further studies. The first clone contained the full length of the gene. The N-terminal region containing the putative signal sequence and the most hydrophobic region of the protein was deleted from the second clone (parRΔ1-23). Three further N-terminally truncated forms (parRΔ2-42, parRΔ2-53, and parRΔ1-84) were expressed in order to identify the minimal structure required for a stable folding and the incorporation of the [2Fe-2S] cluster into the protein. Soluble forms of

the recombinant proteins containing the Rieske [2Fe-2S] cluster were only detectable in the cases of the Δ1-23 and the Δ2-42 forms. No expression of the full length of the gene could be detected, whereas only inclusion bodies were formed in the case of the Δ2-53 and Δ1-84 proteins. No iron-sulfur clusters could be detected in any of the insoluble fractions i.e. the *E. coli* membranes and the inclusion bodies. Attempts to reconstitute the [2Fe-2S] cluster into the Δ1-84 protein were without success.

Since the Δ1-23 protein could be expected to show the strongest similarities to the authentic parR protein it was chosen for further characterization. It was moderately thermostable and could be readily purified by heat precipitation (10 min. at 80 °C) of the *E. coli* proteins (data not shown).

The protein was normally isolated in the fully oxidized state but was completely reduced by the addition of 5 mM ascorbate. The reduced protein displayed the typical EPR spectrum of a Rieske [2Fe-2S] cluster with the g-values g_{xyz} = 1.795, 1.888, 2.030 (Figure 2). The UV/Vis spectra of the protein (Figure 3) are typical for a Rieske protein as well. The reduction resulted in a decrease of the absorption of the [2Fe-2S] cluster in the visible region (Figure 3).

The pH-dependence of the mid-point potential of the parR protein was examined since all archaeal Rieske proteins investigated so far exhibited unusually acidic pKa-values for the first protonation equilibrium (Anemuller *et al.*, 1994, Iwasaki *et al.*, 1996). EPR redox titrations at different pH-values proved the redox potential to be pH dependent above pH 7.5. The data could be best fitted to equation (1) (Clark 1960) assuming two protonation/deprotonation equilibria. pKa-values of 8.1 for the first and 9.8 for the second protonation step and an E_m(low pH) of +224 mV were determined (Figure 4). Fitting the data to equation (2), assuming a single protonation/deprotonation equilibrium, resulted in a significantly weaker correlation (Figure 4).

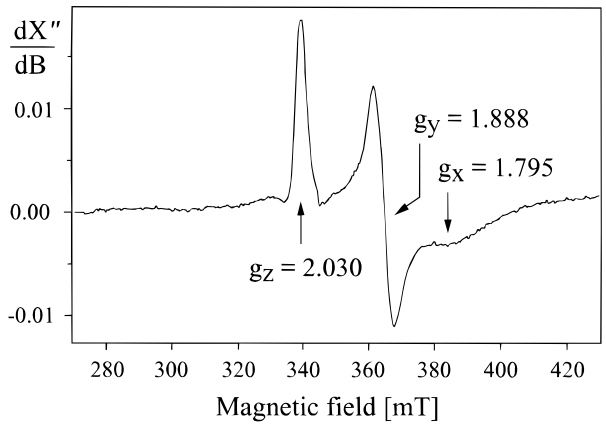

Fig. 2. EPR spectrum of the ascorbate reduced parRΔ1-23 protein. The spectrum was recorded at 15 K, modulation amplitude 0.5 mT, modulation frequency 100 kHz microwave power 2 mW, and micro wave frequency 9.640713 GHz.

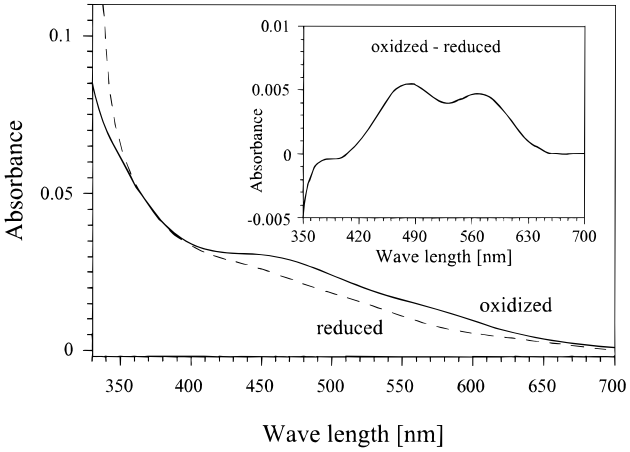

Fig. 3. UV/vis spectrum of the oxidized (solid line) and the ascorbate (2.5 mM) reduced (dashed line) parRΔ1-23 protein. The spectra were recorded in 50 mM Tris/HCl pH 7.5, 1 mM EDTA at a protein concentration of 0.45 mg ml^{-1}. The insert shows the oxidized minus reduced difference spectrum. The maxima are at 475nm and 570 nm. The minimum is at 525 nm.

$$E_m = E_{m\,(low\,pH)} + (RT/nF) * \ln([H^+]^2/([H^+]^2 + K_{0X1} * [H^+] + K_{0X1} * K_{0X2})) \quad (1)$$

$$E_m = E_{m\,(low\,pH)} - (RT/nF) * \ln(1 + K_{OX}/[H^+]) \quad (2)$$

Phylogenetic analysis demonstrates that despite the low similarity to the sequences of the other archaeal Rieske proteins (Schmidt *et al.*, 1996, Castresana *et al.*, 1995) the parR protein forms the lowest branch of the archaeal domain of the Rieske proteins rather than clustering together with the sequences from the thermophilic bacteria *Aquifex*, or *Thermus* (Figure 5).

DISCUSSION

The parR gene from *P. aerophilum* is the third example of a gene coding for an archaeal Rieske iron-sulfur protein. The other sequenced or characterized archaeal Rieske proteins are reported exclusively from *Sulfolobus* species (Schmidt *et al.*, 1996, Castresana *et al.*, 1995, Iwasaki *et al.*, 1996), whereas no gene

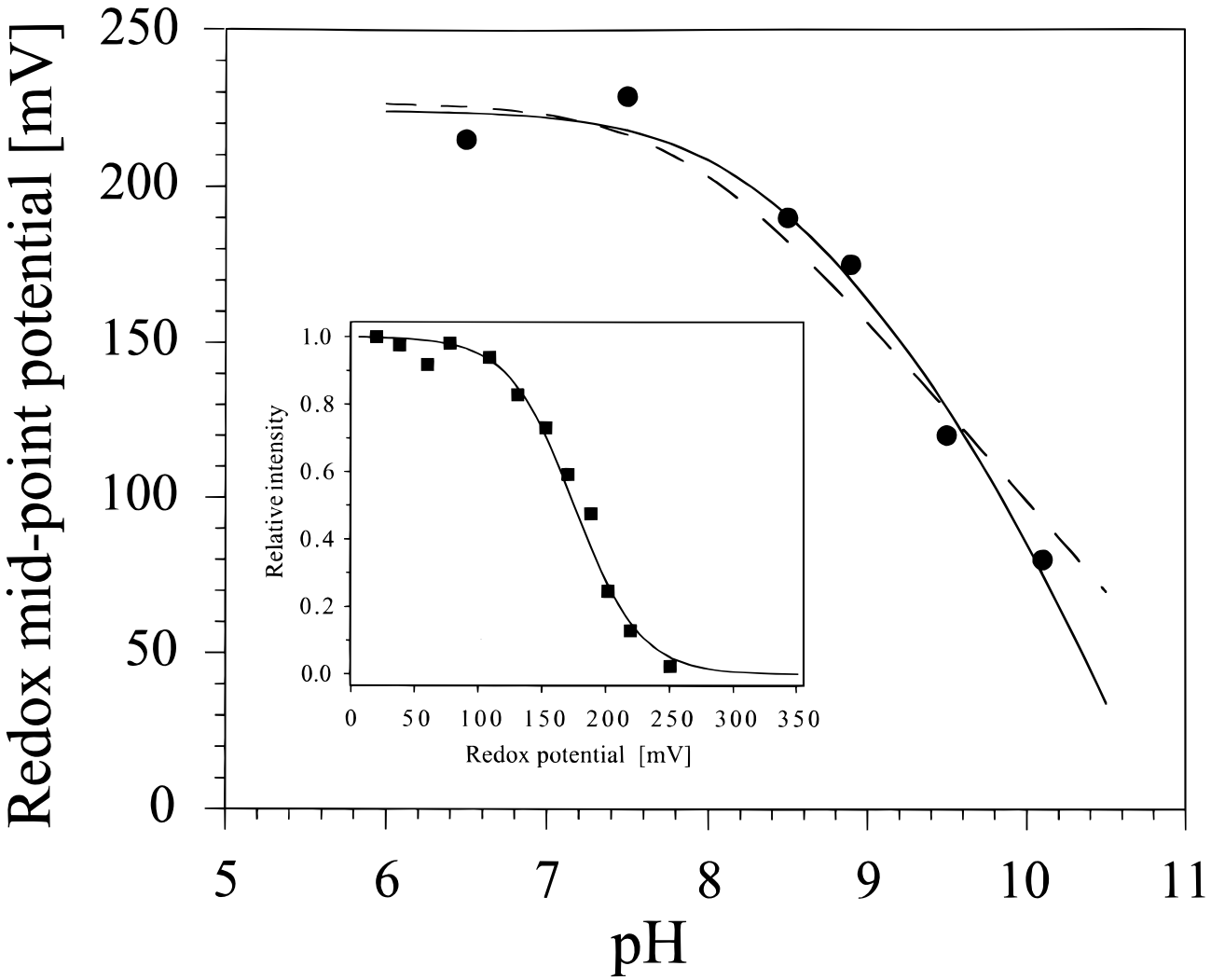

Fig. 4. pH dependence of the redox potential of the parRΔ1-23 protein. The solid line shows the best fit assuming two protonation equilibria with pKa-values of 8.1 and 9.8 and $E_{m(low\ pH)} = +224$ mV. The dashed line represents the best fit assuming a single protonation equilibrium (pKa = 7.8, $E_{m(low\ pH)} = +227$ mV). The insert shows an individual titration at pH 8.9 ($E_m = +175$ mV).

coding for a Rieske protein was detected in the complete genome of *Methanococcus janaschii*. Thus, the discovery of a Rieske protein in a crenarchaeon not belonging to the genus *Sulfolobus* suggests that Rieske proteins are indeed more widely distributed among the archaea, but like in the bacterial domain are not omnipresent. Phylogenetic analysis (Figure 5) reveals four main groups among the Rieske proteins, i.e., the proteins of the cytochrome bc_1 complexes found in mitochondria and bacteria, the Rieske proteins of the b_6f complexes from chloroplasts and cyanobacteria, those of the *Bacillus*-type complexes and the archaeal Rieske proteins. The parR protein together with the two Rieske proteins from *Sulfolobus* (Schmidt *et al.*, 1996, Castresana *et al.*, 1995) forms the archaeal branch of this phylogenetic tree, demonstrating that it is of genuine archaeal origin and was not acquired by horizontal gene transfer. The branching pattern of the three archaeal sequences is consistent with the phylogeny of the archaea as proposed in (Burggraf *et al.*, 1997) on the basis of the 16 S rRNA sequences.

The over all sequence similarity between the parR protein and the other Rieske proteins is rather low. However, all functionally important features can be identified. The N-terminus shows, like that of the soxF protein from *Sulfolobus* and those of many bacterial proteins (Schmidt *et al.*, 1997, Schmidt *et al.*, 1996, van Doren *et al.*, 1993), similarities to signal sequences

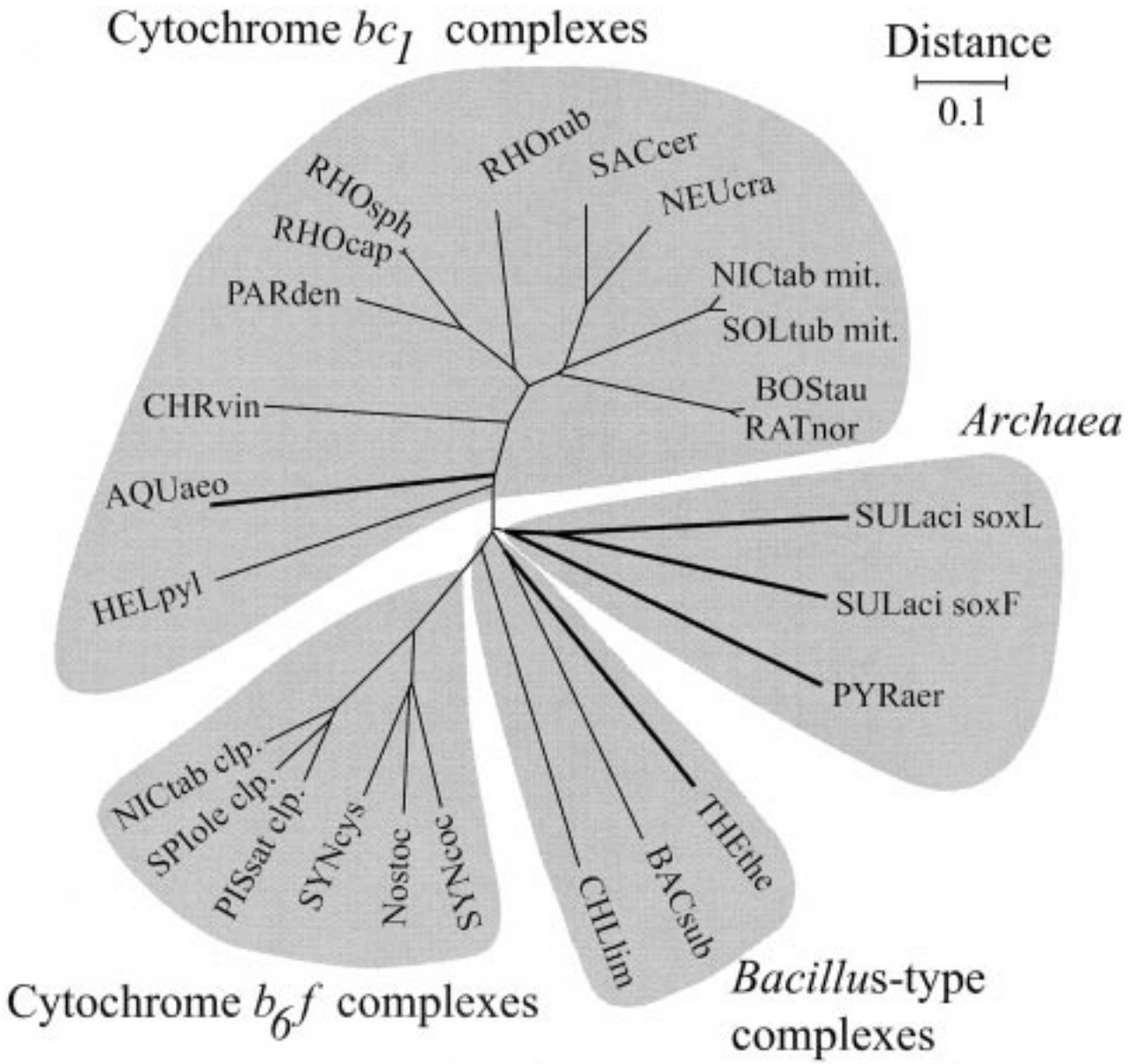

Fig. 5. Unrooted Phylogenetic tree of selected Rieske iron-sulfur proteins. The four main groups among the Rieske proteins, the proteins of the bc_1-, the b_6f-, and the *Bacillus*-type complexes as well as the archaeal proteins are identified by shading. Lineages leading to thermophilic organisms are shown bold. Abbreviations: RATnor, *Rattus norvegicus*; SOLtub, *Solanum tuberosum*; NEUcra, *Neurospora crassa*; HELpyl, *Helicobacter pylori*; PISsat, *Pisum sativum*; all others as indicated under figure 1. The scale bar represents a distance of 0.1.

for the membrane targeting of the proteins. The hydrophobic part of this putative signal sequence (residues 8 to 22) is sufficiently hydrophobic to act as a membrane anchor as demonstrated for the bovine heart mitochondria cytochrome bc_1-complex (Iwata *et al.*, 1998). The following region (residues 30 to 60) resembles the flexible regions of the bovine mitochondrial protein (Iwata *et al.*, 1998) and the spinach chloroplast protein (Carrell *et al.*, 1997). Even though only very few residues are identical, a number of conservative replacements can be identified. Thus, it appears possible that this region of the *Pyrobaculum* protein displays a similar flexibility as the corresponding regions of the mitochondrial and the plastidic proteins.

The iron-sulfur cluster binding motifs in the central part of the protein are well conserved except for

two substitutions (Figure 1c). Likewise conserved is the distance between the two cluster binding motifs. Thus, the insertions between the cluster binding sites found in the soxF and even more prominent in the soxL proteins of *Sulfolobus acidocaldarius* (Schmidt *et al.*, 1996) appear not to be a general characteristic of the archaeal Rieske proteins. The Pro-loop is conserved in the parR protein as well. An unique insertion of 12 amino acids is located between the C-terminal cluster binding motif and the Pro-loop. The functional importance of this sequence element is as yet obscure. However, this region contains two cysteine residues. Molecular modelling (Guex and Peitsch, 1997) of the parR protein on the basis of the coordinates of the bovine heart Rieske protein (Iwata *et al.*, 1996) suggests that these cysteins can form a disulfide bond (data not shown) and thus may contribute to the thermo-stability of the protein. One of the two amino acid exchanges within the cluster binding motifs, a phenylalanine (F113) instead of the leucine normally found in this position (Figure 1c) can be considered a conservative replacement. This is not the case for F137 since the tyrosine normally found in this position in almost all other known Rieske sequences is directly hydrogen bonded to the iron-sulfur cluster (Iwata *et al.*, 1996, Carrell *et al.*, 1997).

Only truncated forms of the parR protein could be expressed in *E. coli*. Soluble proteins containing the [2Fe-2S] cluster were obtained in the cases of the Δ1-23 and the Δ2-42 forms. The failure to express the full lengths of the protein can be explained by the discrepancies of the codon usages of *Pyrobaculum* and *E. coli*. So called rare codons (judged on the basis of the *E. coli* codon usage) occur with the highest frequency in the 5′ region of the gene, especially in a 30 bp stretch coding for the amino acids 6 to 15 (data not shown). All forms of the gene lacking this region (Δ1-23, Δ2-42, Δ2-53, as well as Δ1-84) were readily expressed. Since only the Δ1-23 and the Δ2-42 form, but not the Δ2-53, or the Δ1-84 version of the protein incorporated an [2Fe-2S] cluster, the region between the residues 42 and 53 has to contain sequence elements that are critical for the stability of the iron-sulfur cluster binding domain of the protein. Secondary structure prediction (Rost and Sander, 1994, Rost and Sander, 1993) suggests a β-sheet conformation for the residues 49 to 55 (Figure 6). These residues appear to be homologous to the residues 74 to 78 of the bovine heart Rieske protein which together with the C-terminal residues are forming one of the three β-sheet structures building the backbone of this protein (Iwata *et al.*, 1996). Since the truncation or deletion of this hypothetical structure element of the parR protein (Δ2-53, Δ1-84) leads to an insoluble protein which can no longer incorporate the iron-sulfur cluster it is tempting to assume an overall folding similar to that of the bovine heart protein. However, this conclusion has to be drawn with caution. A reasonable match of the secondary structure predicted for the parR protein with the structure determined for the bovine heart mitochondrial protein is evident only in the immediate vicinity of the iron-sulfur cluster binding sites (Figure 6).

Fig. 6. Comparison of the predicted secondary structure of the parR protein with the secondary structure of the bovine heart mitochondrial Rieske protein derived from the x-ray structures (Iwata et al. 1996, Iwata et al. 1998). β-sheet structures are indicated by capital letters, α-helical regions are shaded grey. The individual strands of the β-sheet structures of the bovine heart protein are annotated according to (Iwata et al. 1996). The N-termini of the different forms of the parR protein expressed in E. coli are marked as follows: m^-: No expression detectable; M24, M42: expression of soluble forms containing the iron-sulfur cluster; m53, m85: expression of inclusion bodies only.

The mid-point potential of the recombinant Δ1-23 form of *Pyrobaculum* Rieske protein is intermediate between those of the proteins from ubi-, plasto- and caldariella quinone containing organisms and the potentials of the proteins from the menaquinone containing organisms (Table I). It is comparable to the potential of the Y185F mutant protein from yeast (Denke *et al.*, 1998) and generally agrees with the conclusions concerning the effects of this amino acid residue on the mid-point potential of the iron-sulfur cluster derived from the site directed mutagenesis experiments on the *Paracoccus* (Schroter *et al.*, 1998) and the yeast (Denke *et al.*, 1998) Rieske proteins. The physiological significance of this amino acid exchange

Table I. Comparison of the C-Terminal Iron-Sulfur Cluster Binding Sites and the Mid-Point Potentials of Selected Rieske Proteins

Sequence	Source	Em [mV] (pH)
CPCHGSxY	*Rhodobacter capsulatus* [1)]	+310 (5.8–8.2)
	Spinach chloroplasts [2)]	+300 (7.5)
	Bovine heart mitochondria [3)]	+312 (7.2)
	Saccharomyces cerevisiae [4)]	+285 (7.0)
	Sulfolobus acidocaldarius (soxF) [5)]	+375 (7.5)
	Paracoccus denitrificans [6)]	+360 (6.0)
CPCHGzxY	*Bacillus firmus* [7)]	+105 (7.5)
	Thermus thermophilus [8)]	+150 (7.5)
	Chlorobium limnicola [1)]	+160 (7.0)
	Bacillus alkalophilus [1)]	+150 (7.0)
	Saccharomyces cerevisiae S183A [4)]	+155 (7.0)
	Paracoccus denitrificans S157A [6)]	+265 (6.0)
CPCHGSxF	*Pyrobaculum aerophilum*	+229 (7.5)
	Saccharomyces cerevisiae Y185F [4)]	+217 (7.0)
	Paracoccus denitrificans Y159F [6)]	+316 (6.0)

x: any amino acid; z: any amino acid except serin.
[1)] Trumpower 1981,
[2)] Zhang *et al.*, 1996,
[3)] Link *et al.*, 1992,
[4)] Denke *et al.*, 1998,
[5)] Schmidt *et al.*, 1997,
[6)] Schroter *et al.*, 1998,
[7)] Riedel *et al.*, 1993,
[8)] Kuila and Fee, 1986.

and the resulting redox potential in *P. aerophilum* remains to be established. Exclusively menaquinones have been reported from *Pyrobaculum* species (Lubben *et al.*, 1995, Gambacorta *et al.*, 1994). Thus, redox potential and iron-sulfur cluster binding motifs similar to those from the menaquinone containing organisms would be expected. A possible explanation for this apparent discrepancy is the adaptation to the high growth temperature of *Pyrobaculum*. Attempts to calculate the redox potential of the parR protein at the growth temperature of *Pyrobaculum* using the temperature dependence of -1.5 mV °C^{-1} as determined for the bovine heart protein (Link *et al.*, 1992) lead to a potential of approximately +115 mV at 100 °C. This would be well in the range of the mid-point potentials of +105 mV to +160 mV determined for the Rieske proteins of mesophilic, menaquinone containing organisms like *Bacillus* or *Chlorobium* (Table I). The only other Rieske protein sequence with a comparable Y → F exchange is reported from a mesophilic organism: *Chromatium vinosum* (GenBank AF034104). Unfortunately no further data about the properties of the latter protein are available. Ubi-and menaquinone at a ratio of 4.3:1 have been reported from *Chromatium* species (Nozawa *et al.*, 1987). This and the reported Pet$^+$ phenotype of the yeast strain containing a Rieske gene with the Y185F substitution (Denke *et al.*, 1998) suggests that in the case of mesophilic organisms this amino acid replacement is generally compatible with ubiquinone as the electron donor for the Rieske protein.

The mid-point potential of the *Pyrobaculum* protein is pH dependent as it was found for all Rieske proteins of the cytochrome bc_1 or b_f complexes as well as the archaeal Rieske proteins investigated so far (Table II). Both pKa-values are slightly higher than those reported for the bovine heart mitochondrial protein. The comparison of the first pKa-values, or were applicable the only determined pKa-value, reveals two groups of Rieske proteins. The first group displays pKa_1-values of approximately 8, whereas these values are shifted to 6.2 for the second group. The distribution into the two groups appears to be independent of the phylogeny and the optimal growth temperature of the organisms. Instead it seems to correlate with the acidophilicity of the organisms.

In summary, the sequencing of the parR gene and the characterization of recombinant protein did not result in the identification of traits specific for archaeal Rieske proteins. Instead it provides, in comparison with already existing data, further insights into ways

Table II. Comparison of the pKa-Values of Various Rieske Iron-Sulfur Proteins and of the Growth Conditions of the Organisms

Organism	pKa_1	pKa_2	thermo-philic	acido-philic
Pyrobaculum aerophilum	8.1	9.8	+	−
Thermus thermophilus [1)]	8.0	−	+	−
Bos taurus mitochondria [2)]	7.6	9.2	−	−
Bacillus firmus [3)]	~8.0	−	−	−
Thiobacillus ferrooxidans [4)]	6.2	−	−	+
Sulfolobus acidocaldarius [5)]	6.2	8.5	+	+
Sulfolobus sp. strain 7 [6)]	6.2	8.6	+	+

[1)] Kuila and Fee, 1986,
[2)] Link *et al.*, 1992,
[3)] Riedel *et al.*, 1993,
[4)] Schoepp *et al.*, 1999,
[5)] Anemuller *et al.*, 1994,
[6)] Iwasaki *et al.*, 1996.

by which the functional properties like the redox potential of iron-sulfur proteins can be adapted to the conditions of extreme environments i.e. high temperature or low pH.

Due to its stability, its small size, the availability in large amounts and easy accessibility to site directed mutagenesis techniques the parR protein will be a valuable model for general studies of the Rieske proteins. It will facilitate the further analysis of the respiratory chain of *Pyrobaculum aerophilum* which is one of the organisms at the very basis of the evolution of aerobic respiration according to its position within the universal phylogenetic tree. The presence of Rieske proteins in archaea like *Pyrobaculum* may indicate that these proteins and thus, potentially the cytochrome bc_1 or b_6f complexes are phylogenetically older than aerobic respiration.

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