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# Expression of the *Solfolobus acidocaldarius* Rieske Iron Sulfur Protein II (SOXF) with the Correctly Inserted [2FE–2S] Cluster in *Escherichia coli*

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**The Rieske protein II (Schmidt *et al.*, 1996, *FEBS Lett.* 388, 43–46) from the thermoacidophilic crenarcheon *Sulfolobus acidocaldarius* (DSM 639) was expressed in *E. coli* cells. The full length protein was strictly bound to the *E. coli* membranes and could only be removed by detergent treatment indicating the presence of a membrane anchor. The iron sulfur cluster was correctly inserted into a fraction of the full length protein and much more effectively into a soluble form created by the deletion of the 45 N-terminal amino acids. The soluble form of the protein displayed the typical spectroscopic properties of a respiratory Rieske protein. The midpoint potential was +375 mV determined by CD redox potentiometry. The presented data demonstrate that the structure of the recombinant protein is very similar or identical to the authentic protein making this a powerful model system for the studies of Rieske proteins by site directed mutagenesis.** 

The Rieske iron sulfur proteins are an unique group of membrane residing, [2Fe-2S] cluster containing proteins. With the exception of the archaeal, i.e. the two Rieske proteins of *Sulfolobus* [1, 2, 3, 4] and the protein from *Thermus thermophilus* [5], they are known as essential subunits of the cytochrome $bc_1/b_6f$ complexes [6, 7, 8]. Against the large group of the other [2Fe-2S] cluster containing proteins they stand out by their unusually high, pH dependent midpoint potentials of +105 to + 350 mV [9, 10] versus about −400 mV of the plant-type ferredoxins [11], their distinctive EPR signals [11] and the presence of two histidins replacing two of the four cystein residues normally found in the coordination sphere of the cluster [11]. An intermediate category of proteins are the soluble Rieske-type proteins reported from many bacterial oxigenases, sharing the EPR spectrum and the histidin coordination with the Rieske proteins, but being clearly distinguishable by their lower (−150 to +5 mV), pH independent midpoint potentials [11, 12]. Recently the x-ray structure of a soluble fragment of the protein from the beef heart $bc_1$ complex has been solved [13] providing detailed structural informations and leading to predictions testable by site directed mutagenesis. However, this approach is mostly limited to microorganisms and non lethal mutations, or organisms like yeast, *Rhodobacter,* or *Chlamydomonas* in which the $bc_1/b_6f$ complexes are dispensable under certain growth conditions [14, 15]. Even in these organisms it can be difficult or impossible to produce sufficient amounts of protein in the case of mutations that interfere with the stability or the assembly of the complexes since the non assembled subunits including the Rieske protein are often subjected to rapid proteolysis [14, 15]. Consequently attempts were made to overexpress Rieske proteins in heterologus systems like *E. coli.* While this was possible for Rieske-type proteins [16 and references cited therein], in the case of the Rieske proteins so far this resulted in the expression of the apo-protein in some cases even containing distorted iron sulfur clusters [14, 16] but not in the production of the native protein with the correctly inserted cluster. The major obstacle appears to be the correct formation of a disulfide bridge between the two cluster binding sites which is critical for the correct positioning of the amino acid residues coordinating the iron ions [13]. Even though the reconstitution of the protein and partial insertion of the cluster *in*

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*vitro* has been reported in one case [16], a system that allowed the expression of the native protein with the correctly inserted cluster remains highly desirable.

We focused our attention on the Rieske proteins of the thermoacidophilic crenarcheon *Sulfolobus acidocaldarius* (DSM 639) because i) they are the only known archaeal members of this group of proteins and ii) they belong to newly detected structures clearly different from classical $bc_1/b_6f$ complexes but never the less share the unique features of the other members of the group [1, 2]. Of these, the Rieske II protein (encoded by the *soxF* gene [4]) is a constituent of a terminal oxidase complex together with a cytochrome *b* homologue suggesting a structure with functional similarity to a $bc_1$ complex [3, 4]. Since this protein could not be isolated so far, its heterologous overexpression reported here provides an ideal opportunity for detailed studies.

In addition to the description of a versatile expression and mutagenesis system for Rieske proteins our aim was 1) to test predictions about the existence of a potential membrane anchor which is under debate in the literature [12, 14, 17, 18], and 2) to test the function of a targeting sequence which has been postulated from the primary structure [2, 14].

## MATERIALS AND METHODS

*Construction of the soxF expression strains.* The *soxF* gene was amplified out of a genomic clone [2] using a standard protocol and a proof reading polymerase. The primer FeSNT (TAA TAT TAA TAA TCA TAT GGA CAG AAG GAC A) and FeSBam (CTC CTT TCT GTG GAT CCA TAT TAA TCA CCC) introduced a NdeI site at the ATG codon and a BamHI site 7 base pairs down stream of the stop codon. The amplified DNA was digested with NdeI/BamHI, purified on an agarose gel and ligated into the equally prepared pet3a vector (Novagen, Madison, WI, USA). The ligated plasmid was transformed into *E. coli* XL-2 Blue cells (Stratagene, Heidelberg, FRG) as described in [20]. Plasmids were isolated from selected colonies [21] and assayed for the presence of the insert by a NdeI/BamHI double digest. The plasmids were subsequently transformed into *E. coli* BL21DE3 cells (Novagen, Madison, WI, USA). Colonies were selected and assayed for the expression of the soxF protein as described below.

*N-terminal deletion mutants were constructed as follows.* Plasmid DNA was isolated from a clone expressing the full length protein and used as template in a PCR reaction utilizing the Expand Long Template PCR System (Boehringer, Mannheim, FRG) with the primer ANTI3a (ATG TAT ATC TCC TTA AAG TTA AAC) in combination with either FNTI23M (ATG AAA CCT GCA TTA GAT TAT GTA) for the $\Delta$ 2 - 23 deletion, or FNTV46M (ATG GCG GAT AAC ACG GAT G) for the $\Delta$ 2 - 46 deletion. The PCR products were treated with Klenow and poly nucleotide kinase [21], purified on an agarose gel, ligated and transformed into XL-2 Blue cells. For each deletion 10 clones were selected, the plasmid DNA isolated and checked by a NdeI/BamHI double digest. 40 - 70% of the tested clones contained an intact NdeI site indicating the incorporation of the full length of both primers into the original PCR product. These plasmids were transformed into BL21DE3 cells.

*Assay for the expression of the soxF proteins.* Cultures were grown over night in 10 ml of LB medium [20] containing 100 $\mu$g/ml ampicillin at 37 °C and rapid shaking (200-250 rpm). 10 ml of fresh medium additionally containing 0.8 mM isopropyl $\beta$-D-thiogalacto-pyranoside (IPTG) were added, the cells incubated for 3 h at 37 °C, harvested by centrifugation and washed twice with 50 mM Tris/HCl pH 7.5, 1 mM EDTA (TE 50/1). Cells equivalent to 100 $\mu$l culture were lysed by addition of sample buffer, incubated 5 min. at 95 °C and loaded onto a SDS poly acrylamide gel.

To test the sub cellular localization of the recombinant proteins the washed cells were broken by sonification. The homogenate was spun for 15 min. at 12,000 g yielding a low speed pellet containing inclusion bodies and large membrane fragments. The supernatant was centrifuged for 1 h at 160,000 g yielding the high speed pellet (membranes) and the soluble fraction containing the cytoplasmatic and periplasmatic proteins. The relative distribution of the membranes between high and low speed pellet showed a considerable variation despite all attempts to standardize the procedure.

*Overproduction of the recombinant soxF proteins.* For the large scale production of the soxF proteins the *E. coli* cells were grown over night as above in 11 flasks initially containing 125 ml of M9 medium [21] supplemented with 0.25 % Yeast extract (GibcoBRL, Eggenstein, FRG), 2 mM L-cystein and ampicillin 100 $\mu$g/ml. 125 ml of fresh medium were added the following morning and the cells incubated for 1 h. The temperature was increased to 42 °C for 30 min. Subsequently the temperature was lowered to 40 °C and 250 ml of YT medium [21] supplemented with 2 mM L-cystein, 1 mM $FeSO_4$, 100 $\mu$g/ml ampicillin and 0.2 mM IPTG were added. After 3 h the cells were harvested by centrifugation, washed, broken and subjected to differential centrifugation as above.

*Enrichment of the full length recombinant soxF protein.* Membranes (high speed pellet) equivalent to 100 mg protein were resuspended at a final concentration of 10-12 mg/ml in solubilization buffer: 25 mM Tris/HCl pH 7.5, 20 mM $\beta$-Dodecylmaltoside (DM) and 10% saturated $(NH_4)_2SO_4$ (AS), slowly stirred for 90 min. at 4 °C and spun for 60 min. at 160,000 g. A saturated AS solution was slowly added to the supernatant to achieve 60% saturation. The solution was loaded onto a hexyl agarose (Sigma, Deisenhofen, FRG) column (6 cm length, 1 cm diameter) equilibrated with 25 mM Tris/HCl pH 7.5, 1 mM DM, 60% saturated AS. The column was washed with 30 ml of equilibration buffer followed by a descending step gradient from 50% to 0% saturated AS in 25 mM Tris/HCl pH 7.5, 0.2 mM DM in 10% steps, 30 ml each step. The partially purified soxF protein was eluted with 40 ml of 25 mM Tris/HCl pH 7.5, 0.3 mM DM and 6 mM N-dodecyl-N,N-dimethyl-ammonio-3-propanesulfonate (SB12).

*Purification of the $\Delta$ 2 - 46 truncated soxF protein.* 5 ml of supernatant containing about 50 mg protein were loaded onto at Sephacryl S300 HR (Pharmacia, Freiburg, FRG) column (1.5 cm diameter, 80 cm length) equilibrated and eluted with 25 mM Tris/HCl pH 7.5, 0.2 mM EDTA and 50 mM NaCl at a flow rate of 0.25 ml/min.. Fractions containing the Rieske protein as assayed by spectrophotometry and SDS-PAGE were pooled, concentrated by ultra filtration on a 10 kD cut-off membrane and the buffer exchanged against 10 mM Tris/HCl pH 7.5, 0.2 mM EDTA. The protein was loaded onto a monoQ HR 5/5 FPLC column (Pharmacia, Freiburg, FRG) equilibrated with the same buffer. The column was washed with 10 ml equilibration buffer and eluted with a gradient from 0 to 300 mM NaCl in buffer. The fractions containing the pure protein as judged by the ratio of the absorbance at 336 nm : 280 nm were pooled, concentrated by ultra filtration and stored at $-20$ °C.

*Miscellaneous techniques.* All other techniques (SDS poly acryl amide gel electrophoresis (SDS-PAGE), determination of iron contend and protein concentrations, EPR spectroscopy and spin quantifications) were performed as described in [1]. CD spectra were recorded and CD redox titrations performed using an OTTLE cell as described in [12].

## RESULTS AND DISCUSSION

The full length as well as two truncated forms of the Rieske protein II (soxF) from the thermo-acidophilic

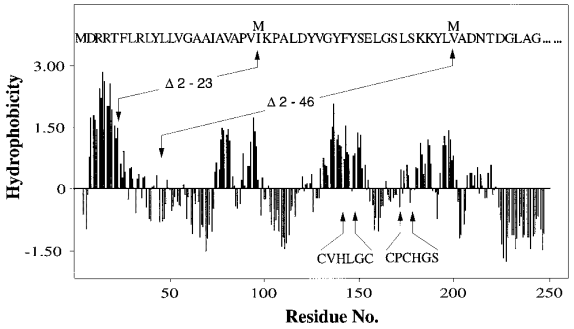


**FIG. 1.** Hydropathy plot and N-terminal amino acid sequence of the soxF protein. The arrows indicate the new N-termini of the truncated proteins as well as the positions of the two conserved iron sulfur cluster binding motifs. The plot was calculated using the MacMolly Tetra program with a 9 residue window.

crenarchaeon *Sulfolobus acidocaldarius* (DSM 639) were expressed in *E. coli* cells. Figure 1 gives an overview of the hydropathy profile of the protein and indicates the N-termini of the truncated forms. The Δ 2 - 23 deletion removes the potential signal sequence for membrane targeting [2] containing most of the N-terminal hydrophobic domain. The Δ 2 - 46 deletion completely removes the hydrophobic domain including the adjacent more amphiphilic part creating a completely hydrophilic N-terminus. All three forms were effectively expressed (fig. 2). The identity of the protein bands on the gel as well as the deletions were confirmed by N-terminal sequencing. Surprisingly the N-terminal methionine was found to be quantitatively removed in

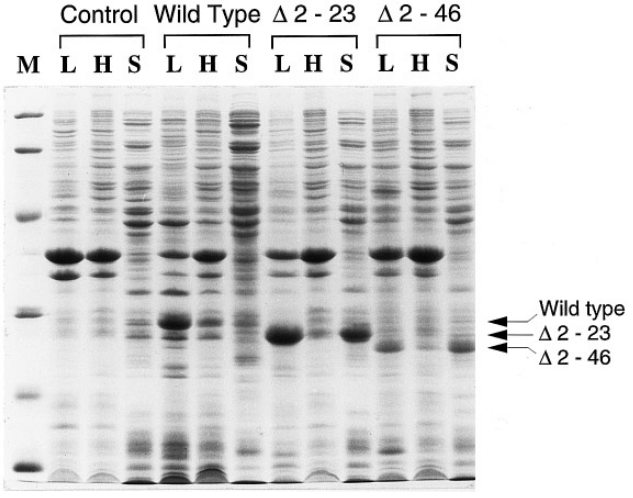


**FIG. 2.** SDS-PAGE analysis of fractions from *E. coli* cells producing the soxF proteins. L: low speed pellet; H: high speed pellet; S: supernatant. Control: Cells containing the pet3a vector without the soxF gene. Wild type: Cells containing the gene encoding the full length of the protein. Δ 2 - 23, Δ 2 - 46: Cells producing the truncated proteins. 40 $\mu$g of protein was loaded on each lane. M: Molecular weight markers, from top to bottom: 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa.

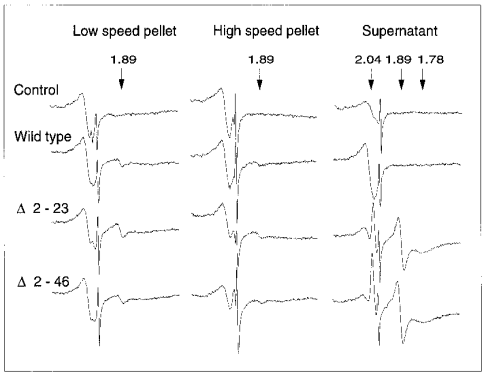


**FIG. 3.** EPR spectra of the *E. coli* cell fractions shown in fig. 2. The samples were reduced by addition of 5 mM ascorbate and the spectra recorded at a temperature of 26.5 K, modulation amplitude 0.5 mT, modulation frequency 100 kHz, microwave frequency 9.6446 GHz and a microwave power of 15.9 mW. Each sample contained 1.9 mg protein for the low and high speed pellet fractions and 1.4 mg protein in the case of the supernatant fractions.

the case of the Δ 2 - 46 mutant. The majority of the wild type and the Δ 2 - 23 mutant protein was found in the low speed pellet mostly in the form of inclusion bodies as inferred from a distinctive insolubility in detergent solutions (1% Triton X 100, 1 hour at 37 °C, data not shown). Contrary to the two deletion mutants no significant amounts of the full length protein were detectable in the soluble fraction. Figure 3 shows EPR spectra of the fractions analysed in fig. 2. The membrane containing fractions of cells expressing the three forms of the protein displayed an additional EPR signal at g= 1.89, typical for Rieske iron sulfur clusters. Much more prominent signals with $g_{xyz}$ = 1.78, 1.89 and 2.04 strictly indicative of a Rieske cluster [11] were observed in the soluble fractions of cells expressing the truncated forms. In the case of the wild type protein about 50% of the g= 1.89 EPR signal could be extracted from the membrane fraction using detergents with DM being most efficient. Chaotropic reagents such as pyrophosphate or compounds like ethylene glycol were entirely unsuccessful (data not shown). Thus, the compound producing this EPR signal had to be tightly bound to the membranes. Following the solubilization by DM we were able to enrich a membrane protein fraction that contained the soxF protein and displayed the typical EPR spectrum of a Rieske cluster (fig. 4). For the first time this proves that *E. coli* cells are able to express, correctly fold and assemble the iron sulfur cluster into a membrane bound Rieske protein as it was hypothesized in [14, 16] and suggested by the findings that soluble proteins containing Rieske-type clusters could be functionally expressed in *E. coli* [16 and references cited therein]. The strict membrane location and the impossibility to solubilize the wild type soxF protein other than by means of detergents suggest that at least in this case the N-terminus of the protein acts as a

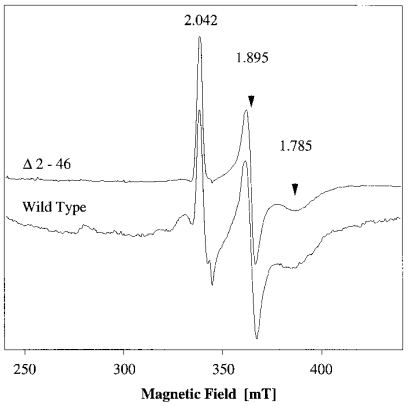


**FIG. 4.** EPR spectra of the reduced, partially purified wild type soxF protein and the purified Δ 2 - 46 mutant. The spectra were recorded in 10 mM Tris/HCl pH 7.5, 0.2 mM EDTA, protein concentration 3.8 mg/ml, for the Δ 2 - 46 form and in the elution buffer from the hexyl agarose column, protein concentration 0.5 mg/ml, in the case of the wild type protein. EPR conditions were as indicated for fig. 3.

membrane anchor interacting directly with the lipid bi-layer. Specific protein-protein interactions resulting in a membrane location of the protein are unlikely since the proteins required for these interactions (e.g. the subunits of the soxM complex) are absent in *E. coli*. The same argument also supports the function of the N-terminus as a signal sequence for membrane targeting of the protein, at least in the *E. coli* system. The amounts of the truncated proteins which are also detectable in the membrane fractions can be explained as the result of non specific interactions between the membrane and other hydrophobic parts on the protein surface promoted by the abnormally high intracellular concentrations of the recombinant proteins. However, additional investigations will be needed to clarify this point.

In contrast to the results reported about the Rieske protein from *Rhodobacter sphaeroides* [14] the iron sulfur cluster was even much more efficiently inserted into the truncated forms of the protein. This may be related to the increased solubility of these proteins as suggested in [16]. Further support of this hypothesis can be derived from the observation that the assembly of the cluster was most effective with the Δ 2 - 46 mutant as indicated by the ratio of the EPR signal to the protein present in the soluble fraction (figs. 2, 3). Concurrently, the ratio of soluble protein versus inclusion bodies (fig. 2) suggests this to be the most soluble form of the protein. However, the amount of soluble protein and the inserted iron sulfur cluster was dependent on the growth conditions. Omitting the heat shock prior to the induction resulted in a significant increase of the insoluble fraction and an about 70% decrease in the incorporation of the cluster (data not shown). Thus, the *E. coli* heat shock proteins induced by this treatment apparently can facilitate the folding of the protein and/or the correct insertion of the cluster, even though the co-expression of the GroEL and GroES chaperonins did not show this effect in the case of the *Nostoc* Rieske protein [16]. The Δ 2 - 46 mutant protein was purified. It is monomeric as judged by the elution volume from the gel filtration column, soluble in absence of detergents, and stable at 4 °C. Iron determination and EPR spin quantification resulted in a ratio of 2.3 Fe / spin. Since we observed no significant EPR signal at g= 4.3 which would be indicative of free iron, or additional EPR signals attributable to a distorted iron sulfur cluster we conclude that all iron in this preparation is incorporated into the Rieske cluster. On a protein basis the ratio of 1.5 mol Fe / mol protein suggests that the preparation contains some apo-protein without the cluster. However, the deviation from the theoretical expected value of 2.0 may also result from inaccuracies of the protein determination due to specific properties of the investigated protein. Furthermore, the presence of the apo-protein does not interfere with most biophysical and spectroscopic studies of the protein. The EPR spectrum of the truncated protein is almost identical to that of the full length recombinant protein (fig. 4). The slight low field shift and broadening of the $g_x$ signal in the latter case may be caused by the presence of the detergent or residual *E. coli* lipids. The observed g values of the Δ 2 - 46 mutant are very similar to those ($g_{xyz}$ = 1.781, 1.889, 2.036) previously observed in preparations of the *Sulfolobus* soxM oxidase complex [3] indicating that the deletion of the N-terminus apparently did not significantly change the structure of the iron sulfur cluster. The uv/vis (oxidized, reduced, and redox) and CD spectra of the purified protein (fig. 5) are very similar to the Rieske proteins investigated so far [1, 5, 12, 22]. Using the redox dependence of the CD signal at 500 nm we have determined the midpoint potential as +375 mV (pH 7.5, 25 °C) versus the standard hydro-

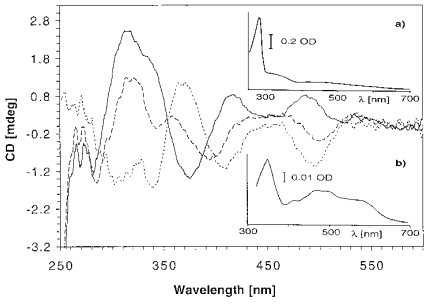


**FIG. 5.** CD spectra of the oxidized (solid line) and reduced (dashed line) Δ 2 - 46 mutant protein. The dotted line shows the difference of the reduced minus the oxidized spectrum. The spectra were recorded at 25 °C in 25 mM Tris/HCl pH 7.5, 0.2 mM EDTA. The protein concentration was 80 mg/ml. Mediators were added as in [12]. The inserts show the uv/vis spectra of the oxidized protein (**a**) at a protein concentration 1.25 mg/ml and the redox difference spectrum (oxidized minus reduced) (**b**).

gen electrode. This is higher than the value of +325 mV (pH 7.4) previously measured by EPR redox titration on *Sulfolobus* membranes [23]. It has to be considered however, that this difference can result from the different methods applied for both measurements as discussed in [24]. In addition the latter value most likely represents the average of the two Rieske clusters present in the membranes in an as yet unknown ratio. Of these, the purified Rieske I protein (*soxL*) showed a more negative potential of +270 mV [2] determined by EPR redox titration. The redox potential and the spectroscopic data strongly demonstrate that the Δ 2 - 46 mutant of the soxF Rieske protein contains a correctly assembled iron sulfur cluster within a tertiary structure of the protein closely resembling or identical to the native protein. This raises the question in which ways the soxF protein differs from the Rieske proteins of *Rhodobacter sphaeroides* [14] or *Nostoc* [16] which could not be expressed in *E. coli* with correctly inserted iron sulfur clusters. Due to the low over all sequence similarities between these proteins [2] it is impossible to attribute this to specific amino acid residues or motifs. The observation that the second Rieske protein of *Sulfolobus* (Rieske I, *soxL* gene [2]), which also shares only little sequence similarity with the soxF protein can be expressed in *E. coli* with the authentic iron sulfur cluster in place as well, (C. L. Schmidt, in preparation) further argues against a specific contribution of individual amino acid residues or motifs. Instead it appears conceivable that the higher intrinsic stability of the tertiary structures of proteins from a thermophilic organism is one of the factors that facilitate the folding of the apo-protein into a structure which can incorporate the iron sulfur cluster.

The overexpression of the soxF protein in *E. coli* provides the opportunity for further investigations into the structure and function of respiratory Rieske proteins. In this respect the *Sulfolobus* proteins deserve special attention since they are part of structures that share significant similarities but are also considerably different from the known $bc_1$ or $b_6f$ complexes [2, 3]. With reference to the x-ray structure of the protein from bovine mitochondria [13] and the emerging structures of the beef heart $bc_1$ complex [25] as well as the spinach chloroplast Rieske protein [26] it will be of special interest to compare these structures to the structure of the *Sulfolobus* protein. Since the system described here allows the straight forward production of the soxF protein independently of other subunits and stability problems, it will provide an excellent tool to test conclusions and predictions derived from the x-ray structures.

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