

Purification and characterization of the Rieske iron-sulfur protein from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*

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Abstract The previously detected Rieske iron-sulfur protein from the membranes of the thermoacidophile *Sulfolobus acidocaldarius* [Anemüller, S., et al. (1993) FEBS Lett. 318, 61–64] was purified to electrophoretic homogeneity and the N-terminal amino acids determined. The apparent molecular weight was estimated to be 32 kDa. The reduced protein displays a rhombic EPR spectrum with $g_{xyz} = 1.768, 1.895, 2.035$. The average g -value of 1.902 is typical for nitrogen ligand-containing clusters. EPR spin quantification and the iron content indicate the presence of one [2Fe-2S] cluster. The purified protein displays ubiquinol cytochrome *c* reductase activity. The pH optimum of this reaction is temperature dependent and was determined to be pH 7 at 56°C. The results presented in this study clearly prove that the *Sulfolobus* Rieske protein belongs to the family of the true Rieske iron-sulfur proteins.

Key words: Rieske; Archaea; Iron-sulfur protein; Ubiquinol cytochrome *c* reductase; EPR

1. Introduction

On the evolutionary tree the archaea of the order *Sulfolobales* form the deepest branch of aerobic organisms [2], and as such are expected to exhibit the most archaic respiratory systems. Since at the postulated origin of autotrophic life in a 'pyrite world' iron-sulfur compounds presumably served as primordial catalysts of biological energy conversion [3,4], the iron-sulfur proteins in contemporary electron transport chains may be viewed as descendants of such ancient catalysts. Here we report the isolation and characterization of a typical Rieske [2Fe-2S] protein from *Sulfolobus acidocaldarius*.

The Rieske iron-sulfur proteins are extrinsic, membrane-residing components [5,6] in the energy-conserving electron transport chains of many bacteria and the related mitochondria and chloroplasts [7,8]. A second class of soluble Rieske-type iron-sulfur protein has been described as components of bacterial oxygenases [9]. Both groups show biophysical and structural similarities, like the presence of histidyl ligands and significant higher reduction potentials compared to other proteins containing [2Fe-2S] clusters. The soluble proteins exhibit pH-independent reduction potentials ranging from –150 to +5 mV [10,11], whereas the potentials of the proteins found in respiratory chains are pH dependent and typically vary from +105 to +350 mV [12,13]. The latter have been exclusively found as components of the bc_1/b_6f complexes together with *b*- and *c*-type cytochromes [7,8]. Within these complexes the function of the Rieske proteins is to abstract the first electron from the

quinol and thus to initiate the reaction sequence of the Q-cycle. Up to now bc_1/b_6f complexes from a variety of different organisms have been studied [8,14], but no structures eventually related to evolutionary predecessors have been found.

EPR studies on the membranes from the extreme thermoacidophilic crenarchaeon *S. acidocaldarius* revealed the presence of a signal reminiscent of the Rieske iron-sulfur proteins [1]. In previous studies we were able to demonstrate that the electrochemical properties of this redox center showed striking similarities to that of the well-known mitochondrial and plastidic Rieske proteins. The reduction potential was determined to be +345 mV (pH 7.0) and was pH dependent in the range from pH 6.0 to 9.5 [15]. However, since *Sulfolobus* does not express any *c*-type cytochromes [16] the existence of a typical bc_1 or b_6f complex in this organism can be excluded. Here we report the isolation and characterization of a Rieske iron-sulfur protein from *Sulfolobus acidocaldarius*. To our knowledge this is the first Rieske protein detected and purified from an archaeal source as well as the first true Rieske iron-sulfur protein that is not part of a classical bc_1 or b_6f complex.

2. Materials and methods

The *Sulfolobus acidocaldarius* (DSM 639) cells used in this study were grown at Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany. The cells were grown, membranes prepared and pre-extracted as described in [17,18]. All purification steps were performed at 4°C. The pre-extracted membranes were suspended in 50 mM Tris HCl, pH 7.5, 20 mM dodecyl maltoside (DM) at a protein concentration of 10 mg/ml, stirred for 90 min and the insoluble components were spun down for 1 h at 120,000 × *g*. A saturated ammonium sulfate (AS) solution was slowly added to the supernatant to achieve 50% saturation. This solution was loaded onto a propyl agarose (Sigma, Deisenhofen, Germany) column (diam. = 1.5 cm; length = 15 cm) equilibrated with 50% saturated AS, 1 mM DM in buffer 1 (25 mM Tris HCl, pH 7.5). The column was washed with the same buffer followed by 40% AS, 0.2 mM DM; 40% AS, 0.5 mM DM, 4 mM *N*-dodecyl-*N,N*-dimethyl-ammonio-3-propane-sulfonate; 30% AS, 0.2 mM DM and 20% AS, 0.2 mM DM; all in buffer 1. The last fraction was concentrated by ultra-filtration on a PM10 membrane (Amincon, Beverly MA, USA), desalted on a Sephadex G-25 (Pharmacia, Freiburg, Germany) column, and loaded onto a hydroxyapatite column (Bio-Rad, München, Germany) (diam. = 2.6 cm; length = 9.5 cm) both equilibrated with 0.2 mM DM in buffer 1. The column was washed with the same buffer and eluted with 25 mM potassium phosphate, pH 7.5, 0.2 mM DM. Fractions were assayed for the presence of the Rieske protein by EPR spectroscopy and SDS-PAGE. The fractions containing the Rieske protein were pooled, concentrated by ultra-filtration and loaded onto a TSK G3000SW HPLC column (LKB, Bromma, Sweden) equilibrated and eluted with 0.2 mM DM in buffer 1.

Ubiquinol cytochrome *c* reductase activity was measured by following cytochrome *c* reduction at 550–540 nm using a Hewlett Packard 8452A diode array spectrophotometer. The assay mixture contained 50 mM bis-Tris-propane buffer of the pH indicated in the figure, 0.2 mM DM, 5 μM horse heart cytochrome *c* (Boehringer, Mannheim,

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Germany), 25 μ M *n*-decyl-ubiquinol (Sigma, Deisenhofen, Germany), and the indicated amounts of protein. The final volume was 0.6 ml and the assays were carried out at 20 or 56°C, as indicated.

EPR spectra were recorded with an X-band Bruker ER 200 D-SRC spectrometer equipped with an ESR 910 continuous-flow helium cryostat from Oxford Instruments. Quantification of EPR signal was carried out by double integration of the experimental spectra, obtained under non-saturating conditions, using a copper standard [19]. The low spin signal of heme *a* ($g_{xy} = 1.45, 2.23, 3.02$) [20] was used to determine the amount of cytochrome *a*₅₈₀/*a*_{a3} oxidase in whole membranes.

Iron content was determined by atomic absorption spectroscopy at 248.3 nm using a Hitachi 180/80 polarised Zeeman atomic absorption spectrophotometer.

The N-terminal amino acid sequence of the Rieske protein was determined by automated microscale Edman degradation of protein blotted onto a polyvinylidene fluoride membrane. SDS-PAGE and silver staining of the gels was performed as described in [18]. Protein concentrations were determined using the Bio-Rad D_C Protein assay (Bio-Rad, München, Germany). Heme content was determined using the pyridine-hemochrome method as described in [20].

Cytochrome *f* from turnip was purchased from Sigma, Deisenhofen, Germany. Cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa* was kindly provided by Prof. M. Brunori (Rome).

3. Results and discussion

The Rieske iron-sulfur protein from *S. acidocaldarius* was purified to electrophoretic homogeneity. The apparent molecular weight of 32 ± 1 kDa, as determined by SDS-PAGE (Fig. 1), is significantly higher than the values of 20–25 kDa determined for the corresponding mitochondrial and plastidic proteins [7,22]. The oxidised protein showed the typical UV/vis spectrum of a Rieske protein (Fig. 2a). The absorption in the visible region was diminished upon reduction by ascorbate (insert, Fig. 2a). The EPR spectrum of the protein was typical for a rhombic [2Fe-2S] cluster with $g_x = 1.768$, $g_y = 1.895$ and $g_z = 2.035$ (Fig. 2b, lower trace). The average *g*-value

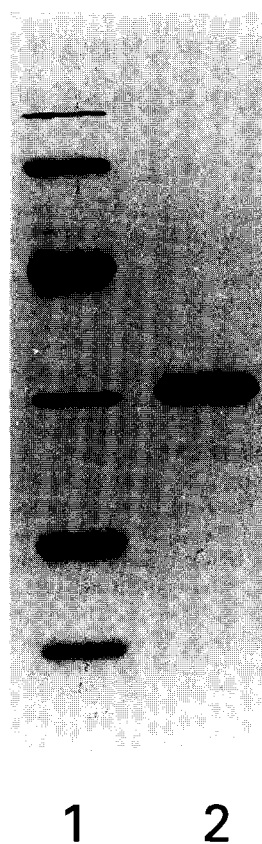


Fig. 1. Silver stained SDS-polyacrylamide gel of the purified Rieske iron-sulfur protein. Lane 1, molecular weight marker: 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa. Lane 2, 1 μ g of the purified protein.

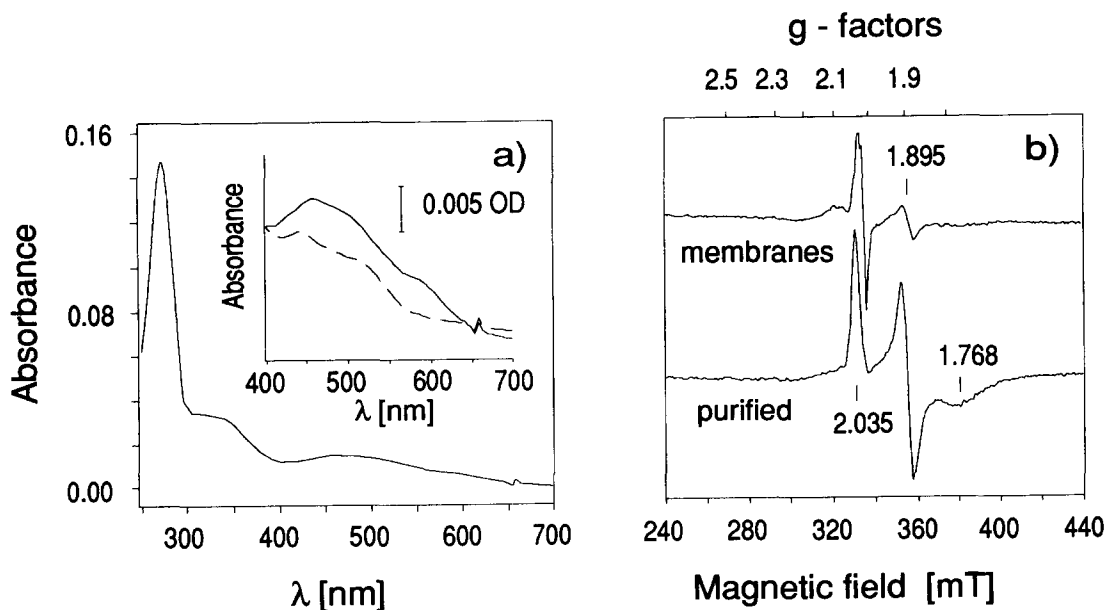


Fig. 2. (a) UV/vis spectrum of the oxidized Rieske protein. The insert shows the expanded visible region of the spectra of the oxidized (solid trace) and the ascorbate reduced (5 mM) protein (dashed trace). The spectra were recorded in 25 mM Tris HCl, pH 7.5, 0.2 mM DM. The protein concentration was 0.13 mg/ml. (b) EPR spectra of *Sulfolobus* membranes (upper trace, 4.7 mg protein) and the partially purified Rieske protein (lower trace, 0.45 mg protein). The spectra were recorded in 25 mM Tris HCl, pH 7.5, supplemented with 0.2 mM DM in the case of the isolated protein. Both samples were reduced by addition of 5 mM ascorbate. The EPR conditions were: temperature, 15 K; microwave power, 2 mW; microwave frequency, 9.4327 GHz; modulation amplitude, 1 mT; and modulation frequency, 100 kHz.

($g_{av} = \sqrt{(g_x^2 + g_y^2 + g_z^2)}/3$) of 1.902 is characteristic for nitrogen ligand-containing [2Fe-2S] clusters and significantly lower than the values found for [2Fe-2S] ferredoxins [8,23]. The g_x - and g_y -values of the purified protein are almost identical to the values determined in membranes ($g_{xyz} = 1.725, 1.890, 2.031$ [1]), whereas the g_x -value shows a significant shift which may reflect the removal of lipids or quinone during the purification. Spin quantification resulted in 0.8 ± 0.3 spins ($S = 1/2$) per protein. The iron content was determined to 1.2 ± 0.2 mol per mol protein. These values are slightly below the values expected for a protein containing one [2Fe-2S] cluster. The deviation from the theoretical values may be explained either by a partial loss of the iron-sulfur cluster during the purification, or by an underestimation of the molecular weight due to anomalous migration on the SDS-PAGE. The N-terminal amino acid sequence of the protein is: VSVLV PXTVT VA..., with the unidentified amino acid at position 7 most likely a proline.

Fig. 3a shows the redox behaviour of the Rieske protein in isolated *Sulfolobus* membranes. It could be reduced by addition of NADH if KCN is present to inhibit the terminal oxidase. The

simultaneous addition of cytochrome *c* diminished the reduction of the Rieske center, suggesting an electron transfer between the Rieske protein and cytochrome *c*. Since analogous results have been observed when NADH was replaced by succinate (data not shown) it is likely that the protein is reduced via the caldariella quinone pool of the *Sulfolobus* membranes [16].

Fig. 3b demonstrates the electron transfer between the Rieske protein and various *c*-type cytochromes. The partially purified Rieske protein eluted from the propyl agarose column could be reduced by addition of ascorbate, and remained reduced after the removal of excess ascorbate by gel-filtration. Basic as well as acidic *c*-type cytochromes were effective in oxidizing the pre-reduced protein.

The ability of the Rieske protein to interact with quinones as well as with cytochromes is further demonstrated by the ubiquinol cytochrome *c* reductase activity of the purified protein (Fig. 4), as was shown for the Rieske protein isolated from beef heart mitochondria [24]. The pH dependence of the catalysed reaction is clearly different from that of the chemical reac-

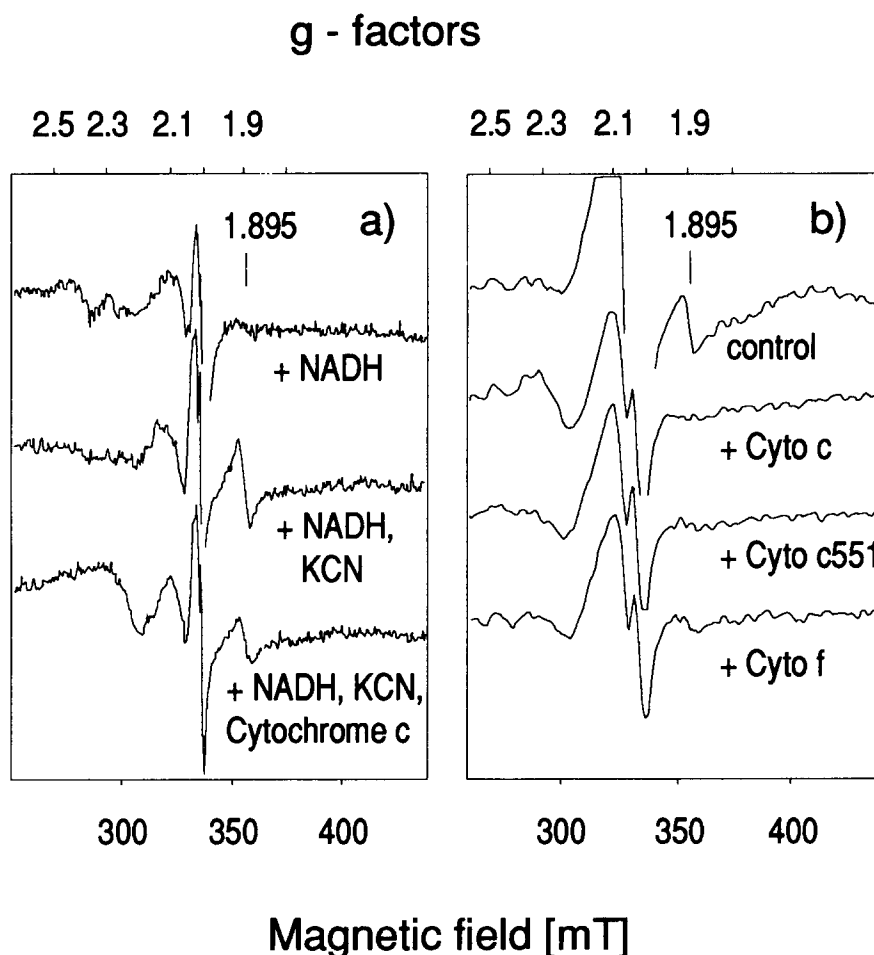


Fig. 3. (a) Redox reactions of the *Sulfolobus* Rieske iron-sulfur protein in membranes. *Sulfolobus* membranes were resuspended at a protein concentration of $60 \text{ mg} \cdot \text{ml}^{-1}$ in a buffer containing 25 mM Tris-HCl, pH 7.5. As indicated in the figure the following compounds were added (the final concentrations are given in parentheses): NADH (2 mM), KCN (5 mM) and horse heart cytochrome *c* ($2 \text{ mg} \cdot \text{ml}^{-1}$). The samples were incubated for 10 min at 37°C , $100 \mu\text{l}$ aliquots transferred into EPR tubes and frozen in liquid nitrogen. (b) Oxidation of the partially purified Rieske protein by *c*-type cytochromes. The Rieske protein-containing fraction eluted from the propyl-agarose column was reduced by addition of 2.9 mM ascorbate and excess reductant removed by gel-filtration. Each EPR sample contained 0.29 mg of protein. As indicated, horse heart cytochrome *c*, or cytochrome *c*₅₅₁ from *Pseudomonas aeruginosa* (each $0.2 \text{ mg} \cdot \text{ml}^{-1}$ final concentration), or cytochrome *f* from turnip ($0.4 \text{ mg} \cdot \text{ml}^{-1}$ final concentration) were added, the samples incubated for 10 min at 37°C , transferred into EPR tubes and frozen. EPR conditions were as indicated for Fig. 2.

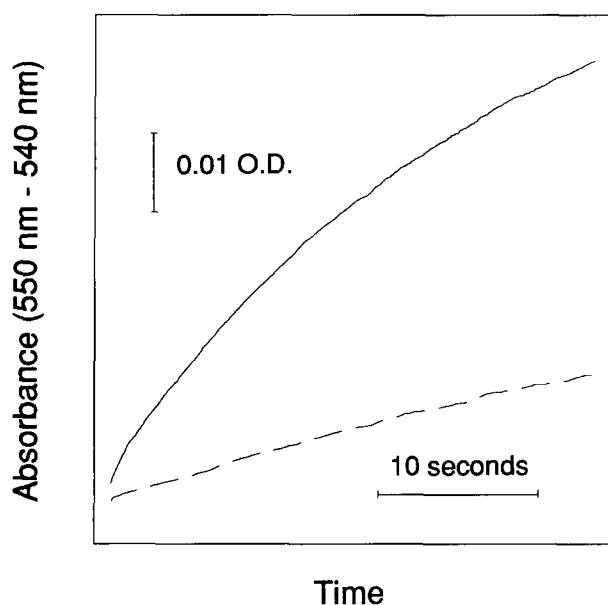


Fig. 4. Ubiquinol-cytochrome *c* reductase activity of the purified Rieske iron-sulfur protein. The figure shows the reduction of cytochrome *c* by *n*-decyl-ubiquinol catalysed by 8.4 µg Rieske protein (solid trace) compared to the chemical reaction in the absence of the protein (dashed trace). The difference between both traces represents a catalytic activity of 746 nmol cytochrome *c*_{reduced} · min⁻¹ · mg protein⁻¹. The assay was performed at 20°C and pH 8.5. All other conditions were as described in section 2.

tion of *n*-decyl-ubiquinol with cytochrome *c* (Fig. 5). Even though this activity is low compared to that of the *bc*₁ or *b*₆*f* complexes [25], it has to be considered that none of the substrates are physiological. However, since caldariella quinone is rather unstable under the assay conditions, the applied model system provides valuable insights into the catalytic function of this first archaeal Rieske protein. The pH optimum of the catalysed reaction was shifted from pH 9 at 20°C to pH 7 at 56°C (Fig. 5), approaching the lower limit of the growth temperature of *Sulfolobus*. Since at physiological temperatures the pH optimum is close to the internal pH of 6.5 determined for *Sulfolobus* cells [26], the *in vivo* function of the Rieske protein is likely to be that of a quinol cytochrome reductase, as is the function of the Rieske proteins in *bc* complexes.

Due to its catalytic properties, the membrane location as well as the high, pH-dependent redox potential, which was demonstrated to be influenced by two protonation equilibria [15], the *Sulfolobus* Rieske protein obviously belongs to the family of the true Rieske proteins found in energy-conserving electron transport systems and not to the Rieske-type proteins of the bacterial oxygenases. This is further substantiated by the detection of an open reading frame in the *Sulfolobus* genome encoding a protein that shows the characteristic hydropathy profile and contains the two typical iron-sulfur cluster binding sites of a true Rieske protein (Schmidt et al., in preparation).

The physiological electron acceptor of the *Sulfolobus* Rieske protein is not yet known. In membranes significant reduction of the Rieske center can only be observed if the activity of the terminal oxidase is inhibited by cyanide (Fig. 3a). Thus in absence of cyanide the electrons of the caldariella quinone pool are either bypassing the Rieske center, or the Rieske protein itself can transfer electrons to the oxidase. However, a direct

electron transfer between the Rieske and the oxidase is very unlikely since the cytochrome *a*₅₈₆/*aa*₃ oxidase of *Sulfolobus* is, despite its superficial similarity to the *caa*₃ oxidases [27,28], a quinol oxidase which was isolated free of any Rieske protein in a highly active form ([17,29], and unpublished data).

There is no indication for the existence of any soluble compounds of the respiratory chain like cytochromes, or copper proteins which could function as intermediate electron acceptors for the Rieske protein in *Sulfolobus*. Therefore the only possible reaction partners for the Rieske protein appear to be other membrane-bound cytochromes. Of these, cytochrome *b*₅₅₈ [18,30] can be excluded since its expression is repressed by high oxygen tensions [30], whereas EPR measurements on membranes demonstrated that the Rieske protein is present independent of the oxygen supply at an almost constant ratio of 1.7–2.0 relative to the cytochrome *a*₅₈₆/*aa*₃ oxidase and thus seems to be an indispensable component of the *Sulfolobus* respiratory chain. A possible reaction partner for the Rieske protein could be cytochrome *a*₅₈₆. This cytochrome spectroscopically seems to be a single and uniform component with a peak at 586 nm in the α -band region of the redox spectrum of *Sulfolobus* membranes. However, careful analysis demonstrates the presence of two pools of this chromophore: a high potential form which can be reduced by ascorbate/*N,N,N',N'*-tetramethyl *p*-phenylenediamine under anaerobic conditions or in presence of cyanide; and a low potential form which can be reduced only by addition of dithionite [31]. The redox titrations of the low potential form resulted in a redox potential of +60 mV for a single component, or alternatively could be fitted for two redox centers with potentials of 20 ± 10 mV and 100 ± 10 mV with contributions of 40% and 60% [30] resembling the *b* cytochromes known from the *bc*₁/*b*₆*f* complexes. Although it may be hypothesised that the *Sulfolobus* Rieske protein forms a cytochrome *a*₅₈₆ complex, functionally related to known *bc*₁*7b*₆*f* complexes, the insensitivity of membrane preparations towards

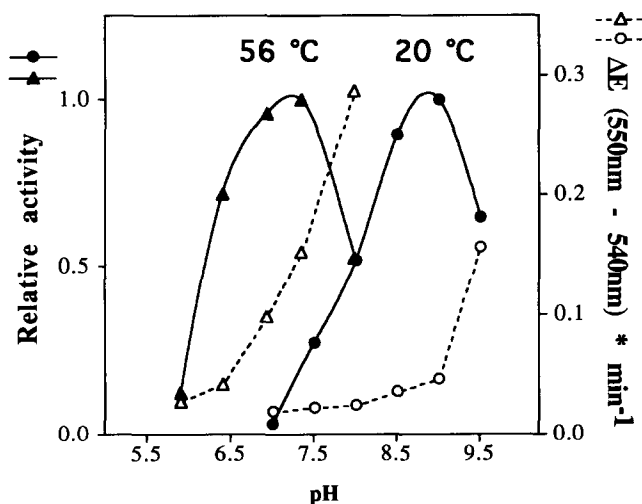


Fig. 5. pH dependence of the *n*-decyl ubiquinol cytochrome *c* reductase activity of the partially purified *Sulfolobus* Rieske protein (●,▲) and of the chemical reaction between *n*-decyl ubiquinol and cytochrome *c* (○,△) at 20°C (●,○) and at 56°C (▲,△). The measurements at both temperatures were performed using independent preparations of the Rieske protein purified on a propyl agarose column. Each point represents the average of two measurements. An activity of 1.0 equals the reduction of 17.28 nmol cytochrome *c* · min⁻¹ · mg protein⁻¹ at 20°C, and 9.00 nmol cytochrome *c* · min⁻¹ · mg protein⁻¹ at 56°C.

stigmatellin and myxothiazole (data not shown) suggests obvious structural differences.

The high potential form of cytochrome a_{586} was identified as the product of the SoxC gene [32]. It is a subunit of the a_{586}/aa_3 terminal oxidase and titrates as a single component with a potential of $+260 \pm 10$ mV (Gleißner et al., unpublished data). It remains to be elucidated whether the two pools of cytochrome a_{586} represent two forms of one protein, or are two entirely different proteins.

Recently Lübben et al. [33] suggested that the Rieske protein could be a subunit of a second terminal oxidase (SoxM) of *Sulfolobus*. This new oxidase is supposed to consist of the Rieske protein and at least two other subunits. However, the protein tentatively assigned to be the Rieske protein by Lübben et al. has an apparent molecular weight significantly below 32 kDa. In addition, the N-terminal amino acid sequence determined for this protein (MDRRT) is completely different from that of the *Sulfolobus* Rieske protein purified in this study.

Although it appears possible, it remains open whether or not *Sulfolobus* indeed contains two different genes encoding Rieske proteins. Actually, the occurrence of auxiliary copies of genes differing only slightly from each other has been found for archaea [34]; their individual activation may be subject to growth conditions.

Independent of these considerations, the results presented above conclusively demonstrate that the Rieske protein from *Sulfolobus acidocaldarius* characterised in this study is a true Rieske protein, like the ones known from the cytochrome bc_1/b_6f complexes. Since it is obvious that it is neither part of a typical bc_1/b_6f complex, nor belongs to an enzyme system comparable to bacterial oxygenases, it may be part of a structure that could be considered as an evolutionary predecessor of the contemporary bc_1/b_6f complexes, or may represent a third line in the evolution of these iron-sulfur proteins.

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