FEBS 24435 FEBS Letters 487 (2001) 351–355

Sulfocyanin and subunit II, two copper proteins with novel features, provide new insight into the archaeal SoxM oxidase supercomplex

Lars Komorowski, Günter Schäfer*

Institute of Biochemistry, Medical University of Lübeck, D-23538 Lübeck, Germany

Received 23 October 2000; revised 24 November 2000; accepted 28 November 2000

First published online 8 December 2000

Edited by Matti Saraste

Abstract The isolation of a fully functional SoxM terminal oxidase supercomplex from the archaeon *Sulfolobus acidocaldarius* has failed thus far and several of its constituents have only been predicted genetically, such as the small Cu protein sulfocyanin and the subunit II bearing a Cu_A center. Here we report the recombinant expression of sulfocyanin and prove its transcription in *Sulfolobus* as well as its presence in the enriched complex. It reveals a redox potential of +300 mV and spectroscopic features that are characteristic of type I copper centers. It is highly thermostable and firmly attached to the complex by one putative transmembrane anchor. Surprisingly, subunit II is completely missing from the isolated complex and behaves as an easily dissociable constituent which is a unique case within the terminal oxidase family. Its loss into the soluble phase upon cell disruption can be considered the reason for the inactivity of the isolated membrane complex.

Key words: Archaeon; Blue copper; Terminal oxidase; Hyperthermophile; Electron transport; *Sulfolobus*

1. Introduction

Like many bacteria the hyperthermo-acidophilic archaeon *Sulfolobus acidocaldarius* has a branched respiratory electron transport system comprising at least two terminal oxidases, the SoxABCD and the SoxM oxidase [1]. However, unlike bacterial or mitochondrial oxidases the latter are genetically and structurally organized as supercomplexes of unusual composition. The SoxABCD complex is a highly active caldariella quinone oxidase combining functional components of a classical complex III with a terminal aa_3-type quinol oxidase [2]; though an iron–sulfur protein is missing, a structural analog to the *b*-type cytochrome of a respiratory complex III is a functional constituent, bearing two hemes A_s, however. It has been proposed that its proton pumping activity may involve a Q cycle-like mechanism [3]. The SoxM supercomplex, in contrast, is composed of an analog to a complex III including a Rieske iron–sulfur center [4] and a typical Cu_A containing cytochrome *c* oxidase, despite the fact that *c*-type cytochromes are totally absent from *S. acidocaldarius*. Instead it was suggested that a putative blue copper protein (sulfocyanin) which is encoded by the *soxE* gene functionally replaces cytochrome *c*. However, so far the SoxM complex could not be purified in catalytically active form [1,5], nor have all of its proposed constituents been identified in the preparations. As a likely reason the loss of essential constituents has been supposed, especially of the copper protein SoxE which hitherto has not been detected by absorption or electron paramagnetic resonance (EPR) spectroscopy in any of the preparations.

Here we report new insight into the structure and properties of the SoxM complex from two aspects. Firstly the blue copper protein SoxE has been heterologically expressed and functionally investigated. Secondly and unexpectedly we could demonstrate that it is a constituent of the isolated complex whereas SoxH, the Cu_A containing subunit II, escapes from the membrane fraction during preparation. These findings contribute to further understanding and pave the way to a functional reconstitution of this unusual archaeal respiratory complex.

2. Materials and methods

2.1. Transcriptional analysis

RT-PCR was performed with a primer pair (MWG Biotech) designed on the basis of the *soxE* sequence (Fig. 1). Reverse transcription was carried out with Superscript II RT (Gibco) following the enzyme manual. The subsequent PCR was carried out in 1×Taq polymerase buffer (MBI Fermentas), 1 mM $MgCl_2$, 200 μM dNTP and 1 μM primer using Taq polymerase (MBI Fermentas) and 100 ng of genomic DNA.

A probe for Northern hybridization was likewise generated from *S. acidocaldarius* DNA but with 200 μM digoxigenin labelled dNTP (DIG DNA labelling mix, Boehringer Mannheim) and a sense primer to antisense primer ratio of 0.9 in order to increase antisense strand production.

RNA was prepared by extraction with guanidinium isothiocyanate and acid phenol using the Roti Quick Kit (Roth). The subsequent Northern analysis was performed as described [6]. Eventually, the labelled DNA was detected by chemiluminescence.

2.2. Construction of the expression vector

A synthetic *soxE* gene with an optimized codon usage for *Escherichia coli* and appropriate restriction sites for the ligation with the pET15b vector including an in frame coding region for a His tag (Novagen) was generated from eight overlapping oligonucleotides (MWG Biotech) (sequence deposited at EMBL gene bank, accession number AJ297962). The oligonucleotides had lengths between 78 and 92 bases. In a first PCR these oligonucleotides were combined to the full length product. The PCR was carried out in 1×Taq polymerase buffer (MBI Fermentas), 1.25 mM $MgCl_2$, 200 μM dNTP, 3 nM oligonucleotides and 600 nM primer using Taq polymerase (MBI Fermentas). In a second PCR with 600 nM primers the full length product was further amplified.

PCR product and pET15b were digested with the appropriate re-

*Corresponding author. Fax: (49)-451-500 4069.
E-mail: schaefer@biochem.mu-luebeck.de

PII: S0014-5793(00)02343-7

striction enzymes (MBI Fermentas). The digested DNA was cleaned with a preparative agarose gel containing 10 μg/ml crystal violet (Sigma-Aldrich) [7] and finally solubilized in 1×TE buffer.

The digested PCR fragment and the corresponding vector fragment were then ligated overnight at 14°C with T4 DNA ligase in the corresponding buffer (MBI Fermentas). The construct was transformed into *E. coli* XL2 Blue (Stratagene) for screening purposes. Screening for the correct construct was accomplished by restriction analysis followed by sequence analysis on an ABI Prism 377 DNA Sequencer (PE Biosystems).

2.3. Expression and purification

The construct was transformed into *E. coli* BL21 (DE3) pLysS (Novagen). At this stage cells were plated on ACCS medium agar (1 g/l $(NH_4)_2SO_4$, 4.5 g/l KH_2PO_4, 10.5 g/l K_2HPO_4, 0.5 g/l Na-citrate·$2H_2O$, 20 mM glucose, 2 mM $MgSO_4$, 1 μg/l thiamine–HCl, 100 μg/ml carbenicillin, 50 μg/ml spectinomycin, 34 μg/ml chloramphenicol+1.5% agar) to minimize basal induction due to sugars in normally used yeast extract containing media. Multiple clones were picked, grown in liquid medium, induced with 1 mM IPTG and then screened for the presence of overexpressed protein with varying induction conditions. Screening was accomplished by SDS gel electrophoresis.

Large scale expression of the His-tagged SoxE was carried out in 6 l culture medium: a colony of BL21pET15synEII was used to inoculate 60 ml ACCS medium. The culture was grown overnight at 37°C. At OD_{600} = 1.5 the culture was used to inoculate 6 l LB medium in three 5 l flasks. The flasks were vigorously shaken at 37°C until OD_{600} reached 0.6. At this time 2 ml 1 M IPTG was added to each flask. The induced cells were shaken at 37°C for 3 h, cooled on ice and then harvested by centrifugation for 20 min at 5000×*g*, 4°C. The sedimented cells were washed in 80 ml 50 mM Tris–HCl (pH 8.0 at 25°C), 2 mM EDTA and centrifuged as above. The pellet was resuspended in 75 ml of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole, 250 U Benzonase (Merck) and the suspension was then freeze-thawed and finally sonicated. The suspension was centrifuged for 30 min at 30 000×*g*, 4°C. The supernatant was applied to a water bath at 62°C for 10 min and then cooled on ice. Eventually the denatured protein was removed by centrifugation for 60 min at 100 000×*g*, 4°C.

2.4. Purification and complementation of the His-tagged SoxE

The heat denatured cytosol (80 ml) was applied to a Ni-NTA column with 5 ml bed volume (Qiagen) equilibrated with 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole. The flow rate was 3 ml/min and a linear gradient from 20 to 250 mM imidazole was developed during 10 column volumes. The fractions containing the SoxE fragment were pooled, concentrated in an ultrafiltration unit using 10 kDa cutoff membranes to approximately 3.5 ml and then loaded onto a Superdex 75 HiLoad gel filtration column (120 ml, Amersham Pharmacia Biotech). The column was equilibrated with 50 mM Tris–HCl (pH 8.0 at 25°C), 300 mM NaCl. The flow rate was 1 ml/min. The SoxE fragment eluted at 70–80 ml. To the eluted fractions $CuCl_2$ was added at a concentration of 200 μM and the blue fractions were pooled.

2.5. Spectroscopy

UV/Vis spectra were recorded with a Hewlett Packard 8453A spectrophotometer equipped with a stirrer at room temperature. EPR spectra were recorded with a Bruker ER 200D-SRC X-band spectrometer. Circular dichroism (CD) spectra were recorded with a Jasco 700 CD spectrometer.

The reaction between reduced cytochrome *c* and the copper binding domain for the determination of E'_0 was carried out in degassed 1×PBS buffer under argon. For this purpose horse heart cytochrome *c* (Sigma) dissolved in 1×PBS buffer was slowly reduced with dissolved $NaBH_4$ just prior to use. The reduction was controlled spectrophotometrically and excess $NaBH_4$ was carefully removed by addition of hydrochloric acid. The final cytochrome *c* concentration was determined spectrophotometrically using an absorption coefficient $\varepsilon_{540-550\,nm}$ = 19.5 mM^{-1} cm^{-1}. Oxidized recombinant SoxE was added stepwise to reduced cytochrome *c* in defined amounts and the UV/Vis spectrum was recorded after each step. From these spectra the oxidized to reduced cytochrome *c* ratio was calculated and subsequently the redox potential of SoxE was determined using the Nernst equation.

2.6. Western analysis

An antiserum against the His-tagged recombinant SoxE was produced from rabbit whole blood using standard procedures [8] with incomplete Freund's adjuvant. For Western analysis SDS gel electrophoresis was performed with different protein fractions from *S. acidocaldarius* which were then electroblotted onto a PVDF membrane which was subsequently developed with a 1:500 diluted antiserum an a protein A–alkaline phosphatase conjugate. Bound antibodies were then detected by chemiluminescence with addition of a chemical enhancer (Sapphire II, Tropix) using a DIANA II system (Raytest).

2.7. Miscellaneous

Protein concentrations were measured with the Bio-Rad D_C protein assay.

3. Results

3.1. Transcription analysis

As a first reference point for the presence of SoxE in *S. acidocaldarius* RT-PCR was conducted with total RNA (Fig. 1) which resulted in a cDNA that was identical to the positive control. With the probe against *soxE* a transcript of about 0.6 kb was identified (Fig. 1). This indicates transcription of soxE as a monocistronic message.

3.2. Expression and purification of the blue copper protein

Vector constructs generated by the commonly used method, PCR amplification of the gene and subsequent ligation to an expression vector, failed because no expression could be detected in *E. coli* at all. Even by cotransfection of a second plasmid which encodes the rare *E. coli* tRNAs, $tRNA^{leu}$ (codon AUA) and $tRNA^{arg}$ (codon AGA) and change to a yeast free medium which boosted the expression of another copper protein from *S. acidocaldarius* (SoxH [9]) no expression was achieved.

Therefore a synthetic N-terminally truncated *soxE* coding region with an optimized codon usage for *E. coli* was generated in two steps using a modified recursive PCR (see Section 2) [10]. In the first step eight overlapping oligonucleotides together with terminally located primers were combined to the full length product which was then further amplified in

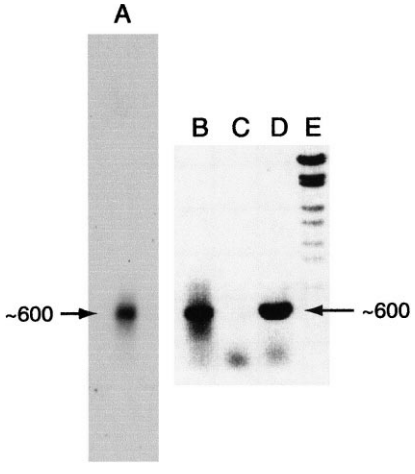

Fig. 1. Transcriptional analysis of *S. acidocaldarius* total RNA. Lane A: Northern analysis of total RNA (8 μg) with a probe against *soxE*; lane B: RT-PCR, positive control with DNA; lane C: RT-PCR, negative control without reverse transcriptase; lane D: RT-PCR, cDNA from *S. acidocaldarius* RNA with primers for *soxE*.

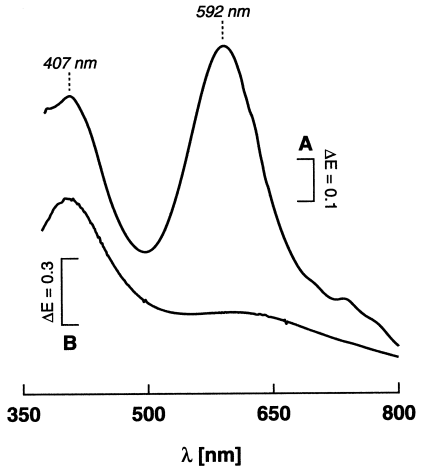

Fig. 2. Optical absorption spectra of the oxidized blue copper protein. A: The purified truncated (Δ2, 33) SoxE of the soluble fraction, 3.8 mg/ml protein in 50 mM Tris–HCl (pH 8.0 at 25°C). B: Enriched and refolded insoluble fraction of truncated (Δ2, 33) SoxE in 50 mM Tris–HCl (pH 8.0 at 25°C).

a second PCR. The PCR product was successfully introduced into a T7 system expression vector adding an N-terminal His tag to the coding region and then transferred into *E. coli* BL21 (DE3) pLysS. This allowed detection by Western analysis with an anti-His tag antibody as well as purification by metal chelate chromatography. The protein was produced in both soluble and insoluble form with a larger extent of the latter but a blue copper signal could not be detected by UV/Vis or by EPR spectroscopy.

The insoluble inclusion body fraction could be partly purified by differential centrifugation. It was then dissolved in 8 M urea under different pH conditions, further purified by metal chelate chromatography, and different refolding procedures were tested. Refolding could be attained with only a small yield and resulted in a green colored form with an absorption maximum at 407 nm that is substantially higher than the typical blue copper maximum at 592 nm (Fig. 2). EPR spectroscopy revealed a nearly axial signal with a slight rhombic distortion with g values $g_{\perp} = 2.24$ and $g_{\parallel} = 2.06$ and a hyperfine splitting $A_{\perp} = 233 \times 10^{-4}$ cm^{-1} (Fig. 3).

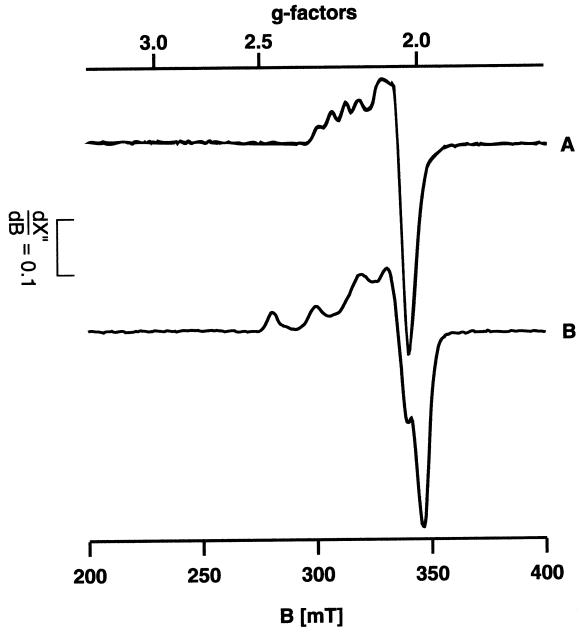

Fig. 3. EPR spectra of the oxidized blue copper protein. A: The purified truncated (Δ2, 33) SoxE of the soluble fraction, 3.8 mg/ml protein in 50 mM Tris–HCl (pH 8.0 at 25°C). B: Enriched and refolded insoluble fraction of truncated (Δ2, 33) SoxE in 50 mM Tris–HCl (pH 8.0 at 25°C). EPR parameters: 9.645 GHz, 2 mW, 20 K.

The soluble form of SoxE was purified from cytosolic proteins by heat treatment, metal chelate chromatography and a final gel filtration step (Fig. 4). During the incubation at 62°C the target protein was separated from most of the contaminating *E. coli* proteins. After centrifugation the supernatant was loaded onto a Ni-NTA column which was eluted with high imidazole concentrations. SoxE eluted at 120 mM imidazole together with one contaminant which could be identified as the histidine rich *E. coli* FKBP-type peptidyl-prolyl *cis-trans* isomerase SLYD by N-terminal protein sequencing. For the final purification the gel filtration step was added. SoxE eluted as a monomer with an apparent molecular mass of 16 kDa as calculated from the protein sequence. Finally the complementation of SoxE was carried out by addition of micromolar concentrations of copper(II); after a few seconds the colorless protein solution assumed the typical color of oxidized blue copper proteins.

3.3. Spectroscopic properties of the blue copper protein

The pure SoxE has a characteristic absorption indicating the oxidized state. At pH 8.0 it exhibits a strong absorbance maximum at 592 nm and only a small one at 407 nm (Fig. 2). Remarkably, the absorption spectrum remains unchanged even on exposure to temperatures above 80°C (not shown). EPR spectroscopy of the pure SoxE shows a typical type 1 copper spectrum (Fig. 3). The g values are $g_{\parallel} = 2.22$ and $g_{\perp} = 2.05$, the hyperfine splitting is small ($A_{\parallel} = 93 \times 10^{-4}$ cm^{-1}) and the signal seems to be nearly axial as far as the resolution of an X-band spectrum allows this conclusion. CD spectroscopy shows a minimum at 218 nm (not shown) and the β-sheet ratio is calculated to be 55%.

3.4. Redox properties of the blue copper protein

SoxE can be reversibly reduced with dithionite, ascorbate, or reduced cytochrome *c* resulting in the loss of its blue color.

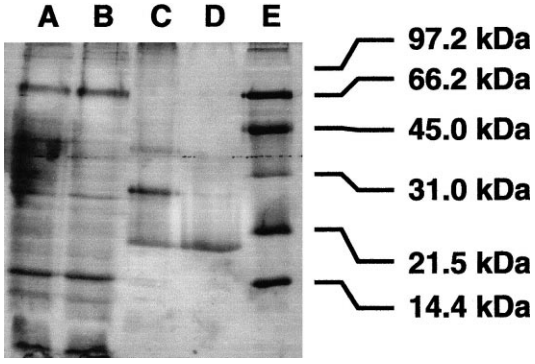

Fig. 4. Laemmli PAGE from purification steps. Lane A: cytosolic fraction (30 μg); lane B: cytosolic fraction after heat precipitation (25 μg); lane C: SoxE after Ni-NTA chromatography (3 μg); lane D: SoxE after final gel filtration (0.9 μg); lane E: Bio-Rad Long Range Marker (1 μg/protein).

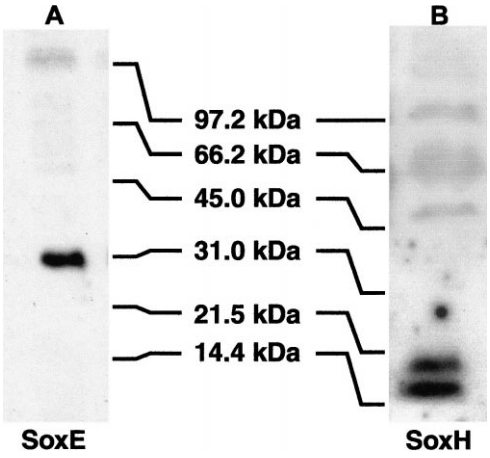

Fig. 5. Western analysis of *S. acidocaldarius* protein fractions. Lane A: 34 μg of enriched dodecylmaltoside solubilized SoxM complex detected with antiserum against SoxE; lane B: 38 μg cytosolic protein detected with antiserum against SoxH.

It is not susceptible to autoxidation at the presence of oxygen. The redox activity with cytochrome *c* was used to determine the midpoint potential of the pure SoxE. For this purpose oxidized SoxE was added stepwise to reduced cytochrome *c* under argon gas to inhibit autoxidation of cytochrome *c*. The protein shows a Nernstian behavior assuming a one electron transition. The equilibrium constant under these circumstances was determined to be 2.35 resulting in a midpoint potential of +300 mV.

3.5. Immunological analysis of SoxE and SoxH

An antiserum against the recombinant SoxE has been raised as described in Section 2. On Western blots of total protein extracts of the overproducing *E. coli* strain BL21pET15sybEII a strong single signal was produced. To investigate the translation of soxE in *S. acidocaldarius*, cells were lysed and membranes separated from the soluble proteins as described [11]. The membrane fraction was solubilized in dodecylmaltoside and the major respiratory complexes SoxABCD and SoxM were separated by hydrophobic interaction chromatography and gel filtration according to [12]. The fraction of interest, containing the SoxM complex, revealed the typical UV/Vis spectra of subunit I (SoxM), of its *b*-type cytochrome component (SoxG), as well as the EPR spectrum of the Rieske FeS protein [5,13]. In addition, with the above antiserum the presence of SoxE, the blue copper protein sulfocyanin, could be clearly detected as a single 30 kDa signal by SDS–PAGE analysis and Western blotting (Fig. 5). The apparent molecular mass is above the calculated value; apparently the native protein migrates irregularly as also observed with several other archaeal membrane proteins from Sulfolobales [2,14,15]. In contrast, sulfocyanin was not detectable in the soluble protein fraction of *S. acidocaldarius* cells.

However, the CuA containing subunit II (SoxH) was unexpectedly detected in the soluble fraction of lysed *S. acidocaldarius* cells as a strong band at 16 kDa by immunostaining with an antiserum against recombinant SoxH (Fig. 5). The strong 16 kDa signal would coincide with the calculated molecular mass of native SoxH whereas a weaker signal at about 19 kDa might result from a modified form, which has not been identified, however. As reported previously, this subunit could not be detected in the isolated SoxM complex by immunostaining, and it had been assumed that the antiserum directed against the recombinant protein for hitherto unknown reasons might be unable to react with the native subunit [9]. The present results clearly demonstrate the opposite, namely that subunit II (SoxH) obviously dissociates from the complex during membrane preparation, a situation never observed before with any terminal oxidase, whereas the rusticyanin-like sulfocyanin remains completely associated with the SoxM complex. The implications of the above findings are discussed below.

4. Discussion

Heterologous expression of both SoxE and SoxH [9] was a prerequisite for investigation of their electrochemical properties as constituents of the archaeal respiratory complex SoxM. According to the gene sequences both copper proteins bear a membrane anchor, which was truncated for expression in *E. coli* in order to obtain the functional domain in soluble form. As described above, SoxE could never be expressed as a full length protein, but a Δ2, 33 truncation allowed expression in sufficient amounts. A majority of this product is insoluble and does not refold properly; it rather results in a green variant as reported for other recombinant or mutagenized type I copper proteins [16]. However, the quota produced as soluble protein forms the correct blue copper center easily as indicated by EPR spectroscopy and the strong blue color compared to aqueous copper(II) complexes. Nevertheless, a slight distortion of the trigonal bipyramidal ligand field towards a yellow colored planar type II copper center is indicated by the maximum at 407 nm in UV/Vis spectroscopy. Though a further truncated Δ2, 99 form could be overproduced in substantially larger amounts, this variant no longer coordinates copper and tends to undergo dimerization by disulfide formation via Cys^{171} (not shown).

Cytochrome *c* interacts unspecifically with SoxE. The redox potential of SoxE as determined by equilibrium titration with cytochrome *c* fits the range to fulfill the proposed function as an intermediate electron carrier between the Rieske protein and the terminal oxidase moiety of the SoxM complex. In addition, its thermostability corresponds to the growth conditions of the hyperthermophile *Sulfolobus*. But most important, the present study for the first time demonstrates SoxE not only to be transcribed but also to remain firmly associated with this respiratory supercomplex. Thus it can no longer be considered, like cytochrome *c*, a loosely attached peripheral constituent of the respiratory chain. Thus the Cu_A center of subunit II, SoxH, should represent the natural electron acceptor for SoxE on the basis of redox potentials.

However, as also discovered in the present study, SoxH is obviously lost from the supercomplex already during disruption of the cells. That explains immediately why the preparations of the SoxM complex so far described were catalytically incompetent. In fact, none of the previous studies achieved a clearcut assignment of a protein band on SDS gels to this subunit [1,5]. Nevertheless, its unexpected solubility is surprising, a property never observed before with a terminal oxidase. However, SoxH exhibits at most only one membrane spanning helix according to the deduced amino acid sequence which, moreover, reveals only a rather short hydrophobic stretch as compared to corresponding subunits from known

cytochrome oxidases. Additionally, partial proteolysis might have occurred because two immunostained bands of SoxH were found. Because expression of a precursor form can be excluded from the gene sequence one would have to assume that the slightly larger band (Fig. 5) corresponds to the native subunit but that both bands exhibit a somewhat retarded migration behavior.

The apparent molecular mass of the native SoxE as calculated from denaturing gels is much too high and remains unexplained. Glycosylation appears possible since the sequence reveals a number of putative glycosylation sites and has indeed been observed with another membrane residing cytochrome from *S. acidocaldarius* [8]. Other reasons could be the presence of covalently bound lipid or a strong association with another peptide; but none of these possibilities has been experimentally proven so far.

In conclusion, the present study has demonstrated that in fact all components of the SoxM gene cluster are present in intact *Sulfolobus* cells and that therefore the previously suggested electron transport pathway [1] is supported as caldariella quinone → cyt a_{587} → Rieske FeS → sulfocyanin → CuA of SoxH → cyt ba_3 of SoxM → oxygen. Further, the recombinant availability of SoxH encourages the full functional reconstitution of the SoxM supercomplex which should then provide two proton pumping energy conservation sites when caldariella quinol serves as the substrate.

Acknowledgements: The authors are indebted to W. Verheyen for helpful technical assistance. The financial support from the Deutsche Forschungsgemeinschaft and from the Fonds der Chemischen Industrie is gratefully acknowledged.

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