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A second terminal oxidase in *Sulfolobus acidocaldarius*

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We previously found that the *soxABCD* operon encodes a quinol oxidase complex in *Sulfolobus acidocaldarius* and this enzyme was purified and characterized. In this study, we have used a cloning procedure based on the conservation of oxidase sequences and the polymerase chain reaction to isolate a new gene (*soxM*) encoding a subunit of another terminal oxidase. This terminal oxidase is a fusion between two central components of cytochrome oxidases, subunits I and III. *soxM* forms a transcriptional unit which is expressed under heterotrophic growth conditions. The corresponding protein was detected by direct protein sequencing in a preparation enriched with a cytochrome absorbing light at 562 nm. This preparation contains a terminal oxidase which is able to oxidize the artificial substrate \( N,N,N',N'-\text{tetramethyl-p-phenylenediamine} \). This preparation also contains SoxC, a protein homologous to the mitochondrial cytochrome *b*, and a Rieske iron-sulphur center. We suggest that SoxM is the core component of a second terminal oxidase complex and that this complex may share a subunit (SoxC) with the SoxABCD complex.

We have previously characterized a terminal oxidase complex from *Sulfolobus acidocaldarius* (Lübben et al., 1992, 1994). This SoxABCD complex contains four molecules of haem A, and absorbs light at 586 nm and 606 nm in its reduced form. In addition to these spectral signals, the *Sulfolobus* membranes contain cytochromes that have absorption maxima at approximately 560 nm (Anemuller and Schäfer, 1990; Becker and Schäfer, 1991). In bacteria, it is common to have several terminal oxidases which are simultaneously expressed in respiring cells. We have used a cloning strategy based on polymerase chain reaction (PCR) to look for genetic loci which would be homologous to *soxB* and encode catalytic subunits of alternative terminal oxidases. This has led to the isolation of a gene which encodes the main catalytic subunit of a novel quinol oxidase, and to the purification of a second terminal oxidase of *S. acidocaldarius*.

The core structure of prokaryotic and eukaryotic cytochrome oxidases contains three common subunits. Subunit I possesses the active site where oxygen is reduced (Saraste, 1990). In contrast, subunit III has no redox centers, and its functional role is not understood (Haltia et al., 1991). In the SoxABCD complex, SoxB and SoxA are related to the subunits I and II of cytochrome oxidase. SoxC is homologous to the cytochrome *b* component of the mitochondrial cytochrome *bc* complex (cytochrome *c* reductase) and the chloroplast cytochrome *bf* complex, and Sox D has no homologous partner in other oxidases (Lübben et al., 1992).

The genes in a typical bacterial operon that codes for a cytochrome *c* or quinol oxidase, have a standard order (Saraste, 1990; Chepuri et al., 1990; Ishizuka et al., 1990; Saraste et al., 1991a,b; Santana et al., 1992; Alge and Peschek, 1993; Quirk et al., 1993; Sone et al., 1993). The first structural gene encodes subunit II, and is followed by genes encoding subunits I and III. The two latter genes are fused into a single reading frame in one of the oxidase operons of *Thermus thermophilus* (Mather et al., 1993). This fusion has also happened in *soxM*. SoxM appears to form a complex at least with two other proteins. One of these is SoxC, and the other seems to be a Rieske-type iron-sulphur protein. Both proteins are normally core subunits of the cytochrome *bc*, and *bf* complexes.

**MATeRIALS AND METHODS**

**Isolation of *soxM***

Genomic DNA from *S. acidocaldarius* DSM 639 was prepared by the procedure described by Marmur (1961). Polymerase chain reaction (PCR) was carried out in a reaction mixture containing 0.2 mM dNTPs (Pharmacia), 100 mM Tris/HCl, pH 8.3, 50 mM KCl, 2−10 μM mixed oligonucleotide primers (Table 1), and 1.5 U Amplitaq DNA polymerase (Perkin-Elmer) in a total volume of 100 μl. The thermal cycling parameters were 30 s denaturation at 92°C, 90 s annealing at 51°C and 40 s extension at 72°C; 25−40 cycles were carried out. A genomic DNA library of *S. acidocaldarius* in λ phage vector (Lübben et al., 1992) was hybridized to a ³²P-labelled PCR product A, a DNA fragment
which was not amplified on the soxABC plasmid template (Fig. 2). A 2.2-kb EcoRI and 3.7-kb and 4.3-kb SacI fragments from an isolated λ clone (λAOS7) were subcloned into pBluescript II using Escherichia coli XL-1 Blue as a host. These plasmids (Fig. 2) were used for dideoxynucleotide sequencing. Other methods for DNA manipulation are described by Ausubel et al. (1993).

RNA analysis

*S. acidocaldarius* was grown at 75°C under vigorous aeration in the medium reported by Lübben and Schäfer (1987) but modified by supplementing with 0.2% (mass/vol.) potassium glutamate. Total RNA was prepared from logarithmically growing cells (absorbance of 0.2–0.5 at 600 nm) as described earlier (Lübben et al., 1992). The probe in Northern blots was the 32P-labelled 2.2-kb insert of pE2.2 (Fig. 3). Primer extension was carried out with oligonucleotides complementary to soxA (nucleotides 404–433 in Fig. 1 of Lübben et al., 1992) and to soxM (nucleotides 279–309 in Fig. 3). A mixture of 5 μg or 10 μg total RNA and 100,000 cpm of 32P-labelled primer in 10 mM Tris/HCl was heated at 95°C for 1 min and annealed for 45 min at 55°C. The extension reaction was carried out by a standard protocol (Ausubel et al., 1993). After RNase treatment and extraction of proteins, 2-μl aliquots of the extension products (total volume 10 μl) were loaded onto 6% sequencing gels. Products of a sequencing reaction with the plasmid pS3.7 were used as size markers.

Purification of the SoxCM complex

*S. acidocaldarius* was grown in the standard medium (see above) at 78°C to the late log phase in a 1500-l fermentor operated by the Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany). Membranes were prepared, washed with cholate and deoxycholate (without potassium thiocyanate) and solubilized with dodecylmaltoside as described (Lübben et al., 1994). The SoxM protein was partially purified by anion-exchange chromatography on DEAE-Sepharose FF and Q Sepharose HP using 0–0.2 M NaCl gradients in a buffer containing 20 mM Bis-Tris propane, pH 6.7, 20% (mass/vol.) ethylene glycol, 0.25 mM EDTA and 0.075% (mass/vol.) dodecylmaltoside. Gel filtration on a Sephacryl S300 HR column was carried out in the same buffer containing 75 mM NaCl.

For protein sequencing, the samples were electrophoretically transferred to poly(vinyldene difluoride) membranes, and there N-terminal sequences were determined by automated Edman degradation using the Applied Biosystems model 477A sequencer.

Immunological methods

The N-terminal peptide MNKIRLKVLYTNTA(C) was coupled to bovine serum albumin from the cysteine residue using *m*-maleimidobenzoyl-N-hydroxysuccinimide ester as the cross-linker (Ausubel et al., 1993). Polyclonal rabbit anti serum against the protein conjugate was raised using a routine immunization protocol. Western blotting and immune specific labelling with an anti-rabbit IgG coupled to alkaline phosphatase were carried out by standard methods (Ausubel et al., 1993).
Redox difference spectra, pyridine haemochromogen spectra and the EPR spectra were measured as described before (Lübben et al., 1994). Cytochrome oxidase activity was determined polarographically as described before (Lübben et al., 1994). The N,N’,N”,N”-tetramethyl-p-phenylenediamine oxidase activity was measured spectroscopically at 546 nm in 20 mM Bistris-propane, 10 mM K$_2$SO$_4$, 1 mM EDTA, pH 6.0, at 40°C using 0.2 mM substrate.

RESULTS

Isolation of a new sox gene

The cloning method is based on the polymerase chain reaction with mixed oligonucleotide primers targeted to the most conserved coding regions within the subunit I gene. We have used the same method to isolate oxidase genes from a number of bacteria (unpublished results; Shapleigh et al., 1992b). A set of mixed oligonucleotide primers (Table 1) was designed complementary to DNA regions encoding highly conserved internal peptides in subunit I. The primers do not match all oxidase sequences found in the database but correspond to the consensus sequences given in Table 1. Moreover, even for the consensus sequence they are not totally degenerate. The locations of targeted positions in a canonical subunit I gene are marked in Fig. 1A. The PCR fragments have predicted sizes because the subunit I genes have only short insertions or deletions. We have constructed a plasmid, pLP4, which carries a 6.5-kb Sall–XbaI fragment containing almost the entire soxABCD operon. This includes the soxB gene which codes for cytochrome oxidase subunit I (Lübben et al., 1992). Fig. 1B shows DNA fragments which have been amplified with PCR using either genomic DNA of S. acidocaldarius or the plasmid pLP4 as a template. A fragment with the predicted size of 0.16 kb can be amplified with primers 2 and 4 (combination D) on both templates. In contrast, combinations A–C result in 0.54-, 0.69- and 0.75-kb fragments, respectively, only on the genomic DNA. Therefore, these fragments must derive from a gene locus which is different from soxB. All primers used in the reactions are not able to hybridize to the plasmid DNA (Table 1).

Fragment A was amplified with primers 1 and 3, which contain EcoRI restriction sites at their 5’ ends, and was cloned using the pBluescript II vector. Nucleotide sequencing revealed that it has an open-reading frame coding for a protein related to cytochrome oxidase subunit I. A genomic library in λEMBL3 was probed with fragment A, and ten positive clones were selected. Southern-blot analyses showed that nine of these clones were identical. The DNA insert was subcloned as described in Fig. 2.

Nucleotide sequencing disclosed a continuous open-reading frame of 2367 bp coding for a protein homologous to subunits I and III of the haem-containing copper oxidases (Fig. 3). No reading frames for other cytochrome oxidase subunits or accessory proteins involved in its biosynthesis (Nobrega et al., 1990; Tzagoloff et al., 1990; Steinrücke et al., 1991; Van der Oost et al., 1991) could be found in the flanking regions 0.5 kb upstream or downstream of soXM. The presence of the soXM gene, with an identical size to the gene in the genomic DNA, was confirmed by a PCR reaction using primers complementary to the ends of the open-reading frame (data not shown). We have called this gene soXM (sequentia oxidases magne) as this gene appears to code for a large fusion protein that is the major subunit of a second terminal oxidase.

Transcriptional unit

The soXM gene is transcribed as a single 2.4-kb mRNA, as shown by Northern-blot analysis (Fig. 4A). The region upstream of soXM has a relatively high AT content which seems to be typical for promoter regions in Sulfolobus. A
Fig. 3. Nucleotide sequence of the boxA and terminator (Term.) sequences and the initiation triplet ATG are underlined. Restriction sites for EcoRI and is present between positions located with primer extension (Fig. 4B) only three nucleotides upstream of the first codon (Fig. 3). Protein sequencing of the mRNA (nucleotide 135 in Fig. 4). This sequence was showed that this ATG codon (at positions 136-138, Fig. 3) begins between a pyrimidine residue and a purine residue. Similarly to the other SulfoZobus transcripts (Hain et al., 1992), transcription initiation sites at nucleotide position 133 is marked (**`). Putative promoter (boxA) and terminator (Term.) sequences and the initiation triplet ATG are underlined. Restriction sites for EcoRI and SacI are shown. The N-terminal peptide obtained by amino acid sequencing is underlined (***). The target sites for the PCR primers (Table 1) are also shown. The sequence has been submitted to the EMBL data library under accession number X73567.
sis and mapping of the 5' end of the mRNA (Fig. 4) indicate that soxM is an independent transcriptional unit. This is supported by the absence of reading frames that would code for other cytochrome oxidase subunits in the flanking regions. The soxM mRNA is expressed in *S. acidocaldarius* grown in the standard medium (see Materials and Methods section). We used primer extension with 5' 32P-labelled oligonucleotides to quantify the soxM and soxABCD transcripts in the cells. With 5 μg total RNA, bands of almost equal intensity are obtained with primers complementary to the regions approximately 130–170 bp from transcriptional initiation sites (Fig. 4C).

**SoxM purifies with cytochrome *b*<sub>sox</sub>**<sub>2</sub>, SoxC and a Rieske Fe-S center

SoxM is predicted to be a protein of 788 amino acids with molecular mass 87082 Da. In the fusion protein, subunit I forms the N-terminal part and subunit III forms the C-terminal part. Most of the amino acids which are invariant in all known cytochrome oxidase sequences (Fig. 5) are found within hydrophobic regions. The conserved histidines in the transmembrane segments II, VI, VII and X are ligands of Cu<sub>b</sub> and the haem irons (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992a; Brown et al., 1993; Calhoun et al., 1993; Hosler et al., 1993). Two characteristic carboxylic acids are marked with arrows in the subunit III part (Fig. 5). The first glutamate residue is homologous to the dicyclohexylcarbodiimide-binding residue in subunit I of several other oxidases, and the aspartate in the highly conserved C-terminal helix is totally invariant (Saraste, 1990; Haltia et al., 1991).

Cysteine residues are present neither in SoxM nor in the SoxABCD complex (Lübben et al., 1992). The calculated isoelectric point of SoxM (pI 9.6) is similar to the values for SoxB and SoxC (pI 10.1 and pI 9.9, respectively). A positive net charge of these proteins at low or even at pH 7 may stabilize binding to the membrane via interactions with negatively charged lipids.

The sequence proposes that SoxM is a cytochrome and should contribute to the optical absorption spectrum of *Sulfolobus* membranes. The absorption maxima at 586 nm and 606 nm have been assigned to the haems in SoxC and SoxB, respectively (Lübben et al., 1992, 1994). Thus it seemed plausible that SoxM could be one of the b-type cytochromes in the membrane (Anemuller and Schafer, 1990; Becker and Schafer, 1991), and we followed the absorption maximum at 562 nm as a guide to isolate the SoxM protein (see Materials and Methods section).

The preparation which has an increased A<sub>562</sub>/protein ratio, contains three major polypeptides (Fig. 6). Protein sequencing showed that the protein band with apparent molecular mass 45 kDa, has the N-terminal sequence MNIKRI. This is identical to the predicted sequence of SoxM (Fig. 3). The same band cross-reacts with a polyclonal antiserum raised against the synthetic N-terminal peptide of SoxM (Fig. 7).

It was difficult to separate cytochrome *b*<sub>sox</sub> from SoxC. This cytochrome is present in all SoxM preparations that we
Fig. 5. A sequence alignment. The sequences of subunits I and III of the haem-containing copper oxidases from *E. coli* (CyoB and CyoC; Chepuri et al., 1990) and *Bacillus subtilis* (CtaC and CtaD; Saraste et al., 1991b) are aligned with SoxM. Putative transmembrane regions are hatched (note that CyoB has an extra segment labelled TM0 at its N-terminus), and amino acids which are invariant in all known haem-containing copper oxidases are boxed. Arrows point the conserved histidines involved in metal binding of subunit I and the conserved carboxylic acid residues of subunit III.

have made. In this complex, the absorption maximum of the SoxC haems is shifted from 586 nm to 592 nm (Fig. 8A). A small peak at 605 nm is also present, but immunoblotting shows that the preparation does not contain SoxB (Fig. 7). We conclude that, like SoxB and SoxC, SoxM and SoxCX tend to form a complex. The third protein which migrates with SoxC and SoxM and with the *N,N,N',N'-tetramethyl-p-phenylenediamine oxidase activity in the final gel filtration (Fig. 6) has an apparent molecular mass of 30 kDa and N-terminal sequence MDRRT. It could be the Rieske Fe-S protein.

**Spectroscopical properties of the SoxCM complex**

The difference spectra of the reduced SoxM complex with and without carbon monoxide give two signals (Fig. 8B). The maximum at 596 nm and the minimum at 447 nm indicate that CO is bound to an a-type cytochrome. Conversely, the signal at 561 nm should derive from a CO bound to a b-type cytochrome. It is therefore difficult to say whether the haem in the active site is a haem A, like that in the SoxABCD complex (Lübben et al., 1994), or a protohaem. Quantitative haem analysis supports a ratio of 3:1 between haem A and protohaem.

The electron paramagnetic resonance spectrum of the SoxCM preparation (Fig. 9A) showed signals with g values 6, 4.3 and 3 (with a shoulder at 2.9), and a Cu(I)-like signal in the g = 2 region. The g = 3 signal and the Cu(I) spectrum completely disappeared upon reduction with ascorbate and *N,N,N',N'-tetramethyl-p-phenylenediamine*, whereas the other signals were not affected. Reduction caused the appearance of a new signal that has the typical characteristics of a Rieske-type Fe-S cluster (g values 1.781, 1.889 and 2.036; Fig. 9B). This signal has recently been found in the *Sulfolobus* membranes (Anämüller et al., 1993).

The EPR spectra clearly show the presence of an ascorbate-reducible low-spin haem with g value 3.0. The broadness at g value 2.9 is presumably also due to a low-spin haem, but this one cannot be reduced with ascorbate. The intensity of the g = 2 copper signal can be estimated to be approximately twice the intensity of the g = 3.0 low-spin haem. A similar copper signal is present in the membrane preparations but it remains to be established whether SoxLM contains a copper site (and an additional subunit), or whether this metal is only adventitiously bound to the complex.

**DISCUSSION**

**Fusion protein**

A gene encoding a similar subunit I/III fusion in *T. thermophilus* is part of an operon which has a similar organization as many other cytochrome oxidase operons in bacteria (Mather et al., 1991, 1993). In contrast, SoxM has no accompanying genes in the transcriptional unit (Figs 3 and 4). We
Fig. 6. Preparation of the SoxCM complex. The elution profile of a Superose 6 HR 10/30 gel-filtration column loaded with cytochromes enriched by three previous ion-exchange chromatography steps (for details see Materials and Methods section) is shown; absorbance at 405 nm (- - - - - - - - - - - -); N,N,N',N'-tetramethyl-p-phenylenediamine oxidase activity at 40°C (W-D). The insert shows a silver-stained gel of the fractions containing N,N,N',N'-tetramethyl-p-phenylenediamine oxidase activity. N-terminal sequences of two major polypeptide components of the complex are shown in brackets.

Fig. 7. Immunoblots of the purified SoxABCD and SoxCM complexes. All antisera have been made against synthetic terminal peptides (see Materials and Methods section and LübBhen et al., 1992). SoxM, SoxA and SoxB, 0.5–2.5 µg total protein was loaded. For SoxC, 10–12.5 µg total protein was loaded for each lane. Gel electrophoresis was carried out in a gel system described by Merle and Kadenbach (1980). The separating gels (12% acrylamide, 0.6% bisacrylamide) contained 13% (mass/vol.) glycerol and 3.6 M urea.

Fig. 8. Optical spectra of the SoxCM complex. (A) The dithionite-reduced minus air-oxidized difference spectrum of the pooled fractions from the gel-filtration column (Fig. 6) is shown. (B) The difference spectrum obtained after binding of carbon monoxide to the reduced sample (CO-reduced minus reduced). The absorbance difference scale (dA) is indicated.

Fig. 9. EPR spectrum of the oxidized and reduced SoxCM complex. (A) The spectrum of the isolated, oxidized enzyme. The EPR conditions were microwave frequency 9428.8 MHz, microwave power 2.6 mW, modulation amplitude 1.27 mT, temperature 15 K. (B) The spectrum of the sample reduced with ascorbate (20 mM) and N,N,N,N'-tetramethyl-p-phenylenediamine (1 mM). The EPR conditions were microwave frequency 9428.6 MHz, microwave power 26 mW, modulation amplitude 1.27 mT, temperature 35 K.
have not been able to raise antibodies to the C-terminus of SoxM. Consequently, we do not know whether the translation product remains stable or whether it is further split into separate subunits I and III. Prokaryotic polyprotein precursors are known to exist (Thöny-Meyer et al., 1992). However, no precursors have been reported for archaea.

A systematic comparison of cytochrome oxidase sequences (Castresana et al., 1994) shows that SoxM forms a separate branch in an evolutionary tree with the CoxI sequence of Halobacterium halobium (Denda et al., 1991). SoxM is clearly separated from the subunit I/III fusion protein of T. thermophilus. This suggests that the fusion events of two (adjacent) genes have occurred independently. An artificial fusion between the homologous subunits of the sequence of the cytochrome bo complex does not destroy the quinol oxidase activity (Ma et al., 1993).

**SoxM in a complex**

SoxM seems to be part of a larger protein complex which also contains SoxC and the Rieske protein, but neither SoxA nor SoxB (Figs 6 and 7). As only protohaem and haem A are present in the Sulfolobus membranes, cytochrome b_{6f} must contain the former. Our interpretation is that this cytochrome is the low-spin haem in SoxM, and the 592 nm absorbance is due to the two haems A, in SoxC. The shoulder at 605 nm may be due to the high-spin haem A, in the active site of SoxM, or to a copper center (Fig. 9).

The EPR spectrum of the reduced sample shows that the preparation contains a Rieske-type Fe-S center, probably the same as previously detected in Sulfolobus membranes (Anemüller et al., 1993). However, the estimated concentration of the [2Fe-2S] cluster was lower than that of the low-spin g = 3, possibly due to its loss during purification. The Rieske center is normally part of the core structure of cytochrome b_{6f} and b_{6f} complexes in eubacteria and eukaryotes. Therefore, it is probable that it binds to SoxC which is homologous to cytochromes b and b_{6f}. It is not clear why the Rieske centre only binds to this protein in the SoxCM complex and not in the SoxABC complex (Lübben et al., 1992, 1994).

**Two terminal oxidases in Sulfolobus**

The cytochrome b subunit of the b_{6f} complexes has not been shown to make a complex with the cytochrome oxidase subunits. We assume that the ability of SoxC to complex with SoxB and SoxM is due to its C-terminal extension (Lübben et al., 1992). It is natural that SoxC can attach to these mutually related proteins, if it has acquired a binding site for the oxidase subunit I. It is apparent that SoxA and the Rieske protein may compete for similar binding site on SoxB and SoxC, and that there may be a specific reason why SoxA is attached to the former and the Rieske protein to the latter. Selection between SoxA and the Rieske protein could have functional implications because the electron transfer into the active sites of SoxB and SoxM may follow different pathways.

The natural substrate of both Sulfolobus oxidases is probably caldariella-quinol (Anemüller and Schäfer, 1990). Protons are transported across the membrane with a $H^+e^-$ ratio of 1, when ascorbate and $N,N',N''$-tetramethyl-p-phenylene-diamine are used as the electron-donating substrate for the intact Sulfolobus cells (unpublished results). It is not known whether both oxidases are responsible for this redox-coupled proton translocation, or whether only one of the oxidases is a proton pump.

It is also not clear why (at least) two terminal oxidases are coexpressed in Sulfolobus during heterotrophic growth. This may reflect the need of the organism to respond flexibly to changes in environmental conditions, such as pH, temperature, substrate supply and oxygen tension. Further studies on oxygen affinity, proton translocation and quinol oxidation of the purified enzymes will be required before this is clarified.

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