Journal of Bioenergetics and Biomembranes, Vol. 33, No. 1, 2001

First Expression and Characterization of a Recombinant Cu_A-Containing Subunit II from an Archaeal Terminal Oxidase Complex

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Received July 21, 2000; accepted September 20, 2000

The branched respiratory chain of the archaeon *Sulfolobus acidocaldarius* contains a supercomplex, SoxM, consisting of a bc_1-like subcomplex and a terminal oxidase moiety, including a subunit II analogous polypeptide, SoxH. However, the latter component has never been identified in preparations of SoxM. We demonstrate the presence of an mRNA transcript by Northern analysis. We succeeded in cloning and expressing the respective gene with truncated N-terminus by deleting a 20 AS membrane anchor, which resulted in a water-soluble purple copper protein, which was further characterized. The recombinant subunit II of the SoxM complex contains a correctly inserted binuclear Cu_A cluster as revealed by UV/vis and EPR spectroscopy. The protein is highly thermostable and displays a redox potential of +237 mV. In recombinant form, the metal interacts with cytochrome *c* as an artificial electron donor; the physiological electron donor is still unknown, since *S. acidocaldarius* does not contain any *c*-type cytochromes. The purple copper center of SoxM shows an interesting pH dependency with a pK_a at 6.4, suggesting protonation of the Cu-ligating histidines. Further lowering the pH causes a reversible transition into another cluster form with concomitant liberation of one copper. It may thus provide a model for the study of cluster rearrangements in response to pH.

KEY WORDS: Archaea; Cu_A; submit II; terminal oxidase; *Sulfolobus*; recombinant expression; hyperthermophiles; spectroscopy; electron transport.

INTRODUCTION

Sulfolobus acidocaldarius is an archaeon growing obligately aerobic at extremely low pH and temperatures between 75–80°C (Brock *et al.*, 1972). As recently reviewed, its respiratory system consists of at least two electron-transducing supercomplexes acting as terminal oxidases (Schäfer *et al.*, 1999); both of these display unusual structures and properties. One complex, SoxABCD, is a quinol oxidase, which has been shown to pump protons, eventually performing a Q-cyclelike mechanism (Gleissner *et al.*, 1994, 1997). Thus far, the other complex, SoxM, could be isolated only in an essentially inactive form (Castresana *et al.*, 1995; Lübben *et al.*, 1994), but was proposed on a genetic basis to consist of a bc_1-like subcomplex associated with a terminal oxidase moiety, with similarities to well-characterized cytochrome *c* oxidases (Iwata *et al.*, 1995; Michel *et al.*, 1998). A significant similarity is the presence of a subunit II analogous component, which, from the deduced amino acid sequence, was concluded to bear a Cu_A-type binuclear center as an electron acceptor for reducing equivalents from cytochrome *c*. However, *Sulfolobus acidocaldarius* does not contain any *c*-type cytochromes and the function of the subunit II analog in this archaeon remains enigmatic. Moreover, neither spectroscopic nor protein-chemical evidence was available on whether or not the putative subunit is really expressed. As yet it has not been identified in purified SoxM preparations and one could also assume that it has been lost or degraded during the isolation procedure.

In the present study, we have cloned the respective gene, *soxH*, and succeeded in expressing it in *Escherichia coli*, which allowed us (1) to demonstrate the insertion of Cu ions forming a typical Cu_A center, (2) to characterize

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its spectroscopic and electrochemical properties, and (3) to produce antibodies for further studies and investigations of the entire oxidase complex *in vivo* and in its solubilized form.

MATERIALS AND METHODS

Cloning and Transcription Analysis

An N-terminally truncated coding region of *soxH* was amplified from *S. acidocaldarius* genomic DNA using PCR. Two sets of primers (MWG Biotech) were designed, introducing appropriate restriction sites for the subsequent ligation with the pET15b plasmid vector (Novagen) resulting in a His-tagged and a non-His-tagged coding region (see Fig. 1). The PCRs were carried out in 1x *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. The program consisted of a 4-min 30-sec denaturing step at 95°C, 35 cycles of 30-sec at 95°C, 20-sec at 53°C, 40-sec at 72°C, and a 5-min final elongation step at 72°C.

A probe for Northern hybridization was similarly generated, but with 200 μM digoxigenin-labeled dNTP (DIG DNA labeling mix, Boehringer Mannheim) and a sense primer-to-antisense primer ratio of 0.9 in order to increase antisense strand production. The subsequent Northern analysis was performed as described (van Miltenburg *et al.*, 1995). The labeled DNA was eventually detected by chemiluminiscence.

In order to generate the expression vectors, 5 μg of PCR product, or 10 μg pET15b, respectively, were digested with the appropriate restriction enzymes (MBI Fermentas). The digested DNA was cleaned with a preparative agarose gel containing 10 μg/ml crystal violet (Sigma-Aldrich), and finally solubilized in 1x TE buffer.

The digested PCR fragment (150 ng) and 250 ng of the corresponding vector fragment were then ligated overnight at 14°C with T4 DNA ligase (MBI Fermentas) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 μg/ml BSA. The ligated construct was transformed into *E. coli* XL2Blue (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.

Expression and Purification

Both resulting vector constructs were transformed into *E. coli* BL21 (DE3) pLysS (Novagen) bearing the additional pSJS1244 plasmid, which codes for *E. coli* rare codon tRNAs AUA, and AGG (Kim *et al.*, 1998). At this stage, cells were plated on ACCS medium agar [1 g/liter $(NH_4)_2SO_4$, 4.5 g/liter KH_2PO_4, 10.5 g/liter K_2HPO_4, 0.5 g/liter Na-citrate $\times$ 2 H_2O, 20 mM glucose, 2 mM $MgSO_4$, 1 μg/liter thiamin-HCl, 100 μg/ml carbenicillin, 50 μg/ml spectinomycin, and 34 μg/ml chloramphenicol + 1.5% agar) to minimize basal induction. 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 periods, temperature, and media. The screening was accomplished by SDS gel electrophoresis.

Large-scale expression of the non His-tagged SoxH fragment was carried out in 2.4-liter culture medium. A single colony of BL21pET15H was used to inoculate 5 ml ACCS medium. The culture was grown overnight at 30°C. At $OD_{600} = 1.5$, 4 ml of this culture were used to inoculate 4 $\times$ 100 ml ACCS medium in 1-liter flasks. The flasks were vigorously shaken overnight at 37°C until the cultures reached stationary phase after about 16 h. ACCS medium (500 ml) were added to each flask and the cultures shaken for 2 more h at 37°C prior to addition of 600 μl 1 M IPTG. Induction was continued at 37°C for 5 h and the cells harvested by centrifugation for 20 min at 5.000 $\times$ *g*, 4°C.

The sediment was suspended in 40 ml 50 mM Tris-HCl (pH 8.0 at 25°C), 2 mM EDTA, and 1 mg/ml lysozyme, followed by sonification after which the lysed cell suspension was centrifuged 30 min at 5.000 $\times$ *g*, 4°C. The sediment was resuspended in 40 ml 50 mM Tris-HCl (pH 8.0 at 25°C), 2 mM EDTA, and centrifuged as above. This second pellet contained mainly the overexpressed inclusion bodies. The pellet was suspended in 10 ml 50 mM citrate (pH 5.5), 8 M urea, and 200 μM $CuCl_2$. This suspension was heated in a water bath at 80°C for 30 min and then cooled to room temperature overnight followed by a centrifugation for 20 min at 20,000 $\times$ *g*, 20°C. The supernatant was added to 48 ml 50 mM Tris-Cl (pH 8.0 at 25°C), 4 mM $MgSO_4$ under rapid stirring, and the solution then concentrated to 5 ml by ultrafiltration using 10-kDa cutoff membranes (Millipore). Benzonase (250 units; Merck) was added to the concentrated solution, which was left at room temperature overnight.

Of the SoxH-containing solution 2.5 ml were applied to a POROS HQ/M column with 1.8 ml bed volume (PE Biosystems), equilibrated with 50 mM Tris-Cl (pH 8.0 at 25°C) and 200 μM $CuCl_2$. A linear gradient from 0 to 2 M NaCl was applied. Fractions containing the Cu_A-binding domain eluted at 300 to 500 mM NaCl and were pooled, concentrated as above to approximately 2 ml, and loaded onto a Superdex 75 HiLoad gel filtration column (120 ml, Amersham Pharmacia Biotech). The column

was equilibrated with 50 mM Tris-Cl (pH 8.0 at 25°C), 200 mM NaCl, and 50 μM $CuCl_2$. The copper binding domain eluted at 75 to 100 ml.

The His-tagged form of SoxH could easily be purified by Ni-chelating chromatography, but only under denaturing conditions. Complete removal of urea caused aggregation and precipitation of the protein. Both forms could be complemented with copper as described in the section on Results.

Spectroscopy

UV/visible spectra were recorded at room temperature with a Hewlett Packard 8453A spectrophotometer equipped with a stirrer. EPR spectra were recorded with a Bruker ER 200D-SRC X-band spectrometer.

Copper concentrations were determined by atomic absorption on a Hitachi Zeeman atomic absorption spectrophotometer, model 180-80.

The reaction between reduced cytochrome *c* and the copper-binding domain for the determination of E_0' was carried out in degased lx PBS buffer under argon. For this purpose, horse heart cytochrome *c* (Sigma) dissolved in 1x 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 $\varepsilon_{540nm-550nm} = 19.5$ (mM^{-1}/cm^{-1}).

Miscellaneous

An antiserum against the truncated recombinant SoxH form with the His-tag was produced from rabbit whole blood using standard procedures (Harlowe and Lane, 1988) with incomplete Freud's adjuvant.

Protein concentrations were measured with the Biorad D_C protein assay.

RESULTS

Cloning of soxH and Transcription Analysis

Figure 1 illustrates the structure of the *soxH* gene together with the cloning scheme. The short membrane anchor (AS 6–20; shaded N-terminal sequence stretch) has been truncated in order to obtain a water-soluble gene product, as indicated by the primer positions. Also indicated as shaded symbols are the amino acids ligating

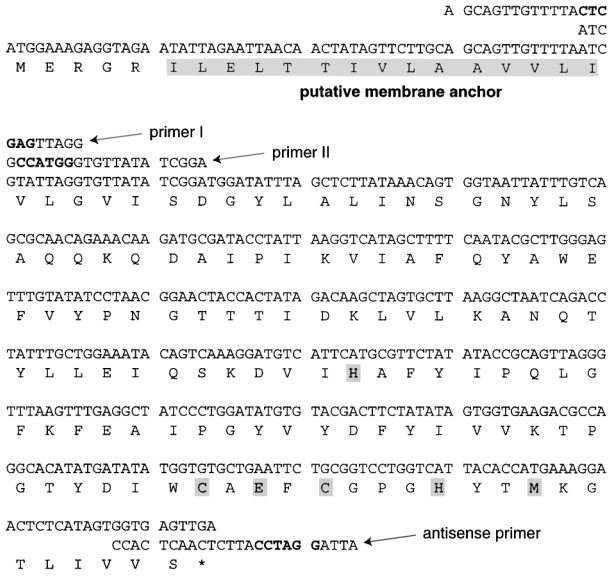

Fig. 1. DNA and protein sequence of SoxH from *S. acidocaldarius* (Castresana *et al*., 1995). Putative ligands for the Cu_A center are shaded in gray. In addition, primer sequences for PCR amplification are given. Primer I adds a His tag in combination with pET15b.

the Cu_A binuclear metal cluster. This set of essentially C-terminal located positions is almost invariantly conserved in typical Cu_A centers, whereas the N-terminal half of the sequence shows only scarce similarities to other subunits II of terminal oxidases. It should be emphasized that the sequence indicates the presence of only one putative transmembrane helix and, moreover, that this helix might not even span the membrane completely with only 17 hydrophobic residues. As described in the section on Methods, a digoxigenin labeled probe for Northern Blotting was derived with the same primers as used for construction of expression clones.

As a first reference point for the presence of *SoxH* in *S. acidocaldarius*, Northern analysis was conducted with genomic DNA. With the probe against *soxH*, two transcripts of 3.3 and 0.8 kb, respectively, were identified (Fig. 2). Of these, the smaller one corresponds to a monocystronic message, whereas the gene also appears to be cotranscribed with adjacent genes from the same cluster (Castresana *et al.*, 1995).

Expression of the Cu_A Binding Domain

Two N-terminally truncated constructs were generated in order to express *soxH* (subunit II) from *S. acidocaldarius* in *E. coli* (Fig. 1). One of them added an N-terminal His-tag to the gene, which allowed detection by Western Blot with an anti-His-tag antibody and purification by

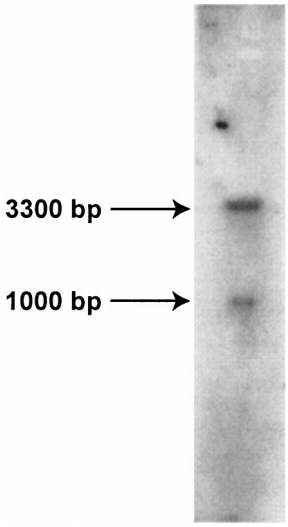

Fig. 2. Northern analysis of *S. acidocaldarius* whole RNA with a probe against *soxH*. Eight micrograms were analyzed electrophoretically on a denaturing agarose gel and compared with a standard containing λ DNA digested with *Sty*I on the same gel.

affinity chromatography. The constructs were successfully introduced into T7 system expression vectors and then transferred into *E. coli* BL21 (DE3) pLysS. First expression experiments with the selected clones failed in spite of a large variety of media and induction conditions. Only by cotransfection of a second plasmid, which encodes for the rare *E. coli* tRNAs, $tRNA^{ile}$ (codon AUA) and $tRNA^{arg}$ (codon AGA) and by change to a yeast-free medium, could expression of the Cu_A-binding domain of SoxH be attained. Both forms were expressed mainly as inclusion bodies, which could be easily separated from cytosolic and membrane proteins by differential centrifugation. At this stage, purple copper could neither be detected by EPR nor by UV/visible spectroscopy indicating an incorrectly folded or modified Cu_A-binding domain.

Complementation and Purification of the Cu_A Binding SoxH Domain

The inclusion body fractions were denatured in 8 M urea and different refolding procedures were tested. Both the His-tagged and the non-His-tagged protein could be complemented with copper by addition of micromolecular concentrations of $CuCl_2$ to the unfolded proteins at pH 5.5. At this stage, EPR and UV/visible spectroscopy showed the presence of a native purple copper center. However, both protein forms precipitated when urea was removed either by rapid dilution combined with ultrafiltration, or by dialysis, respectively. Addition of different detergents during the refolding could not prevent precipitation. Only after incubation at 80°C for 30 min followed by slow cooling to room temperature, prior to the removal of urea, could the non-His-tagged protein be obtained in a soluble form; however, the procedure failed with the His-tagged protein.

The renatured Cu_A-binding domain was further purified by anion-exchange chromatography on a POROS HQ column followed by gel filtration on a Superdex 75 column (Fig. 3). The anion-exchange chromatography separated the Cu_A-binding domain from major contaminants, including lysozyme, which had been added during cell lysis. At this stage, the protein was pure enough for most spectroscopic experiments. As a final purification step and for the determination of the apparent molecular mass, a second gel filtration step was added. Two fractions containing the purple copper protein eluted from the gel filtration column. A high-molecular weight fraction within the exclusion volume exhibited a high E_{260}/E_{280} ratio due to bound nucleotides. The other fraction eluted after 75 ml, proving the recombinant Cu_A-binding domain to be a monomer with an apparent molecular mass of 14 kDa. This agrees with the calculated molecular mass and the apparent molecular mass determined from gel electrophoresis (cf., Figs. 1 and 3). The copper content determined by atomic absorption spectroscopy of dialyzed samples was typically about 1.3 Cu atoms/SoxH molecule corresponding to 65% occupancy of the refolded recombinant protein.

Spectroscopic Properties of the Cu_A Domain

The pure Cu_A-binding domain has a characteristic purple color indicating its oxidized state. At pH 5.8, it exhibits two strong absorbance maxima at 538 and 478 nm,

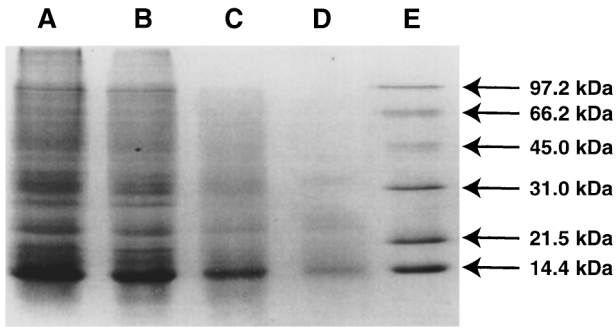

Fig. 3. Laemmli PAGE from purification steps. Lane A, Purified inclusion body fraction (100 μg); lane B, inclusion body fraction after heat precipitation (37 μg); lane C, refolded SoxH after anion-exchange chromatography (18 μg); lane D, refolded SoxH after final gel filtration (4.4 μg); lane E, BioRad long-range marker (1 μg/protein).

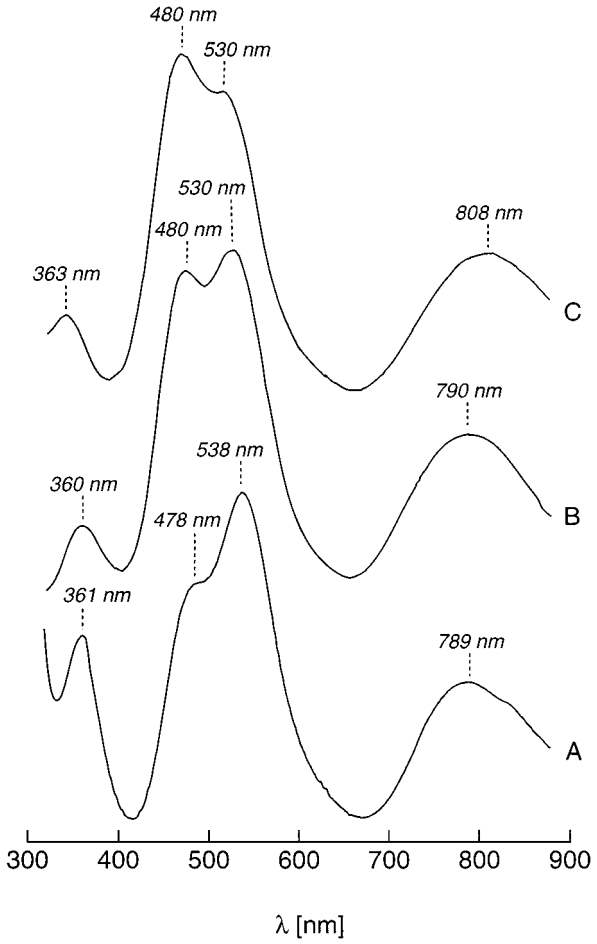

Fig. 4. Optical absorption spectra of (A) the purified recombinant SoxH at pH 5.8; (B) the soluble Cu_A-binding domain of *T. thermophilus* at pH 8.0 (Williams *et al*., 1999); and (C) the soluble Cu_A-binding domain of *P. denitrificans* at pH 7.0 (Lappalainen *et al*., 1993). The positions of absorption maxima are given.

respectively. Two additional maxima are present at 361 and 789 nm (Fig. 4). Extinction coefficients at the maxima are given in Table I. The spectrum is closely similar to those found for the Cu_A domains from *P. denitrificans* (Lappalainen *et al.*, 1993) and *T. thermophilus* (Slutter

Table I. Extinction Coefficients for Maxima of the Cu_A Center of Subunit II of the SoxM Complex at pH 5.8 Derived from the Spectrum in Fig. 3

λ(nm)	$\varepsilon(mM^{-1}/cm^{-1})$
361	2.3
478	3.2
538	3.7
789	2.4

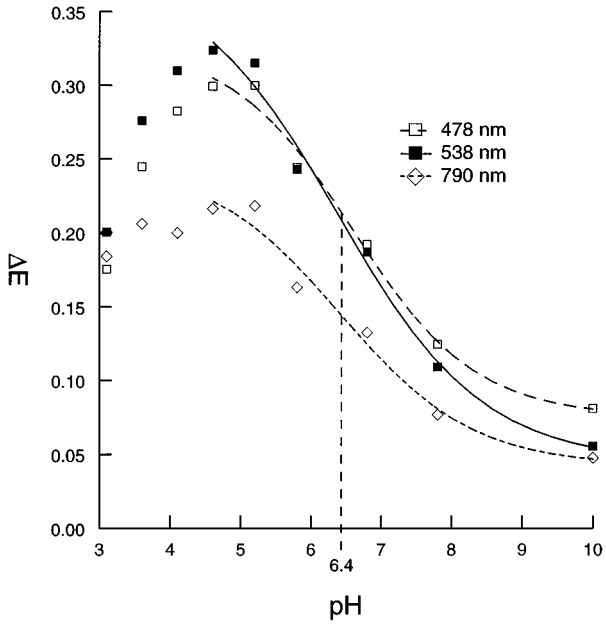

Fig. 5. pH titration of the absorption maxima of the Cu_A center. Parameters: 52 μM Cu_A (by EPR and AAS quantification) after renaturation in 50 mM citrate and 8 M urea. pH was adjusted with small aliquots of HCl or NaOH, respectively.

et al., 1996), but the absorption ratios at peak maxima are different. Remarkably, the absorption spectrum remains unchanged, even at temperatures of 80°C (not shown). The identical spectrum can already be obtained after the addition of copper to the urea-solubilized inclusion bodies (8 M urea). The presence of urea seems to have no effect on these properties, but can prevent precipitation at low pH (<6).

Moreover, the spectrum shows an interesting, fully reversible pH dependence. At the above mentioned maxima, the absorption intensities (oxidized *minus* reduced differences) were titrated over a wide pH range, as illustrated in Fig. 5. This titration unanimously indicates a pK_a between 6.4–7, suggesting the protonation of the cluster-ligating histidines to be responsible for the spectral change. At low pH, the titration displays a maximum, with a dramatic loss of spectral absorption between pH 5 to 3. While at pH $\geq$ 10 the spectral changes become irreversible by denaturation, this is not the case at low pH. The bell-shaped curve may even suggest an additional pK_a at pH values below the experimentally tested range.

Extreme spectral states from the titration of Fig. 5 are shown in Fig. 6. Whereas the typical signature of the purple copper center deteriorates completely at pH 10, under acidic conditions a shift of the 538 nm peak to 560 nm occurs concommitantly with intensification of the near IR absorption and a peak shift to 784 nm. In this state, the

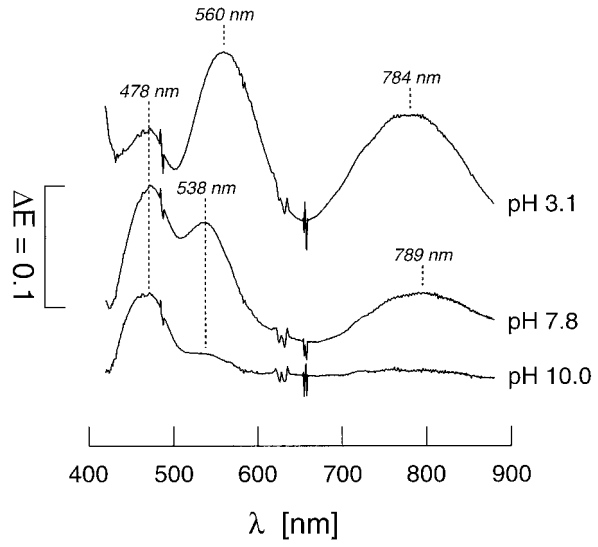

Fig. 6. UV/vis difference spectra of the Cu_A center at different pH values. Parameters: 52 μM Cu_A (by EPR and AAS quantification) after renaturation in 50 mM citrate and 8 M urea. pH was adjusted with small aliquots of HCl or NaOH, respectively. The locations of spectral maxima are given.

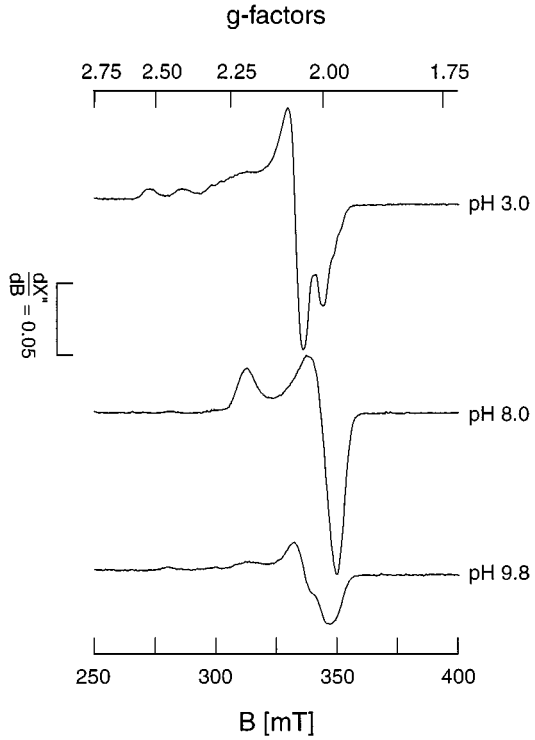

Fig. 7. EPR spectra of the Cu_A center at different pH values. Parameters: 52 μM Cu_A (by EPR and AAS quantification) after renaturation in 50 mM citrate and 8 M urea. pH was adjusted with small aliquots of HCl or NaOH, respectively. EPR parameters: 9.644 GHz, 2 mW, 17 K.

color turns bluish, reminiscent much more of a blue than a purple copper protein.

These spectral properties, which reveal a reversible pH-dependent modification of the ligand field in the binuclear cluster, are reflected also by the EPR spectra. The X-band EPR spectrum of the pure Cu_A-binding domain is similar to the spectrum of the native Cu_A site in cytochrome *c* oxidase (Fig. 7). At pH 8.0 the values are $g = 2.20$ and $g = 2.01$. As above, an identical spectrum could be found at high urea concentrations. Importantly, the metal quantification revealed a ratio of 0.52 for EPR, detectable over chemically determined copper. This is direct evidence for the presence of a true Cu_A, *i.e.*, a mixed valence binuclear metal cluster with formally one EPR-silent Cu ion.

Actually, at pH 9.8, all spectral signatures of a Cu_A center are lost, because of irreversible denaturation. In contrast, at pH 3.0, a completely new spectrum emerges, which clearly contains signatures of the free Cu^{2+} type (4-line hyperfine split *g* value), whereas the Cu_A-type signals are replaced by novel resonances. The latter may, in part, result from superimposed residual Cu_A signals as to be expected in a reversible equilibrium system.

Therefore, an unequivocal deconvolution of the low-pH spectra could not be achieved. However, taking the occurence of free copper into account, a reversible transformation between a Cu_A site and a blue copper-type metal center appears possible, as discussed further below.

Redox Properties of the Cu_A Domain

The Cu_A-binding domain can be reversibly reduced with dithionite, ascorbate, and reduced cytochrome *c*, resulting in the loss of its purple color. At stoichiometric concentrations of the reductants and in the presence of oxygen, the protein shows very fast auto-oxidation rates. The redox activity with cytochrome *c* was used to determine the midpoint potential of the pure Cu_A-binding domain. For this purpose, the oxidized Cu_A-binding domain was added stepwise to reduced cytochrome *c* under argon gas to avoid auto-oxidation. The protein shows a Nernstian behavior, assuming a one-electron transition. The equilibrium constant, under these circumstances, was determined to be 0.8, resulting in a midpoint potential of +237 mV and a respective ΔG_0 value of 23 kJ/mol.

Immunological Interactions

Though transcription of the *soxH* gene is evident, demonstration of the presence of the translation product is an important issue from two aspects: one is the illustration of all putative components suggested from the

respective gene cluster to exist in the integrated SoxM oxidase complex; the other is the aim to set up an affinity method for copurification of the integrated complex without loss of constituents and for interaction studies with soluble components as, for example, SoxE. However, the antiserum raised against the recombinant SoxH was not able to detect native SoxH either in *S. acidocaldarius* membranes or in detergent solubilized membrane protein fractions, although Western analysis of the recombinant protein showed a strong signal (not shown).

DISCUSSION

The binuclear Cu_A center in subunit II of cytochrome *c* oxidases serves as the entrance site for electrons from cytochrome *c* and is missing in otherwise structurally analogous quinol oxidases (Trumpower and Gennis, 1994). This study is the first report on a purple copper center from a terminal oxidase of an archaeal hyperthermophilic organism, *S. acidocaldarius*. By expression of a truncated form of the *soxH* gene, devoid of the hydrophobic N-terminal membrane anchor, a soluble form was obtained, which allowed investigations of its properties. Despite deletion of the first 22 amino acid residues, the engineered subunit II has a great tendency to aggregate at low pH.

The UV/visible and EPR-spectral features largely resemble those of the soluble Cu_A domain from *P. denitrificans* expressed in *E. coli* (Lappalainen *et al*., 1993) and the engineered Cu_A sites of CyoA (Wilmanns *et al*., 1995) or from *Th. thermophilus* (Slutter *et al*., 1996). Notably, the quantification of copper by EPR versus chemical determination yields a ratio of 0.52, indicating that the equivalent of 1 Cu atom in the cluster is EPR silent. From that we conclude that the mixed-valence structure of this binuclear metal ion cluster is preserved in the archaeal domain as well, representing an ancient evolutionary catalytic element also present in nitrous oxide reductases (Farrar *et al*., 1996; Van der Oost *et al*., 1994), which are evolutionary related to heme copper oxidases (Hendriks *et al*., 1998). Actually, modeling of the soluble SoxH domain from *Sulfolobus* on the basis of coordinates of subunit II of bovine cytochrome *c* oxidase (Tsukihara *et al*., 1995) supports this conclusion, suggesting H92, C127, E129, C131, H135, and M138 (cf. Fig. 1) as ligands to the binuclear Cu_A site.

For the first time, the UV/visible spectra reveal the typical long-wavelength absorption at 789 nm not seen as yet with preparations of the detergent-solubilized archaeal SoxM oxidase complex. Interestingly, the absorption ratio at the maxima between 480–540 nm is reversed as compared to the Cu_A spectra of *P. denitrificans* and *Th. thermophilus*, suggesting a slightly different geometry of the ligand field. However, no significant differences to the latter can be seen from the EPR spectra. In fact, when copper ions are added to the apoprotein, the formation of the Cu_A cluster by appropriate organization of the peptide chain obviously proceeds spontaneously, since the correct EPR and UV/visible spectra already occur in 8 M urea under conditions where at least partial unfolding of the protein has to be assumed. In contrast, completion of the folding process during removal of urea appears to be slow and needs pre-incubation at high temperature, a typical condition for hyperthermophilic proteins. The high thermostability of the soluble form of SoxH is also documented by the persistence of the unaltered UV/vis spectrum even at 80°C.

The reversible dependence of visible and EPR spectra on pH is remarkable, because it suggests a reversible transition between two different forms of copper centers, a binuclear Cu_A-type center and a mononuclear copper center, respectively. The reversible occurrence of free copper detectable by EPR (cf. Fig. 7) particularly argues in favor of the liberation of one Cu ion from the binuclear cluster upon acidification. Lowering the pH first causes a protonation of the histidines ligating the Cu ions (Fig. 5). Further acidification obviously results in a more drastic conformational change, presumably due to alterations of salt bridges or hydrogen-bond networks. From modeling studies, it emerges that it needs only a movement by about 1.5–1.8 Å of a β-strand to bring a carboxyl (E129) into position to generate an alternate structure, which has similarities to the metal coordination in blue copper proteins, provided that one bridging thiol ligand [C131] and one Cu ion move out. This may be facilitated by protonation of the His ligands. Although this hypothesis appears attractive, it requires further study and preferentially crystallization for final proof. As an additional perspective, site-directed mutagenesis might be suitable to test the hypothesis with the above protein.

Whereas the measured redox potential of the SoxH domain of +237 mV is in excellent agreement with other soluble Cu_A domains (Lappalainen *et al*., 1993; Slutter *et al*., 1996) its rapid interaction with cytochrome *c* differs from expectations, based on its physiological function. Because *Sulfolobus* does not synthesize *c*-type cytochromes, the latter can not be its natural reductant *in vivo*. It is very likely that in the recombinant SoxH, the Cu_A domain is much more exposed than in the intact oxidase complex, thus allowing nonspecific interaction with cytochrome *c*. On the other hand, the question of the natural reductant is still open; it has been proposed that a putative blue copper protein, the product of the *soxE* gene, might assume this role *in vivo* (Schäfer *et al*., 1999); however, this remains to be demonstrated.

The transcription of SoxH has been shown by Northern blotting. Nevertheless, the translation product could not be detected in SoxM complex preparations by Western blotting with an antiserum which perfectly and specifically detects the recombinant SoxH. A possible reason may be chemical modification, like glycosylation. Because, in analogy to known cytochrome *c* oxidase structures, an orientation of SoxH toward the outer membrane surface has to be expected, glycosylation is, indeed, an interesting aspect. In the intact terminal oxidase, the binuclear copper center may be shielded by glycosylation as a protective against the extremely acidic environment at the membrane surface of *S. acidocaldarius*. This would resemble the situation with cytochrome $b_{558/566}$, which is also oriented toward the outer surface of the plasma membrane and exhibits an extraordinary high degree of glycosylation (Hettmann *et al*., 1998). In fact, homology models of the SoxH structure, based on the coordinates of subunits II from sequences in *P. denitrificans, B. taurus*, and *Th. thermophilus* (Iwata *et al*., 1995; Tsukihara *et al*., 1995; Williams *et al*., 1999) reveal a number of possible glycosylation sites located in a beltlike array on a level located above the Cu_A center with respect to the membrane surface. They appear oriented preferentially to one side of the protein; thus the opposite site might represent a contact site for the still unknown physiological reductant. This remains an interesting, but speculative, suggestion until experimental proof can be achieved.

In summary, the availability of recombinant SoxH offers the possibility of crystallization and elucidation of the three-dimensional structure of this archaeal Cu_A center. In addition, work is in progress on functional reconstitution with other components of the branched respiratory chains in order to fully resolve the complex respiratory system of *Sulfolobus acidocaldarius.*

ACKNOWLEDGMENTS

The authors are indebted to W. Verheyen for helpful technical assistance and to Dr. W. G. Purschke for stimulating discussions and advice. The financial support from the Deutsche Forschungsgemeinschaft (Grant Scha 125/17-3) and from the Fonds der Chemischen Industrie is gratefully acknowledged.

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