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Expression, Isolation, and Crystallization of the Catalytic Domain of CopB, a Putative Copper Transporting ATPase From the Thermoacidophilic Archaeon *Sulfolobus solfataricus*

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The P-type CPX-ATPases are responsible for the transport of heavy metal ions in archaea, bacteria, and eukaryotes. We have chosen one of the two CPX-ATPases of the thermophile *Sulfolobus solfataricus*, CopB (=SSO2896) for the investigation of the molecular mechanism of this integral membrane protein. We recombinately expressed three different soluble domains of this protein (named CopB-A, CopB-B, and CopB-C) in *Escherichia coli* and purified them to homogeneity. 3D crystals of CopB-B, the 29 kDa catalytic ATP binding/phosphorylation domain were produced, which diffracted to a resolution of 2.2 Å. CopB-B has heavy metal stimulated phosphatase activity, which was half maximal in the presence of 80 μM Cu^{2+}. The protein forms a phosphorylated intermediate with the substrate γ-(^{32}P)-ATP. No specific activation of the polypeptide was observed, when CopB-B phosphatase activity was tested in the presence of the purified CopB-C and CopB-A proteins, which provide the cation binding and the phosphatase domains. We conclude that CopB is a putatively copper translocating ATPase, in which structural elements integrally located in the membrane are required for full, coordinated activation of the catalytic ATP binding domain.

KEY WORDS: Transport; heavy metal; CPX-ATPase; P-type ATPase; copper; *Sulfolobus solfataricus*; thermophile; archaeon; CopB; CopA.

INTRODUCTION

Copper is a trace element that serves as a cofactor for various enzymes in cell metabolism, e.g., for the superoxide dismutase and cytochrome *c* oxidase. While small amounts of copper are vital for every living organism, an excess of copper is extremely toxic (Strausak and Solioz, 1997). Therefore a strong regulation of intracellular copper concentration is required, which is maintained largely by specific transporters, the so-called CPX-ATPases (Pena *et al.*, 1999; Solioz and Vulpe, 1996).

Copper transporting ATPases belong to a large superfamily of P-type ATPases, which translocate mono- and divalent ions across biological membranes (Axelsen and Palmgren, 1998). Heavy metal transporting ATPases are known as CPX-type ATPases, named after a conserved intramembrane CPX motif, which forms a heavy metal ion channel through the membrane (Fig. 1). Their single catalytic subunit is an integral membrane protein, in which pairs of transmembrane segments are conntected by three different functional domains facing the cytosol (Lutsenko and Kaplan, 1995), see Fig. 1. The ATPases have a molecular mass in the range of 70–200 kDa; they form a phosphorylated intermediate during their reaction cycle by transferring the γ-phosphate from ATP to an aspartic acid residue of the conserved sequence DKTGS/T (Fig. 1). In addition, the catalytic domain has a nucleotide binding site (GDGXNPXP-motif), and additionally a HP

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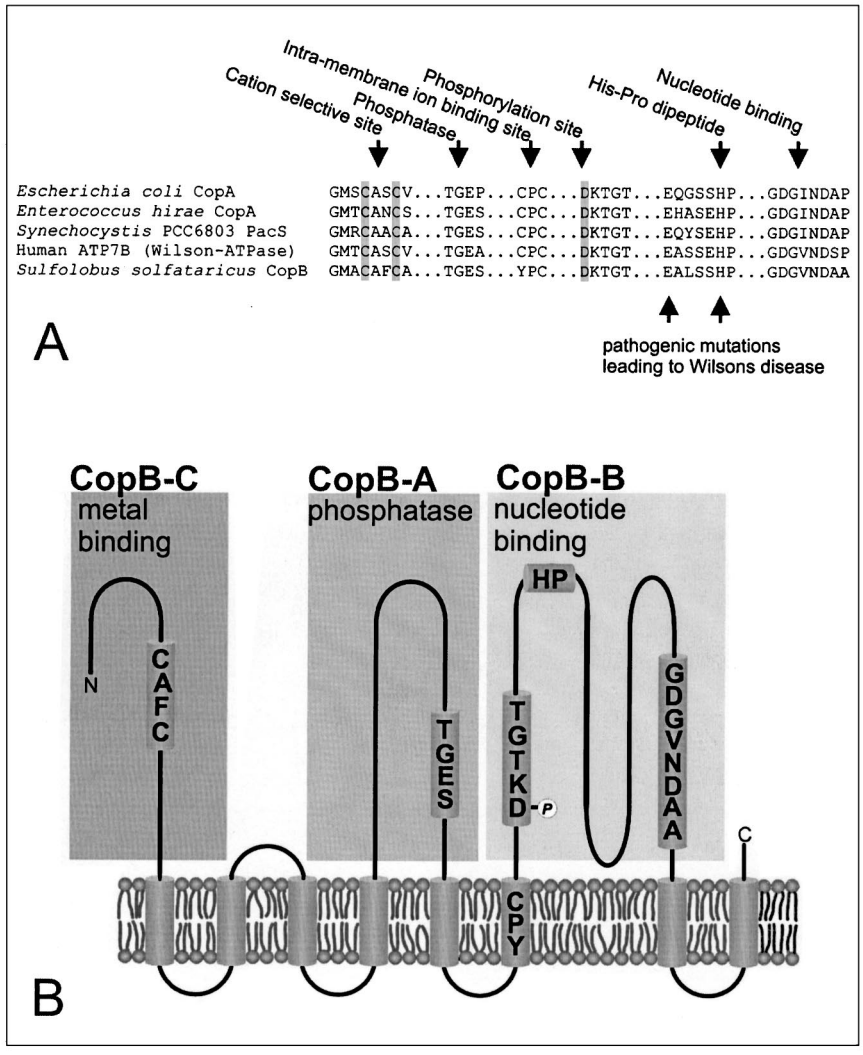

Fig. 1. (A) Partial sequence alignment restricted to significant regions of interest from various copper translocating ATPases, indicating the affiliation of CopB to the CPX-ATPases. CopA of *Escherichia coli* (SwissProt Q59385), CopA of *Enterococcus hirae* (SwissProt P32113), PacS of *Synechocystis* sp. strain PCC6803 (SwissProt P73241), Human WND-ATPase ATP7B (SwissProt P35670), CopB (SSO2896) of *Sulfolobus solfataricus* P2 (TrEMBL Q97UU7). The alignment was done with the program ClustalW (Thompson *et al.*, 1994). (B) Topology of CopB as inferred from sequence comparison with other CPX homologues (Solioz and Vulpe, 1996) and from secondary structure prediction (Sonnhammer *et al.*, 1998). The hydrophilic domains CopB-A, CopB-B, and CopB-C, which were expressed and purified, are specifically highlighted in this diagram.

signature. The phosphatase domain is characterized by its typical TGES-motif (Solioz and Vulpe, 1996). Another prominent feature is the so-called heavy metal-associated domain (HMA-domain), which is present in 1–6 copies at the N-terminus; cysteine ligands from the sequence motif GMXCXXC (Fig. 1) provide the binding partners of heavy metals (Solioz and Vulpe, 1996). HMA domains from bacterial and from eukaryotic copper translocating ATPases, which are involved in copper transport diseases, have been determined (Banci *et al.*, 2001, 2002; Gitschier *et al.*, 1998).

The knowledge of the 3D structure of the entire protein is of great interest for the understanding of the molecular mechanism of the transmembrane copper transport. The 3D structure of the eukaryotic Ca^{++}-ATPase isolated from sarcoplasmic reticulum of rabbit is known (Toyoshima *et al.*, 2000). In principle, this can be regarded as a rough model for the superfamily of P-type ATPases.

However, for a detailed comparison the structure of the Ca^{++}-ATPase is of limited value due to the low sequence similarity to copper-ATPases.

To elucidate the molecular mechanisms of an integral membrane CPX-ATPase, we devised a strategy by which the holo protein was dissected into smaller, functional domains for easier study. The first part of this approach consists of the heterologous expression of the hydrophilic domains of the CPX-ATPase CopB of the archaeon *Sulfolobus solfataricus* P2, which we have named CopB-A, CopB-B, and CopB-C.

MATERIALS AND METHODS

Construction of Expression Plasmid pCOPB-B

The gene fragment for expression of CopB-B (amino acids 383–645 of SSO2896) was amplified by the polymerase chain reaction with the oligonucleotide primers GCGCCATGGCTCTTTCATTATAC and GCGGGATCCTCAATTAGATGGAATAGCATTAC (restriction sites for *Bam*HI and *Nco*I are underlined) with genomic DNA of *Sulfolobus solfataricus* strain P2 (gift from Dr C. Schleper, Darmstadt) as template. The fragment was cut with *Nco*I and *Bam*HI and ligated into the vector pET-27bmod (kind gift from Dr M. Engelhard, Dortmund), in which the *pelB* leader sequence was deleted by replacement of the 369 nt *Sph*I/*Nco*I fragment of pET-27b (Novagen) with the 302 nt *Sph*I/*Nco*I fragment from pET-19b (Hohenfeld *et al.*, 1999).

Expression of the Soluble CopB-B Domain of *Sulfolobus* ATPase in *E. coli*

The plasmid encoding CopB-B was transformed into *Escherichia coli* strain BL21 (DE3) and selected for on kanamycin plates at a concentration of 50 μg/mL^{-1}. Liquid cultures (LB medium, 1–5 L total volume) were grown using indented conical flasks at 37°C and 150 rpm after inoculation with 1/100 volume of preculture. When the cell density reached an OD_{600} of 0.5–0.7, expression was induced by addition of 0.5 mM isopropyl thio-β-D-galactoside. After 5 h, the cells were harvested by centrifugation at 4°C using a SLA 3000 rotor (Kendro) at 6000 rpm for 15 min, suspended in MES-buffer (60 mM MES, 1 mM EDTA, pH 6.2) and frozen at −20°C.

Purification of the Expressed ATPase Domain CopB-B

Frozen cells were resuspended in MES-buffer and broken by ultrasonication for 1 h at level 8 and 50% pulse length (Branson sonifier 250). Cell debris was pelleted by ultra-centrifugation (70 Ti rotor, Beckman) at $100{,}000 \cdot g$ for 1 h. For heat aggregation of *E. coli* proteins the supernatant was incubated in a water bath for 30 min at 70°C. Precipitated material was separated by ultra-centrifugation at $100{,}000 \cdot g$ for 30 min and the soluble proteins of the supernatant were concentrated by 60% ammonium sulfate precipitation. After sedimentation by ultra-centrifugation at $100{,}000 \cdot g$ for 30 min, the precipitate was resuspended in 3–5 mL MES-buffer and dialysed for 12 h against the same buffer.

Cation-exchange chromatography—The dialysed protein solution was loaded onto a 20 mL S-Sepharose FF (Amersham Biosciences) cation-exchange column, equilibrated with MES-buffer. After a washing step with 4 volumes of MES-buffer, the proteins were eluted by a 0–500 mM NaCl gradient (total volume 200 mL). The eluate was monitored at 280 nm and peak fractions were analysed by SDS-PAGE. Fractions containing pure CopB-B were pooled and concentrated using Vivaspin spin concentration tubes (Sartorius) with a pore diameter at 10 kDa. The buffer was exchanged by dilution with 5 mM MES, pH 6.2 and reconcentration to 20–30 mg/mL as described before. The protein was stored at −20°C.

Expression and Purification of CopB-A and CopB-C

The fragments encoding CopB-C (amino acids 1–91) and CopB-A (amino acids 194–326) were amplified, cloned, and expressed as described above, using the primers GCGCCATGGTGTCACAAGAACAAAATAATATAAC and GCGGGATCCTCAACTCCAACTAATTATAGATC TTCTCC (CopB-C) and GCGCCATGGGAGCAATATT AAATAAT and GCGGGATCCTCAAATTACTTTATCA AAGAAAG (CopB-A). The fragments were purified as described for CopB-B, except that for CopB-C an anion exchange column (Q-Sepharose FF) was used.

Activity Assay: *para*-Nitrophenyl Phosphate (pNPP) Hydrolysis

The catalytic activity of the CopB-B domain was determined by using *para*-nitrophenyl phosphate (pNPP) as the substrate instead of ATP. The yellow decomposition product *para*-nitrophenol (pNP) can be colorimetrically quantified. Reactions were performed in a volume of 200 μL at 65°C for 20 min. About 4 μM enzyme was incubated with 10 mM pNPP in reaction buffer (60 mM MES, 30 mM NaCl, pH 6.2) and various metal salt (e.g., copper (II) chloride or others) concentrations varied from

0 to 1000 μM). The reaction was started by addition of the substrate and stopped with 1 mL 0.2 N NaOH. After storage on ice for 30 min, the samples were measured in a photometer at 410 nm with H_2O as reference. Identical samples without enzyme were performed as blank values (which represented the autohydrolysis of pNPP). pNP concentration was calculated according to the Lambert-Beer law and the activities were given in nanomole of enzymatically hydrolysed pNPP per milligram of protein per minute, using $\epsilon_{410} = 16.200\,M^{-1}/cm^{-1}$. Cu^{1+} was generated by inclusion of 1 mM sodium ascorbate from a freshly prepared stock solution (50 mM, pH adjusted to 6.2 with NaOH) and 0.5 mM $CuCl_2$ to the assay mixture.

Phosphorylation of CopB-B With ^{32}P-ATP

Purified CopB-B was diluted to 0.18 mg/mL in 50 mM MES, pH 6.2, 5 mM $MgCl_2$. About 50 μL of this solution was placed in an Eppendorf tube and 10 μCi of γ-^{32}P-ATP (30 Ci/mmol) was added. The mixtures were either incubated at room temperature for 1 min (negative control) or at 60°C for 1 or 2 min. The incubations were stopped by addition of 1 mL ice-cold 10% trichloroacetic acid, kept on ice for 5 min and then centrifuged for 5 min at 13,000 rpm with a bench-top centrifuge. The supernatants were carefully removed and the pellets were washed first with 1 mL of 10% trichloroacetic acid solution centrifuged as above and finally washed with 1 mL of ice-cold water. The pellets were dissolved in 20 μL of 10% SDS solution, and mixed with the same volume of SDS-PAGE loading buffer. About 20 μL of samples were loaded on 12.5% gels without heating. The gels were run for 2 h, rapidly stained with Coomassie Brilliant Blue R250, destained, dried, and exposed to a phospho-imager.

Crystallization and Diffraction Analysis of CopB-B

Hanging drop vapor diffusion crystallization was performed in 24 well plates (Linbro, ICN) using glass cover slides of appropriate size. The thawed protein solution was diluted to 12 mg/mL in 5 mM Na-MES buffer, pH 6.2. About 2 μL of protein solution was placed on a glass cover slide and the same amount of crystallization solution was added. The glass plate was placed on top of a well containing 0.5 mL crystallization solution and sealed with vacuum grease (medium viscosity, Bayer). Initial crystallization conditions were found using crystal screens 1 and 2 from Hampton Research. The crystallization condition was further optimized using additive screens 1 and 2 from Hampton Research. The final crystallization solution consists of 20–24% (w/v) PEG-6000 (Fluka) in 100 mM sodium cacodylate (pH 6.5), 200 mM ammonium sulfate, 10 mM DTT, and 1 mM sodium azide. At 18°C crystals appeared after 2 weeks and continued to grow for additional 3 weeks. The domain CopB-B forms very thin plates which are highly intergrown (Fig. 2(A)). For data collection, the crystals were separated from each other and were transferred to cryosolution consisting of 28% (w/v)

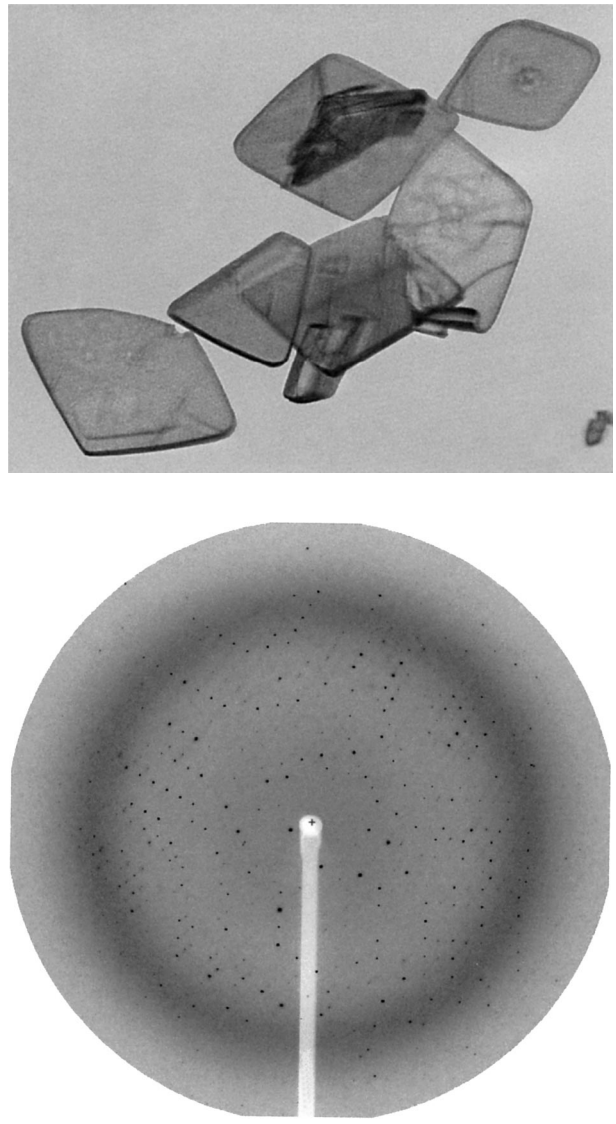

Fig. 2. Crystals (A) and diffraction image (B) of the catalytic domain CopB-B. The protein forms thin, intergrown plates which have to be separated for X-ray data collection. The diffraction pattern for a 1° rotation image (exposure time 5 s) was collected with a MarCCD detector at beamline X06SA of the Swiss Light Source (SLS, Villigen) using a wavelength of 0.978 Å at a crystal-to-film distance of 200 mm (giving a Bragg spacing of 2.6 Å at the edge of the detector).

PEG-6000 and 17.5% (v/v) glycerol in 100 mM sodium cacodylate (pH 6.5), 200 mM ammonium sulfate, 10 mM DTT, and 1 mM sodium azide, and were frozen in liquid nitrogen after 2–3 min incubation. Different native and derivative data sets were collected at beamline ID 14-4 at the ESRF in Grenoble and the PX beamline X06SA at the SLS in Villigen (Fig. 2(B)).

Various Biochemical Methods

Proteins were analyzed with SDS-PAGE on 12.5% gels according to Laemmli (1970). Protein concentration was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

RESULTS

The Hydrophilic Domains of the CPX-ATPase CopB From *Sulfolobus solfataricus*

The strategy for our experiments was based on the expectation that thermophilic proteins might be better candidates for mechanistic studies than their mesophilic counterparts. Genome sequencing of *S. solfataricus* P2 (She *et al.*, 2001) had indicated the existence of only two genes encoding P-type ATPases in this organism, CopA (SSO2651) and CopB (SSO2896), which have high sequence similarity to heavy-metal CPX-ATPases (Fig. 1). The presence of eight membrane-embedded segments of CopB was predicted using the computer program MPex[5] and TM-HMM[6] (Sonnhammer *et al.*, 1998). On the basis of the published secondary structural model of other CPX-ATPases (Solioz and Vulpe, 1996), we could roughly derive the topology of CopB. The read-out of the domain borders allowed the construction of the fragments CopB-A (phosphatase domain), CopB-B (ATP-binding and phosphorylation domain), and CopB-C (heavy metal binding domain), which were heterologously expressed in *Escherichia coli*.

Heterologous Expression and Purification of CopB-A, CopB-B, and CopB-C

Applying the information gained by the structure predictions of CopB, we cloned the respective gene fragments in a sligthly modified expression plasmid pET-27b. The protein fragments were expressed at high levels by application of standard protocols (Rosenberg *et al.*, 1987; Studier and Moffatt, 1986). Inclusion bodies were absent and the expression yields of CopB-B (compare Fig. 3, lanes 1 and 2) and CopB-C were 6–8 mg/L culture media; Cop-A was obtained in smaller amounts. Expressed CopB-A and CopB-B were verified by means of mass fingerprinting using MALDI mass spectrometry after trypsin digestion of excised protein bands from gel electrophoresis of *E. coli* cell homogenates (Shevchenko *et al.*, 1996) (data not shown).

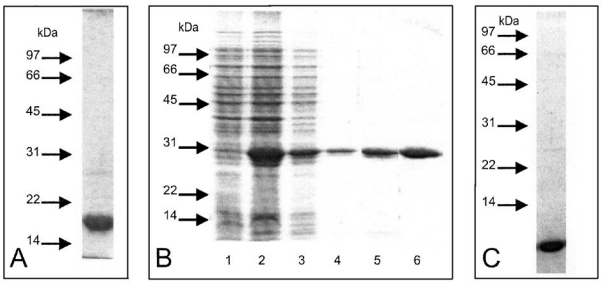

Fig. 3. (A) SDS-PAGE documenting the purity of the fragment CopB-A: Peak fraction eluted by S-Sepharose FF. (B) Stepwise purification of the catalytic fragment CopB-B. Lanes 1—extract of cells before induction (12 μg protein); 2—cells 5 h after induction (31 μg); 3—supernatant after homogenization and ultracentrifugation (10 μg); 4—supernatant after heat precipitation (1.6 μg); 5—concentrate after ammonium sulphate precipitation and dialysis (5.3 μg); 6—concentrate after S-Sepharose FF (3.5 μg). (C) Purity of CopB-C: Peak fraction eluted by Q-Sepharose FF.

After cell disruption, all three expressed proteins are soluble and were prepared by a three-step protocol, consisting of a heat precipitation to largely remove the proteins of *E. coli*, and an ammonium sulphate fractionation. Further purification of CopB-C was performed by application of anion exchange chromatography, whereas the fragments CopB-A and CopB-B were purified to homogeneity using cation exchange columns. The samples loaded on columns were still strongly contaminated by nucleic acids, which were completely removed in this purification step (as indicated by absorbance spectra; not shown). It is assumed that this material represents a complex aggregate also consisting of unfolded domains. CopB-B (regarded to be in the folded state) was eluted at a salt concentration of 250–300 mM NaCl. The fraction of folded CopB-B was estimated to be at least 40% of the totally applied material. The stepwise purification of the 29 kDa polypeptide is shown in Fig. 3(B). Column chromatography was an indispensable step to the removal of a slightly smaller contaminant (about 28 kDa), which is seen in Fig. 2(B), lanes 5 and 6. The molecular mass of CopB-B was determined by electrospray ionization mass spectrometry to 28,655 Da,

[5] S. Jaysinghe, K. Hristova, and S. H. White (2000). http://blanco.biomol.uci.edu/mpex

[6] http://www.cbs.dtu.dk/services/TMHMM/

which is in exact agreement with the value calculated from the protein sequence. The other fragments, CopB-A and CopB-C, were purified by the same procedure except that in case of CopB-C, an anion exchanger matrix was employed. Column eluates of the products indicate that this procedure yielded pure CopB-A and CopB-C (Fig. 3(A) and (C)).

Catalytic Properties of CopB-B

The purified CopB-B fragment was capable of hydrolysing ATP at a low rate as measured from determination of liberated inorganic phosphate (Heinonen and Lahti, 1981). Therefore, we investigated the biological function of the domain by its potential to form a phosphorylated reaction intermediate, which is a common property of P-type ATPases (Pedersen and Carafoli, 1987; Solioz and Camakaris, 1997). The purified protein was exposed to γ-^{32}P-ATP at various temperatures. The complex was formed after 1–2 min incubation at the catalytic temperature of 60°C, which is obvious from autoradiography of SDS polyacrylamide gels (Fig. 4(B), lanes 2 and 3). As a negative control, the sample was kept for a minute at room temperature in the presence of the radioligand (Fig. 4(B), lane 1), demonstrating that no reaction had taken place.

The enzymatic hydrolase assay of P-type ATPases can be readily tested with the chromogenic substrate *para*-nitrophenyl phosphate instead of ATP (Campos *et al.*, 1988; Hasselbach and Stephan, 1987; Ray and Nandi, 1986), which allows the performance of enzyme activity tests at higher throughput, which was necessary for the optimization of test conditions. The optimum pH of 6.2 at a salt concentration of 30 mM was determined. The phosphatase requires high temperatures, as reflected by the living conditions of the thermophile *Sulfolobus*. The optimal test temperature is 65°C because under these conditions the temperature-dependent nonenzymatic hydrolysis rate of *para*-nitrophenyl phosphate (background) is kept lower than about 50% of the total hydrolysis rate.

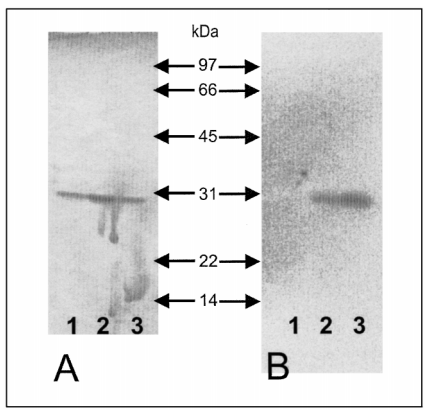

Fig. 4. Phosphorylation of CopB-B with radioactively labeled ATP. After incubation of 4 μg of purified CopB-B with γ-^{32}P-ATP at various temperatures for different times, unbound ligand was removed by subsequent precipitation/wash steps as outlined in Materials and Methods section. The recovered samples were loaded without heating on a 15% SDS gel, which was quickly run, briefly stained/destained and vacuum dried. (A) Coomassie Brilliant Blue stain, (B) autoradiography as obtained after exposition of the same gel to phospho-imager. Lanes 1—1 min incubation at room temperature; 2—1 min incubation at 60°C; 3—2 min at 60°C.

Table I. Cation Specificity of CopB-B[a]

Metal ion	Activity (nmol mg^{-1} min^{-1})	Standard deviation ($n = 6$)
None	1.6	±0.9
Ca^{2+}	1.5	±0.9
Cd^{2+}	1.6	±1.4
Cu^{+}	1.8	±0.5
Ag^{+}	3.9	±2.8
Zn^{2+}	6.1	±0.9
Cu^{2+}	14.5	±1.4

[a] Activities were measured as described in Materials and Methods sections, using 0.5 mM of chloride salts of the respective metals. Blanks containing the same mixture without enzyme were measured for each of the respective metals. The activities represent the nanomoles of nitrophenol produced per minute per milligram of enzyme, which is equivalent to the amount of liberated phosphate. Cu^{+} was generated by addition of 1 mM sodium ascorbate to the reaction mixture.

The hydrolase activity of CopB-B was fairly low in the absence of activating cations or in the presence of non-activators like Ca^{++} (Table 1). Cu^{+} did not stimulate, but Ag^{+}, Zn^{++}, or Cu^{++} at a concentration of 0.5 mM lead to a severalfold increase. The metal-induced phosphatase activity could not be inhibited by *ortho*-vanadate at concentrations up to 100 μM. The activating effect of Cu^{++} is seen from Fig. 5. The cation induction is half-maximal at 80 μM, and it could be saturated, which clearly indicates that the heavy metal activation of CopB-B is specific. Furthermore, binding assays (Hemdan *et al.*, 1989) with immobilized metal affinity chromatography (IMAC) confirmed the specificity of CopB-B for Cu^{++} (not shown). It was also tested whether the presence of the other hydrophilic fragments exert a stimulatory effect of the catalytic domain. The activation profile by increasing Cu^{++} concentration of equimolar mixtures of CopB-A, CopB-B at 4 μM each was similar to that of CopB-B alone (data not shown). Additional inclusion of CopB-C did not alter this conclusion. Comparable experiments using Zn^{++} instead also did not show specific activation of CopB-B in presence of the other domains.

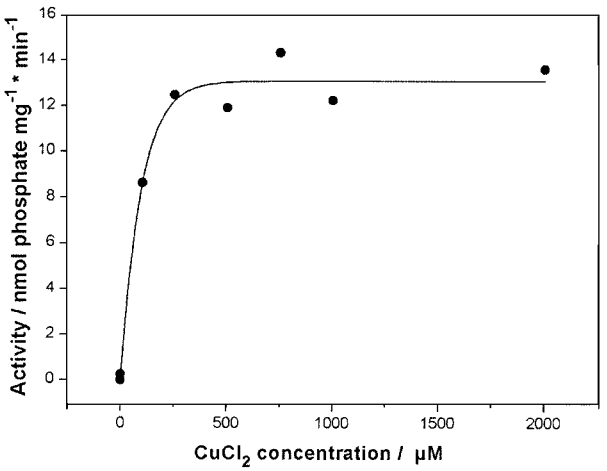

Fig. 5. Stimulation of the *p*-nitrophenyl phosphatase activity of the CopB-B fragment by addition of Cu^{++}.

Crystallization of CopB-B

The successful crystallization of the catalytic domain of CopB provides further evidence for its conformational homogeneity. The crystals form thin plates but do diffract the X-ray beam to high resolution (maximum X-ray diffraction was observed at bragg spacing of around 2.2 Å at beamline ID14-4 (ESRF, Grenoble)) indicating well-ordered packing within the crystal lattice. The protein crystallizes in the orthorhombic space group $P2_12_12$ ($a = 71.4$ Å, $b = 52.1$ Å, $c = 68.9$ Å). On the basis of Matthews parameter (Matthews, 1968; Collaborative Computational Project, Number 4, 1994), we estimated one monomer in the asymmetric unit (Matthews coefficient 2.3, 45.8% solvent). We currently solve the structure of CopB-B using seleno-methionine substituted protein for MAD-phasing.

DISCUSSION

A number of heavy metal translocating ATPases from both bacteria and eukaryotes have been characterized (e.g., Fan and Rosen, 2002; Møller *et al.*, 1996; Rensing *et al.*, 2000; Wunderli-Ye and Solioz, 2001; Wyler-Duda and Solioz, 1996). We have chosen the archaeal prokaryote *Sulfolobus solfataricus* to examine CPX-ATPases, because the inventory of P-type ATPases is clearly arranged and consists of the two representatives CopA and CopB; moreover, thermophilic enzymes are likely more suitable for structural studies than their mesophilic counterparts due to their apparently diminished flexibility and enhanced stability at ambient temperature. To our knowledge, there is only one report on the functional characterization of the ATPase CopA of another hyperthermophile, *Archaeoglobus fulgidus* (Mandal *et al.*, 2002). A soluble protein with high sequence similarity to the catalytic region of CPX-ATPases from *Methanococcus jannaschii* (Ogawa *et al.*, 2000) has been characterized, which was recently been identified to be a phosphatase (Bramkamp *et al.*, 2003).

The *Sulfolobus solfataricus* homologue CopB was expressed at very low levels under physiological growth conditions (Grogan, 1989; Zillig *et al.*, 1980), as it could be demonstrated by Western blotting using polyclonal rabbit antisera raised against the CopB-B fragment (data not shown). For this organism the biological role of CopB is not known, because no mutants are available for the study of its function in vivo. We have, however, determined the functional presence of a copper-dependent phosphatase using the aforementioned *para*-nitrophenylphosphate assay on isolated *Sulfolobus* membranes (not shown). Further characterization of the holo protein in vivo will obviously be a high priority. In this paper, we describe a straightforward protocol for the expression and purification of the three hydrophilic domains of the integral membrane protein CopB. A great advantage of the experimental strategy is the relative ease of fragment enrichment based on heat denaturation of *Escherichia* proteins whereas the heterologously expressed thermophilic proteins remain soluble. After ammonium sulphate precipitation, only one additional chromatographic step was needed to achieve final purification.

The catalytic domain CopB-B is a heat-stable protein, which exhibits relatively small phosphatase activity at high temperatures. It was anticipated that CopB-B, being part of a typical P-type ATPase, transfers the γ-phosphate of its genuine substrate ATP to the protein to produce a covalent acyl phosphate complex with a conserved aspartyl residue. Successful formation of this intermediate can be regarded as indicator for the existence of the functional protein. For the optimal mechanistic investigation of CopB-B, we replaced the substrate ATP with the pseudosubstrate *para*-nitrophenyl phosphate, which has been used in assays of various P-type ATPases (Campos *et al.*, 1988; Hasselbach and Stephan, 1987; Ray and Nandi, 1986). Its chromophoric character allows for the use of rather simple test conditions, which permits the throughput of large numbers of samples. In addition, this substrate bypasses troublesome problems related to the formation of complexes of heavy metals, such as copper, with ATP (Li *et al.*, 1996). CPX-type ATPases have been reported to have low turnover in the nanomole per milligram protein range (Fan and Rosen, 2002; Mandal *et al.*, 2002; Wyler-Duda and Solioz, 1996). Nevertheless, the observation of

even little enzymatic activity in case of CopB-B alone is surprising, because one would expect a single turnover reaction resulting in the formation of aspartyl phosphate protein in the absence of the phosphatase domain thought to be located on CopB-A. Yet the performance of multiple reaction cycles may be explained by the intrinsic instability of the aspartyl phosphate, which could be readily cleaved nonenzymatically at the high incubation temperature. The noninhibitory effect of the transition-state analogue *ortho*-vanadate can also be explained by the special geometry provided by CopB-B.

CopB-B has been shown to have a basically low *p*-nitrophenyl phosphatase activity, which is strongly activated by the presence of low concentrations of zink or copper. The substrate (i.e., ion translocation) specificity of CopB is not known. This may in part be due to the fact that some of the ion binding parts are located in the membrane region (e.g., the YPC sequence motif, Fig. 1). Other ion binding sites, which may be involved in conferring the specificity, are perhaps placed on a hydrophilic region of CopB. We therefore tested the dependance of CopB-B on ion activation and observed the highest rates in presence of Cu^{++} or Zn^{++}, which suggest that these metals are the natural substrates of CopB. Cu^{+}, regarded to be the properly translocated ionic species of copper translocating ATPases (Fan and Rosen, 2002), did not activate the CopB-B domain. It has to be noted that other heavy metal ions exerted activation effects (data not shown): The variability of Co^{++}, Ni^{++}, Mn^{++}, and Pb^{++} might be explained by cation activation effected by contaminating ions.

The different domains of the P-type ATPase interact with each other and work together during the catalytic cycle (Scarborough, 2002; Toyoshima and Nomura, 2002). The surprisingly low phosphatase activity of CopB-B was therefore thought to be increased by addition of the other functional domains CopB-A and CopB-C. However, no activation of Cu^{++} or Zn^{++}-induced phosphatase activity was observed in presence of the other polypeptides. This indicates the special importance of the membrane-integral protein elements in contributing to the putative interaction partners of CopB-B. CopB-C thus plays most likely a regulatory role, as is the case in the similar metal binding domains of the Menkes protein (Voskoboinik *et al.*, 2001).

The functional role of the two heavy metal transporters CopA and CopB ATPase of *Sulfolobus* is of great interest (i.e., whether they act as import or export translocators for a certain heavy metal species). If both the sequence homology of CopB and the catalytic activation of CopB-B by copper (or zink) are taken into account, the protein clearly belongs to the superfamily of CPX-ATPases; CopB is a putative copper translocating ATPase of this thermoacidophilic archaeon. We are currently trying to solve the problem by investigation of the transport activity using intact *Sulfolobus solfataricus* cells. Because of the high sequence similarity of the hydrophilic domains of CopB, these are appropriate models for structural and mechanistic studies of CPX-ATPases. The next step will be the elucidation of the 3D structure of the catalytic domain CopB-B, which is currently underway in our laboratory.

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