Phosphoenzyme formation by purified, reconstituted copper ATPase of *Enterococcus hirae*

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Received 2 October 1996; revised version received 28 October 1996

Abstract **The *Enterococcus hirae* CopB ATPase serves in the secretion of excess copper from cells and belongs to the recently discovered, new class of heavy metal transport ATPases. We here report the affinity purification of CopB to near homogeneity and its reconstitution into phospholipid vesicles. In these proteoliposomes, the ATPase formed an acylphosphate reaction intermediate with the γ-phosphate of ATP. ATPase activity and phosphoenzyme formation were inhibited by vanadate with an I_{50} of 0.1 mM. Our results suggest that heavy metal and non-heavy metal ATPases operate by the same underlying mechanism.**

Key words: Copper; P-type ATPase; Purification; Phosphorylation; Vanadate; (*Enterococcus hirae*)

1. Introduction

Copper is an essential element for life due to its function as a cofactor in over 40 known enzymes. However, copper can also cause cell damage by means of radical formation and subsequent oxidative damage to biomolecules. In spite of the essential role of copper and its possible toxicity, very little is known about copper homeostasis. In *Enterococcus hirae*, intracellular copper appears to be regulated by the action of the four genes *cop*Y, *cop*Z, *cop*A and *cop*B, encoded by the *cop* operon [1–3]. *Cop*Y and *cop*Z encode proteins that regulate the expression of the *cop* operon, while *cop*A and *cop*B code for copper ATPases. To effect copper homeostasis, CopA serves in the uptake of copper under copper-limiting conditions and CopB in its extrusion if cytoplasmic copper reaches toxic levels [4,5].

Putative copper ATPases with high sequence similarity to CopA and CopB of *E. hirae* have also been described for humans [6–10], yeast [11,12] and other bacteria [13–17] and this fast growing group of heavy metal pumps now numbers over 20 cloned species [18]. By several criteria, these newly discovered enzymes belong to the family of P-type ATPases, classically represented by Ca^{2+}- and Na^+K^+-ATPases. The features shared by heavy metal and non-heavy metal ATPases are: (i) a single or major catalytic subunit of 70–200 kDa; (ii) an invariant DKTGT; and (iii) inhibition by micromolar concentrations of vanadate [19,20].

With the wealth of sequence information now available on known and putative copper ATPases, it became clear that these enzymes form a distinct evolutionary branch. They contain conserved structural features not present in other P-type ATPases. Most apparent is the different predicted membrane topology of heavy metal ATPases: transmembranous helices (m) are interspersed with cytoplasmic domains (C) from N- to C-terminus in the order C-m-m-m-m-C-m-m-C-m-m-C in these enzymes, compared to C-m-m-C-m-m-C-m-m-m-m-C for Na^+K^+-ATPases, Ca^{2+}-ATPases and related enzymes [18,21]. In addition, heavy metal ATPases feature, in their long, polar N-termini, one to six conserved heavy metal binding sites with the consensus motif CXXC or DHXXMXG(H;M). Finally, a conserved HP near the phosphorylation site and an intramembranous CPC or CPH motif is unique to heavy metal ATPases. Based on this latter feature, believed to be part of the ion channel through the membrane, it has been proposed to call these enzymes CPX-type ATPases [18,22].

The evolutionary distinction and the gross structural difference between ATPases pumping heavy metals and those pumping Mg^{2+}, Ca^{2+}, Na^+, K^+, or H^+ raised the issue of whether all these pumps function by the same underlying mechanism. To address this question, we elaborated the purification and functional reconstitution of the CopB copper ATPase of *E. hirae*. Here we show that CopB in proteoliposomes forms an acylphosphate intermediate with the γ-phosphate of ATP as part of the reaction cycle and that this phosphorylation as well as ATP hydrolysis is inhibited by vanadate.

2. Materials and methods

2.1. Materials

Lysozyme and Na_2ATP were supplied by Sigma and DNase I by Boehringer Mannheim. Dodecyl-β-D-maltoside was purchased from AFC Chemicals (Bnei Brak, Israel) for protein extraction and from BioMol (Hamburg, Germany) for chromatography. Growth medium additives were supplied by Becton Dickinson and [γ-^{32}P]ATP (1000 mCi/μmol) by DuPont NEN. All other chemicals were from Merck, Darmstadt, Germany, or from Sigma Chemical Corp., St. Louis, MO, and were of the highest grade available.

2.2. Preparation of membranes from E. hirae

The *E. hirae* mutant Y1 that lacks the repressor CopY and thus overexpresses the *cop* operon about 50-fold [3] was used for the purification of CopB. Cells were grown semi-anaerobically in a 16 l fermenter in media containing 60 mM Na_2HPO_4, 1% Bacto-peptone, 0.5% yeast extract, 1% glucose, 7.5 mM DL-tyrosine, pH 7.8–8. Cultures were grown at 37°C to an OD_{546} of 3.5–4 and cells collected by centrifugation for 12 min at 8000×*g* at room temperature. The cells were washed twice in 1/50 volume of 2 mM $MgSO_4$. The wet cell weight was determined and this reference value used as g cells in all subsequent steps. The cell pellets were resuspended in 6 ml/g cells of lysis buffer (250 mM K_2SO_4, 2 mM $MgSO_4$, 50 mM glycyl-glycine, pH 7.2) incubated with 4 mg/g cells of lysozyme and incubated in a

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Abbreviations: MES, *N*-morpholinoethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; C_8E_{5-7}, hexaethylene glycol mono-*n*-(pentyl,hexyl,heptyl) octyl ether; Zwittergent 3-10, *n*-decyl-*N,N*-dimethyl-3-ammonio-1-propenesulfonate; BCDS, bathocuproine disulfonate.

PII S0014-5793(96)01306-3

shaking water bath at 37°C for 45 min. 0.5 mg/g cells of DNase I was added and incubation continued for a further 15 min. All subsequent steps were performed at 4°C. The protoplasts were centrifuged at 23 000×*g* for 12 min and the pellet resuspended in 2 ml/g cells of buffer G (20 mM Tris-SO_4, 5 mM $MgSO_4$, 25 mM Na_2SO_4 25 mM K_2SO_4, 1 mM β-mercaptoethanol, 1 μM $CuSO_4$, 20% glycerol, pH 7.5). The cells were homogenized in a Potter-Elvehjem homogenizer and passed through a French press at 20 MPa. Membranes were finally collected by centrifugation at 90 000×*g* for 60 min, resuspended in 0.66 ml/g cells of buffer G and stored frozen at −70°C. Membrane protein concentration was determined by the method of Bradford [23], using bovine serum albumin as a standard.

2.3. Purification of CopB from E. hirae membranes

1 g of membrane protein in 90 ml of buffer G were supplemented with 1/50 volume of protease inhibitor mixture (100 mM *N*-α-*p*-tosyl-L-lysine-chloromethylketone, 100 mM *N*-*p*-tosyl-L-phenylalanine-chloromethylketone, 100 mM *p*-aminobenzamidine-HCl, 100 mM phenylmethylsulfonylfluoride in dimethylsulfoxide) and extracted with 1 g of dodecyl-β-D-maltoside under stirring on ice for 1 h. The suspension was centrifuged at 90 000×*g* for 45 min and 10 mM imidazole-SO_4, pH 7.5, was added to the supernatant, which was passed through a 1.6×5 cm Ni-NTA agarose column (Quiagen), pre-equilibrated with buffer JD (20 mM Tris-SO_4, 5 mM $MgSO_4$, 1 mM β-mercaptoethanol, 1 μM $CuSO_4$, 20% glycerol, 0.05% dodecyl-β-D-maltoside, pH 7.5) containing 10 mM imidazole-SO_4. The column was washed with 40 column volumes of buffer JD containing 10 mM imidazole-SO_4, and CopB eluted with buffer JD containing 50 mM imidazole-SO_4. Elution was monitored by UV absorption at 260 nm; CopB eluted at 5–25 ml. Fractions were analyzed by electrophoresis on 7.5% SDS polyacrylamide gels [24] and visualization of proteins by silver staining [25]. The fractions containing CopB were pooled and diluted with an equal volume of 0.05% dodecyl-β-D-maltoside in H_2O prior to loading onto a 0.5×5 cm MonoQ column (Pharmacia), pre-equilibrated with buffer JD. Elution was performed with a 30 ml linear gradient of 0–250 mM Na_2SO_4 in buffer JD and highly pure CopB eluted at 18–22 ml. Purified CopB could be stored frozen at −70°C for several months without detectable loss of activity.

2.4. Reconstitution of CopB into liposomes

Soy bean phospholipids (Asolectin, Associated Concentrates, Woodside, NY) were purified as described [26] and stored dissolved in ether (50 mg/ml) at −20°C. For reconstitution, 0.2 ml of this stock (10 mg of phospholipid) were dried under a stream of N_2, followed by drying in vacuo for 30 min. The lipids were then suspended in dialysis buffer (20 mM Tris-SO_4, pH 7.4) and dissolved by addition of 20 mg β-D-octylglucoside from a 200 mg/ml stock in H_2O. 100 μg of purified CopB were added from frozen stock (1 mg/ml) and the final volume adjusted to 1 ml with dialysis buffer. The mixture was immediately transferred to dialysis tubing (Visking, Type 20/32, Viskase Corp., Chicago, IL) pretreated with EDTA and dialyzed for 150 min against 250 ml of dialysis buffer at 4°C. The buffer was then changed to 20 mM Tris-SO_4, 1 mM DTT, pH 7.4, and dialysis continued overnight.

2.5. ATPase assays

Vesicles containing 5 μg of reconstituted CopB were preincubated in assay buffer (50 mM K-MES, pH 6, 5 mM $MgSO_4$, 4 μM $CuSO_4$, 20% glycerol, 2.5 mM dithiothreitol), with or without Na_3VO_4 as indicated, for 10 min at 37°C in a total volume of 500 μl. The reaction was started by the addition of 1 mM Na_2ATP from a 200 mM stock, pH 6.5. Aliquots of 100 μl were taken at 0, 30, 60 and 90 min and transferred to cuvettes containing 10 μl of 0.5 M Na-EDTA to stop the reactions. Free P_i was assessed by a colorimetric assay as described [27].

2.6. Detection of the acylphosphate intermediate

1 μg of reconstituted CopB was preincubated in a total volume of 100 μl of assay buffer (above) for 10 min at 37°C with or without Na_3VO_4 and the reaction started by addition of 1 μM (5 μCi) of [γ-^{32}P]ATP. After 10 s the reaction was stopped by the addition of 135 μl of methanol/chloroform (4:1) and vortexing for 5 s. Lipids were removed essentially as described [28]: the samples were centrifuged for 1 min at 6000×*g* and to the protein pellet, 135 μl of methanol were added. Following vortexing for 5 s, the samples were centrifuged for 5 min at 6000×*g* and the resultant pellets air-dried and resuspended in 40 μl of acidic SDS sample buffer and separated by acidic 7.5% SDS polyacrylamide gel electrophoresis [29]. The gels were run at 100 mA for 3.5 h. Free labeled ATP was removed by floating the gel in 20% ethanol, 10% acetic acid, three times for 20 min. The gel was dried and exposed to a phosphoimaging screen. Quantification of the band intensities was conducted with the ImageQuant software. For pulse-chase experiments, 1 mM cold ATP was added 10 s after the start of the labeling reaction and the incubation continued for 30 s. Subsequent steps were as above.

To assess the base lability of phosphoenzyme intermediates, the final pellets from the above phosphorylation reactions were resuspended in 0.5 M KOH and kept on ice for 5 min. Sensitivity to hydroxylamine was tested by resuspending the pellet in 100 mM Na-acetate, pH 5.6, followed by the addition of 250 mM NH_2OH and incubation at room temperature for 10 min. KOH and NH_2OH-treated samples were again subjected to organic extraction/precipitation as described above.

3. Results and discussion

To study the functional aspects of the CopB copper ATPase of *E. hirae* on a homogeneous preparation, a purification procedure was elaborated. A previously constructed mutant, Y1, deficient in the CopY repressor overproduces CopB approx. 50-fold compared to uninduced wild type [3]. Membranes were prepared from this strain as described under Section 2. Extraction of membranes with dodecyl-β-D-maltoside at a detergent/protein ratio of one resulted in the preferential extraction of CopB. From this extract, CopB could be purified to greater than 90% purity in one step by adsorption to a Ni-NTA agarose column, followed by elution with imidazole (Fig. 1). CopA, which shares 35% sequence identity to CopB and is also overexpressed in Y1, does not bind to Ni-NTA agarose under the conditions used, as confirmed by Western blot analysis with antibodies against CopA and CopB (not shown). This is probably due to the difference in the N-termi-

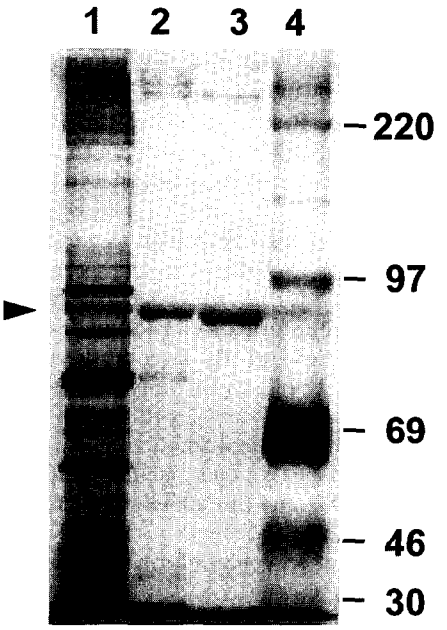

Fig. 1. Purification of CopB from *E. hirae* membranes. Lanes: 1, 10 μg of total *E. hirae* membrane protein; 2, 1 μg of pooled CopB-containing fractions eluted from Ni-NTA agarose; 3, 0.5 μg of the CopB peak fraction eluted from the MonoQ column; 4, protein size markers of the indicated molecular masses (kDa). Samples were separated onto a 7.5% SDS-polyacrylamide gel and visualized by silver staining as described under Section 2.

Table 1
Summary of the purification of CopB ATPase

Fraction	Protein (mg)	Specific activity (nmol/min per mg)	Yield (%)	Purification factor
Membranes	700[a]	–[b]	100	1
Extract	500	–[b]	90[c]	1.3[c]
Ni-NTA peak	4	18	80[c]	126[c]
MonoQ peak	1.9	20	38	140

[a]From 16 l of culture, corresponding to 70 g of wet cells.
[b]CopB ATPase activity cannot be assessed in crude preparations due to the presence of 10^3-fold higher background activity from other ATPases.
[c]Estimate from Western blot developed with antiserum against CopB.

ni of the two proteins: CopB has three repeats of a histidine-rich consensus heavy metal binding motif while CopA has a cysteine-containing heavy metal binding motif [2]. Further purification of CopB by FPLC on a MonoQ anion exchange column eliminated minor contaminating proteins. The purification procedure is summarized in Table 1. Purified ATPase in detergent solution had a specific activity of typically 20 nmol/min per mg.

To assess the properties of CopB ATPase in a membrane-bound form, a procedure for the functional reconstitution of purified CopB was developed. When the enzyme was reconstituted into crude soy bean phospholipid vesicles from a octylglucoside/dodecylmaltoside detergent mixture by dialysis, 2–3-fold stimulated ATPase activity was recovered in the proteoliposomes as compared to detergent solubilized enzyme. ATPase activity was linear for at least 120 min (data not shown). This activity appears low compared to non-heavy metal ATPases, but is higher than expected based on the copper transport rates observed in native vesicles of 0.07 nmol/min per mg [5] and the estimated purification factor of 140-fold observed here. The stimulation of the ATPase activity upon reconstitution indicates that the enzyme was not fully active in dodecylmaltoside. However, other detergents tested, namely CHAPS, decylglucoside, decylmaltoside, nonylglucoside, C_8E_{5-7}, and Zwittergent 3-10, resulted in the isolation of considerably less active or even inactive CopB.

The ATPase activity of reconstituted CopB responded to vanadate, a universal inhibitor of P-type ATPases, in a biphasic manner. While 30 μM VO_4^{3-} stimulated the ATPase activity by 50–60%, higher vanadate concentrations inhibited with an apparent I_{50} of 100 μM (Fig. 2). Another response pattern of CopB to vanadate inhibition had been observed in copper transport experiments with native membrane vesicles: copper transport was inhibited maximally by 40 μM vanadate, but this inhibition was relieved at higher concentrations [5]. This difference may be due to the very different experimental conditions in these experiments, paired with the complex chemistry of vanadate.

Since CopB is a Cu^+ and Ag^+-pump, ATP hydrolysis would be expected to be stimulated by Cu^+ and Ag^+, but we did not observe such stimulation of the ATPase activity. However, 60% inhibition of ATP hydrolysis was observed in the presence of 0.5 mM BCDS, a chelator specific for cuprous ions [30]. Whether there was still sufficient contaminating copper to stimulate ATPase activity under these conditions or whether the enzyme was partially uncoupled (artifactually or intrinsically) remains to be assessed.

The formation of an acylphosphate intermediate is the hallmark of P-type ATPases. Since CopB and other heavy metal ATPases form a separate evolutionary branch and have a structure quite distinct from that of non-heavy metal ATPases, it was of interest to examine phosphoenzyme formation. CopB could be labeled with [γ-^{32}P]ATP in proteoliposomes (Fig. 3). The reaction had reached steady state after 10 s and the label could be chased with cold ATP, indicating turnover of the phosphoenzyme intermediate. The enzyme phosphate intermediate was sensitive to base and hydroxylamine, which are considered to be reliable criteria for the presence of an acylphosphate bond [31]. Thus, CopB shares the underlying reaction step of phosphoenzyme formation with the non-heavy metal ATPases. Vanadate inhibition of this reaction exhibited the same I_{50}, as had been observed for ATP hydrolysis (data not shown). This contrasts with the high vanadate sensitivity of non-heavy metal ATPases, which are inhibited by 10–100-fold lower vanadate concentrations.

CadA, the cadmium pumping ATPase of *Staphylococcus aureus*, is the only other heavy metal ATPase for which biochemical data are currently available. It confers cadmium resistance to bacteria by expelling Cd^{2+}. In contrast to CopB, phosphoenzyme formation by CadA was refractory to inhibition by up to 2 mM vanadate [32]. Whether this difference in vanadate sensitivity represents a fundamental mechanistic difference between heavy metal and non-heavy metal ATPases remains to be established. The similarity of CadA and CopB prompted us to look at the effect of cadmium on CopB. Cd^{2+} proved to inhibit strongly both ATP hydrolysis (P. Duda, in

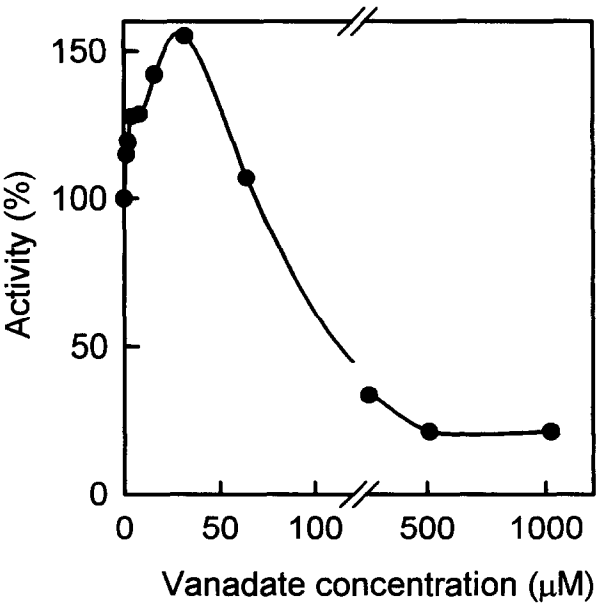

Fig. 2. Inhibition of the ATPase activity of CopB by Na_3VO_4. ATP hydrolysis by proteoliposomes was assayed as detailed under Section 2. 100% activity corresponded to 52 nmol/min per mg.

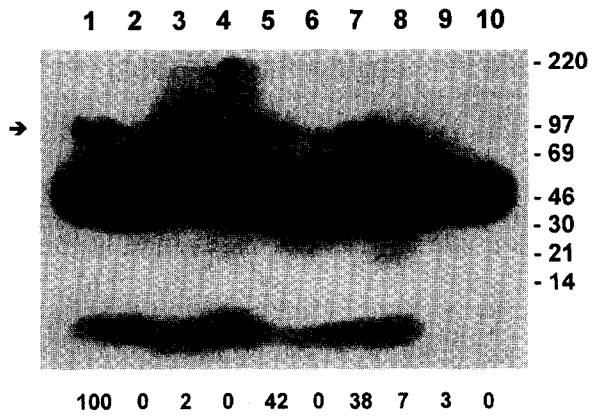

Fig. 3. Formation of an acylphosphate intermediate by reconstituted CopB ATPase. 1 μg of reconstituted CopB was phosphorylated with 1 μM (5 μCi) [γ-^{32}P]ATP in the presence of 5 μM $CuSO_4$ for 10 s, preceded or followed by the treatments indicated. Lanes 1–8 were pairwise experiments with and without chase. Lanes: 1, no treatment; 2, chase (chase was always labeling followed by 30 s incubation with 1 mM unlabeled ATP); 3, preincubation with 500 μM Na_3VO_4; 4, chase; 5, preincubation with 500 μM BCDS; 6, chase; 7, preincubation with 10 μM $CdSO_4$; 8, chase; 9, treatment with KOH; 10, treatment with NH_2OH. Numbers below the gel give the relative quantities of phosphoenzyme formed and the scale to the side indicates the relative mobilities of molecular mass markers in kDa. The arrow indicates the position of CopB; the blurred dark areas are due to free radiolabeled ATP and derivatives. Other details are described under Section 2.

preparation) and phosphoenzyme formation by CopB, thus clearly demonstrating the different nature of these two enzymes.

Analogous to the lack of stimulation of ATP hydrolysis by copper or silver ions, acylphosphate formation could not be activated by copper or silver ions. However, 0.5 mM of the copper chelator BCDS inhibited acylphosphate formation by CopB by 60%, as had been observed for ATP hydrolysis and although we could not demonstrate complete inhibition, this result would suggest that cuprous ions are required for phosphoenzyme formation.

Our procedures for the preparation of highly purified CopB and its functional reconstitution into proteoliposome will form the basis for further studies of the function of this copper pumping ATPase.

Acknowledgements: This work was supported by Grant 32-37527.93 of the Swiss National Foundation and by a Grant from the Roche Research Foundation. P.D. is recipient of a fellowship from the Helmut Horten Foundation.

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