Tetrathiomolybdate inhibition of the *Enterococcus hirae* CopB copper ATPase

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Abstract Tetrathiomolybdate (TTM) avidly interacts with copper and has recently been employed to reduce excess copper in patients with Wilson disease. We found that TTM inhibits the purified *Enterococcus hirae* CopB copper ATPase with an IC_{50} of 34 nM. Dithiomolybdate and trithiomolybdate, which commonly contaminate TTM, inhibited the copper ATPases with similar potency. Inhibition could be reversed by copper or silver, suggesting inhibition by substrate binding. These findings for the first time allowed an estimate of the high affinity of CopB for copper and silver. TTM is a new tool for the study of copper ATPases. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Tetrathiomolybdate; Copper ATPase; Inhibitor; Enzyme kinetics; *Enterococcus hirae*

1. Introduction

Copper is essential and serves as cofactor for more than 30 enzymes, many of which are of key importance, such as superoxide dismutase to combat radical formation, cytochrome *c* oxidase for cellular respiration, tyrosine oxidase in pigmentation, or lysyloxidase for connective tissue maturation [1]. Yet, a surplus of copper is toxic and leads to radical formation and oxidation of biomolecules. Therefore, copper homeostasis is a key requisite for every organism.

The Gram-positive bacterium *Enterococcus hirae* has a well-characterized copper homeostatic system. It is encoded by the *cop* operon, which is composed of a promoter/operator region followed by four genes that function in the control of cellular copper: a repressor, a copper chaperone, and two copper pumps [2–4]. The CopY repressor regulates the expression of all four *cop* genes by binding to the promoter/operator in a copper sensitive fashion [5] and the CopZ chaperone is involved in intracellular copper routing [6]. The two copper ATPases, CopA and CopB, belong to the CPx-type ATPases. This class of heavy metal ATPases is highly conserved and encountered from bacteria to humans [7]. CopA appears to serve in the uptake of copper under copper limiting conditions, and CopB in the extrusion of copper when it approaches toxic levels [8,9]. CopA and CopB share 35–40% sequence identity with the human Menkes and Wilson copper ATPases. Mutations in the genes encoding these ATPases can result in severe copper dysbalance. In the case of Menkes disease, this leads to a systemic copper deficiency. In Wilson, toxic copper overload results in liver cirrhosis and neurological symptoms. The human copper ATPases have so far not been purified. To investigate the molecular basis of copper transport and the effect of disease mutations, we have previously employed purified CopB from *E. hirae* as a model system [10].

Purified CopB is fully active without the addition of copper to the system. This suggested that the affinity of the enzyme for copper is very high and that sufficient copper is contaminating the water and the reagents to fully activate CopB. We tested a range of copper binding compounds in their ability to scavenge residual copper and to inhibit CopB. Tetrathiomolybdate (TTM) was thus found to be a potent inhibitor of CopB. Thiomolybdates have the ability to complex copper in mammals and to deplete their copper stores. This had first been recognized in ruminants grazing in areas with molybdenum-rich soil [11]. Molybdenum ingested by way of molybdenum-rich grasses appears to be transformed, together with sulfides from dietary sulfur compounds, to thiomolybdates by rumen bacteria [12]. The thiomolybdates subsequently complex ingested as well as stored copper, leading to copper deficiency. This observation has recently led to the use of TTM in humans to combat copper overload in Wilson disease [13]. TTM has also been reported to inhibit a variety of cupro-enzymes in vitro, including ceruloplasmin, cytochrome *c* oxidase, superoxide dismutase, and tyrosine oxidase [12].

We here show that di- tri-, and tetrathiomolybdate are potent inhibitors of the CopB copper ATPase. The reversibility of the inhibition by copper and silver ions points towards metal chelation as the inhibitory mechanism and the low IC_{50} of 34 nM suggests a very high affinity (low K_m) of CopB for copper and silver. Thiomolybdates may thus be a valuable new tool in the study of copper ATPases.

2. Materials and methods

2.1. Materials

ATP and ammonium tetrathiomolybdate were supplied by Sigma-Aldrich (St. Louis, MO, USA). Dodecyl-β-D-maltoside was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Growth media additives were supplied by Becton Dickinson (Franklin Lakes, NJ, USA). Chemicals that were not specified are from Merck (Darmstadt, Germany) or from Sigma-Aldrich, and were of analytical grade.

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Abbreviations: BCA, bicinchoninic acid; BCS, bathocuproindisulfonate; DTT, dithiothreitol; TTM, tetrathiomolybdate

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2.2. Purification of thiomolybdates

Commercial TTM was separated by Sephadex column chromatography into di-, tri- and tetrathiomolybdate, essentially as described by Kelleher and Mason [14]. In brief, 2 ml of 0.15 M ammonium tetrathiomolybdate in 20 mM Tris–SO_4, pH 7.5, was passed through a 2×15 cm Sephadex G-25 column (Amersham Pharmacia Biotech Europe, Freiburg, Germany). Fractions having the characteristic spectra of di-, tri-, and tetrathiomolybdate were collected and stored frozen at −20°C. They were used for experiments within 40 days of column purification. Published extinction coefficients were used to calculate the concentrations [15].

2.3. Purification of CopB

Y1 cells overexpressing CopB [4] were grown in 16 l of N-media in a fermenter to an OD_{546} of 0.5–0.7, induced with 0.1 mM of *o*-phenanthroline for 1 h and collected by centrifugation for 10 min at 8000×*g*. The subsequent steps for the preparation of membranes from *E. hirae* were carried out as previously described [16]. Batches of 1 g of membrane protein were extracted with 1 g of dodecyl-β-D-maltoside in 90 ml buffer G (20 mM Tris–SO_4, pH 7.5, 5 mM $MgSO_4$, 25 mM Na_2SO_4, 1 mM β-mercaptoethanol, 1 μM $CuSO_4$, 20% (v/v) glycerol), supplemented with 1/50 volume of a protease inhibitor cocktail [16] on ice for 90 min with constant stirring. The suspension was centrifuged at 90 000×*g* for 45 min and the supernatant loaded on a 2×11 cm Ni-NTA column (Qiagen AG, Basel, Switzerland), followed by elution with a 32 ml linear 0–100 mM imidazole gradient in G buffer without Na_2SO_4 and $CuSO_4$, but containing 0.05% (w/v) of dodecyl-β-D-maltoside. Purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [17] followed by staining with Coomassie blue. Enzyme assays were performed with CopB fractions of greater than 90% purity. Protein concentrations were determined by the method of Bradford [18], using bovine serum albumin as standard.

2.4. ATPase assays

Purified CopB (5 μg) was preincubated in 200 μl of assay buffer (20 mM Tris–SO_4, 5 mM $MgS0_4$, 1 mM ascorbic acid, 0.05% dodecyl-β-D-maltoside, pH 7.5) and thiomolybdates or other complexing agents added as indicated for 5 min at 37°C. The reactions were started by the addition of 1 mM Na-ATP. After 10 min, 100 μl of assay mix were transferred to tubes containing 10 μl of 0.5 M Na-EDTA to stop the reaction. Released phosphate was subsequently determined with a colorimetric assay [19]. Metal ion reversion assays were performed in 0.1 μM TTM as described above, except that increasing amounts of $CuSO_4$ or $AgSO_4$ (0.3–100 μM) were included in the preincubation. To test the reversibility of inhibition by dilution, 25 μl of purified CopB (150 μg) containing 1 μl of 2.5 μM di-, tri-, or tetrathiomolybdate were preincubated for 5 min at 37°C, followed by a 100-fold dilution into assay buffer. Activity measurements were started with 1 mM ATP and phosphate release assessed after 45 min as described above.

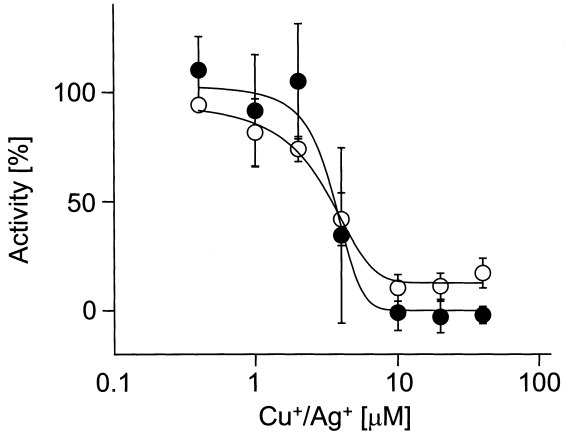

Fig. 1. Inhibition of CopB by copper and silver ions. Copper (○) and silver (●) were added to the assay as indicated in the figure. The activity of CopB was measured as described in Section 2 and is shown as the mean ± standard deviation of the relative activity of three experiments.

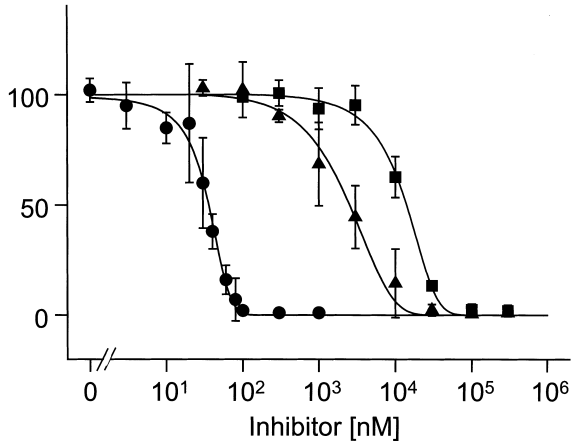

Fig. 2. Inhibition of CopB by copper chelators. CopB was preincubated with TTM (●), DTT (▲), or BCA (■), followed by activity measurements as described in Section 2. The relative activity of CopB is shown as the mean ± standard deviation of three experiments.

3. Results and discussion

3.1. Inhibition of CopB by copper and silver

It is a fundamental concept of ATP driven ion pumps that ATP hydrolysis and ion transport are coupled processes. Thus, no ATP hydrolysis by CopB should be observable in the absence of copper. Using purified CopB, a V_{max} of 1.3 μmol/min/mg and a K_m for ATP of 0.5 mM had previously been determined for ATP hydrolysis, but no stimulation by copper could be observed [10]. To the contrary and as shown here, copper and silver inhibited ATP hydrolysis (Fig. 1). The lack of stimulation was ascribed to a very high affinity of CopB for copper in conjunction with significant contaminating copper in the assay system. By graphite-furnace atomic absorption measurements we determined a mean concentration of 0.1 μM contaminating copper in the complete assay system. At metal ion concentrations above 3 μM, substrate inhibition ensued. While these metal ion concentrations can only be taken as relative values due to the presence of different complexing agents (β-mercaptoethanol, Tris, ATP), the results suggested that the affinity of CopB for metal ions is very high.

3.2. Inhibition of CopB by copper scavengers

To deplete the assay system of copper, we employed the common copper(I) complexing agents bicinchoninic acid (BCA) and bathocuproindisulfonate (BCS). These chelators inhibited CopB activity with similar potency (Fig. 2). The observed IC_{50} values were 14 μM for BCA, and 4 μM for BCS. Dithiothreitol (DTT) which forms copperthiolates similarly exhibited an IC_{50} of 3 μM. These IC_{50} values are clearly in excess of the contaminating copper level of 0.1 μM and these copper scavengers were thus not able to compete efficiently with CopB for copper.

TTM (MoS_4^{2-}) is a potent copper binding agent which is known to remove copper from metallothionein in vivo [20] and which inhibits several cuproenzymes in vitro [14,21–23]. However, TTM is usually contaminated by the oxidation products trithiomolybdate ($MoOS_3^{2-}$) and dithiomolybdate ($MoO_2S_2^{2-}$), as well as by uncharacterized contaminants [14]. Using chromatographic means (see Section 2) we purified di-,

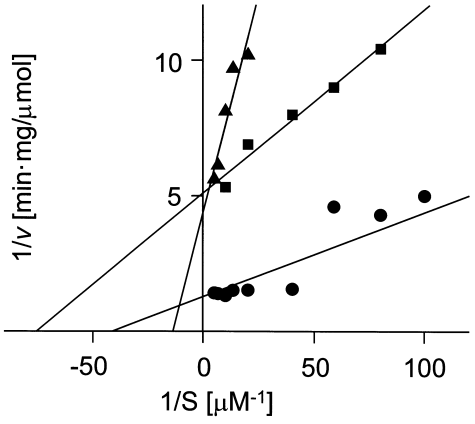

Fig. 3. Effect of ATP concentration on TTM inhibition. Lineweaver–Burk plot of the rate of ATP hydrolysis (v) by CopB at varying ATP concentrations (S) and in the presence of 0 (●), 34 nM (■), and 40 nM TTM (▲). The copper concentration was 0.1 μM. Other details were as described in Section 2.

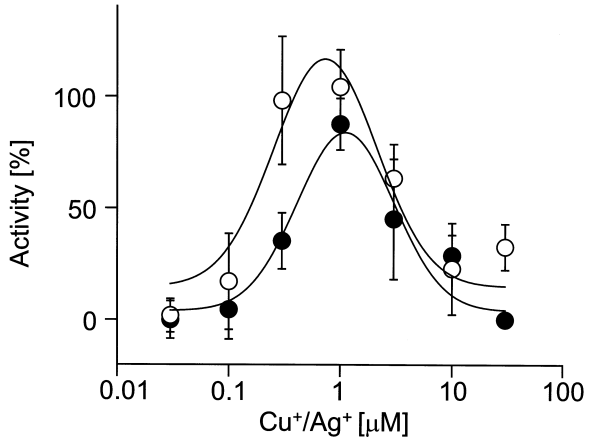

Fig. 4. Reversion of TTM inhibition by copper and silver. CopB in the presence of 0.1 μM TTM, resulting in >95% inhibition, was incubated with increasing concentration of copper (○) and silver (●). The relative activity of CopB was measured as described in Section 2 and is shown as the mean ± standard deviation of three experiments.

tri- and tetrathiomolybdate, characterized by their typical colors (yellow, orange and red, respectively) and their distinct spectra. The contamination of the commercial ammonium tetrathiomolybdate preparation that we used with di- and trithiomolybdate was 0.3 and 1.6%, respectively. In addition, it contained a brown, insoluble fraction that remained on the top of the column.

Purified TTM inhibited CopB with an IC_{50} of 34 nM (Fig. 2). Di- and trithiomolybdate were as potent as TTM (not shown). The stoichiometry of copper–TTM complexes has previously been reported to be one [20]. Assuming that TTM inhibits CopB by sequestering copper, an upper limit of the K_m of CopB for copper of 66 nM can be approximated (100 nM contaminating copper−34 nM TTM-bound copper = 66 nM residual copper; assuming negligible dissociation of copper from Cu–TTM). The true K_m is most likely even lower, since part of the contaminating copper will be tightly bound to other components of the assay system. In yeast, it has been estimated that there is less than one free copper ion per cell [24]. It appears likely that a similar situation prevails in bacteria. Since CopB secretes copper from the cell, it must be endowed with a very high affinity for copper and a K_m for copper of or below 66 nM appears apt. It should be noted that due to the acid sensitivity of the molybdates, all measurements were conducted at pH 7.5 rather than at the pH optimum of CopB of pH 5.5 [10].

3.3. Kinetic analysis of thiomolybdate inhibition

Kinetic analysis of the inhibition by TTM exhibited mixed uncompetitive/non-competitive kinetics (Fig. 3). This nature of the inhibition clearly argues against an interaction of TTM with the ATP binding site, which would result in inhibition that is competitive with ATP. In all likelihood, TTM inhibition arises from complexation of copper, either in solution or by formation of tertiary TTM–Cu–CopB complexes. The formation of such complexes is suggested by the capability of TTM to extract tightly bound copper from proteins and has been demonstrated for metallothionein [20]. To fully understand the mode of TTM inhibition would require a more thorough kinetic analysis which is beyond the realm of this work.

3.4. Reversion of thiomolybdate inhibition by dilution

The reversibility of the inhibition of CopB by thiomolybdates was analyzed by dilution experiments. CopB was essentially fully inhibited by 100 nM di-, tri-, or tetrathiomolybdate. This inhibition could be reversed on average to 50% by lowering the inhibitor concentration to 1 nM by a 100-fold dilution (Table 1). In comparison, thiomolybdates directly added at 1 nM only caused an approximately 15% inhibition. Thus, the inhibition of CopB by thiomolybdates is at least partially reversible. Bovine and ovine ceruloplasmin oxidase inhibited by TTM could be fully reactivated by removal of the drug [14,22]. Full reversibility of the CopB inhibition by thiomolybdates may be compromised by chemical interaction of CopB with the inhibitors or by their partial oxidation and/or aggregation in our in vitro assay system substrate between the enzyme binding site of CopB and TTM. If the commercial TTM preparation was used without purification, it inhibited with similar potency, but the inhibition was largely irreversible, probably due to aggregation phenomena with the insoluble fraction in the TTM preparation.

Table 1
Inhibition of CopB by thiomolybdates and reactivation by dilution

Inhibitor	% ATPase activity[a]		
	100 nM added	re-diluted to 1 nM[b]	1 nM added
Tetrathiomolybdate	<5	41 ± 4	81 ± 12
Trithiomolybdate	<5	52 ± 7	85 ± 6
Dithiomolybdate	<5	43 ± 17	86 ± 7

[a]The data are the mean ± standard deviation of three experiments, expressed relative to an untreated control.
[b]CopB was preincubated with 100 nM thiomolybdate, followed by 100-fold dilution with assay buffer.

3.5. Reversion of thiomolybdate inhibition by copper and silver

If the inhibition of CopB by thiomolybdates is indeed due to the complexation of copper required for enzyme turn-over, it should be reversible by added copper or silver ions. As shown in Fig. 4, increasing amounts of copper or silver ions reversed the inhibition by TTM. Complete reversal of inhibition was observed with a three-fold molar excess of copper and a 10-fold molar excess of silver ions over inhibitor. However, high copper and silver concentrations themselves inhibited the ATPase, as shown in Fig. 1, accounting for the drop in ATPase activity at high metal ion concentrations. It thus appears that copper–thiomolybdate complexes are not inhibitory.

Taken together, TTM inhibition has allowed us to assign to the CopB copper ATPase of *E. hirae* a maximal value of the K_m for copper of 66 nM. In addition, TTM represents a novel, potent inhibitor of CopB and may be of wider use as a new research tool in the investigation of copper ATPases. Our findings are also of interest in the realm of TTM being evaluated as a novel drug in the treatment of Wilson patients with neurological symptoms [13]. If TTM is over-dosed and thus not entirely complexed by copper, it could possibly inhibit any residual activity of mutant Wilson ATPase or the Menkes copper ATPase, ATP7A, in Wilson patients and introduce a therapeutic risk factor.

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