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Characterization of a P-type Copper-Stimulated ATPase from Mouse Liver

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Abstract. Mouse liver microsomes treated with octylthioglucoside (OTG-microsomes) were examined for copper-stimulated ATPase activity. The activity was about 1 μmol Pi/mg protein/hr under optimal conditions [300 mM KCl, 3 mM $MgSO_4$, 10 mM GSH, 0.5 μM $CuSO_4$, 3 mM ATP and 50 mM acetate buffer at pH5.0]. A reducing agent such as GSH or dithiothreitol was required for the activity, and removal of Cu^+ from the reaction mixture by bathocuporinedisulfonate resulted in a complete loss of copper-stimulated ATPase activity. Vanadate inhibited the copper-stimulated ATPase activity. The OTG-microsomes were phosphorylated in a hydroxylamine-sensitive and copper-stimulated way. Iron used instead of copper also stimulated both ATPase and phosphorylation. These results suggest that microsomes from mouse liver contain copper/iron-stimulated P-type ATPase.

Key words: Copper — P-type ATPase — ATPase activity — Acylphosphate formation — Iron — Liver

Introduction

Copper is required as a cofactor for many oxidative enzymes, but is generally toxic in excess. Menkes and Wilson diseases are human inherited disorders caused by genetic defects in copper metabolism. The cDNAs responsible for Menkes and Wilson diseases have been cloned and identified as putative copper transporting P-type ATPases (for review, *see* Bull & Cox, 1994; Vulpe & Packman, 1995; Solioz & Vulpe, 1996; Harris & Gitlin, 1996). While indirect evidence for their function in copper transport has been shown (Sambongi et al., 1997; Hung et al., 1997; Zhou & Gitschier, 1997; Lutsenko et al., 1997; DiDonato et al., 1997), reports on direct measurements of ATPase activity and acylphosphate formation are limited (Usta et al., 1997; Solioz & Camakaris, 1997).

In this study copper-stimulated ATPase was identified in OTG-treated mouse liver microsomes; the enzyme hydrolyzed ATP at acidic pH in a vanadate-sensitive way. The microsomes were also shown to be phosphorylated in a hydroxylamine-sensitive way. These results suggest the presence of a P-type copper-stimulated ATPase in mouse liver.

Materials and Methods

Materials

Bathocuporinedisulfonate, HEPES and OTG were obtained from Dojindo Laboratories (Kumamoto, Japan). Malachite Green was from Sigma Chemical.

Membrane Preparations

The mice (ddy or El strain) were maintained on standard laboratory food, anesthetized and killed by decapitation. Livers were removed and were homogenized with Dounce homogenizer (loose fitting) in 0.25 M sucrose containing 1 mM EGTA and 5 mM HEPES at pH7.4. After centrifugation for 20 min at 7,600 × *g* twice, the microsomal fractions were recovered from the supernatant by centrifugation for 60 min at 10,5000 × *g*. The resulting pellets were suspended in 10 mM HEPES at pH7.4. The activities of glucose-6-phosphatase, 5′-nucleotidase and acid phosphatase in the microsomes were 100–150, 15–20 and 10–15 nmol Pi/mg/min at 37°C, respectively. To obtain OTG-microsomes, the microsomes (3 mg/ml) were treated with 1% OTG in 10 mM HEPES buffer at pH7.4 for 15 min at room temperature and were centrifuged for 90 min at 10,5000 × *g*. The pellets were suspended in 10 mM HEPES buffer at pH7.4.

ATPase Assay

Unless otherwise stated, ATPase activity was measured in triplicate at 37°C in 50 mM acetate buffer, pH5.0 (100 μl) containing 300 mM KCl,

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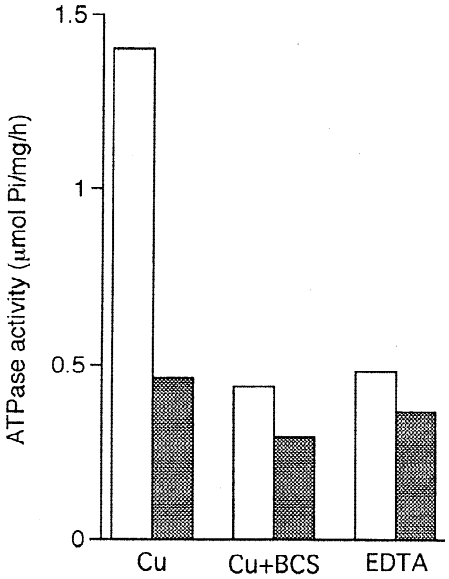

Fig. 1. Copper-stimulated ATPase activity in OTG-microsomes. The ATPase activity was assayed at 37°C in the presence of 0.5 μM $CuSO_4$, 0.5 μM $CuSO_4$ plus 0.5 mM bathocuporinedisulfonate or 1 mM EDTA either without (closed bar) or with (Open bar) 10 mM GSH. EDTA was added before adding GSH. The reaction was started by adding ATP and was terminated with 5% perchloric acid. The inorganic phosphate released was measured colorimetrically as described in (Lanzetta et al., 1979).

3 mM $MgSO_4$, 10 mM dithiothreitol or reduced glutathione (GSH), and either 0.5 μM $CuSO_4$ or 0.5 mM bathocuporinedisulfonate. Following incubation at 37°C for 15 min, the reaction was started by adding 3 mM ATP and was stopped by adding 5% perchloric acid. The amount of inorganic phosphate released was measured colorimetrically (Lanzetta et al., 1979). The copper-stimulated ATPase activity was estimated by subtracting the activity with bathocuporinedisulfonate from that with $CuSO_4$. When iron-stimulated ATPase was assayed, $FeCl_3$ was added to replace $CuSO_4$.

Acylphosphate Formation

OTG-microsomes (1 mg protein) were phosphorylated at 0°C in the reaction mixture (0.5 ml) used for the ATPase assay. The reaction was started by adding 40 μM of [γ-^{32P}] ATP (2×10^3 Bq/nmol, Amersham) and terminated by adding 10% perchloric acid containing 0.1 M phosphoric acid and 10 mM ATP. After centrifuging at 10,000 × *g* for 5 min, the pellet was washed twice with 5% perchloric acid containing 0.1 M phosphoric acid and subsequently once with water. The final pellet was suspended in water and its radioactivity was counted.

Results and Discussion

ATPase Activity

A convenient way of detecting functional P-type ATPase is to assay ATPase activity. However, mammalian copper-stimulated ATPase activity has not been reported, except for a preliminary report (Usta et al., 1997).

We detected copper-stimulated ATPase activity in mouse liver microsomes treated with 1% OTG. As shown in Fig. 1, copper-stimulated ATPase activity was detectable in the presence of GSH. No obvious copper-stimulated activity could be detected without GSH. The GSH can be replaced with dithiothreitol or dithioerythritol. Removal of Cu^+ with bathocuporinedisulfonate completely eliminated the copper-stimulated activity, and the residual activity was nearly the same level as the activity without GSH. EDTA also eliminated the copper-stimulated ATPase activity when added to the reaction mixture before adding GSH or dithiothreitol. These results indicate that the copper-stimulated ATPase is dependent on Cu^+.

The ATPase activity was increased with increasing salt concentrations of KCl and NaCl, while $NaNO_3$ and NaBr reduced the activity at higher concentrations (from about 240 mM) (Fig. 2). These results suggest that a high ionic strength is favorable for copper-stimulated ATPase activity. In this study we routinely included 300 mM KCl to the reaction mixture.

The optimum pH of the copper-stimulated ATPase activity was about at 5.0 (Fig. 3). Copper-stimulated ATPase activity was hardly detectable at neutral pH. In contrast, Usta et al. (1997) detected similar activity at neutral pH. Judging from the copper concentration dependency (*see below*) together with the pH dependency, our activity may be different from theirs.

The activity was very sensitive to copper concentration and the maximal activity was observed around 1 μM (Fig. 4).

Vanadate, a specific inhibitor of P-type ATPases, inhibited the ATPase activity (Table). Bafilomycin, an inhibitor of V-type ATPase, and ouabain had no effect on copper-stimulated ATPase activity.

It was of interest to determine whether copper can be replaced by other heavy metal ions. Among ions tested at 1 μM, iron stimulated ATPase (Fig. 5). When iron concentration was elevated to 100 μM, the activity reached about 5 μmol Pi/mg/hr and was higher than that of copper-stimulated ATPase activity observed under optimal conditions (~1 μmol Pi/mg/hr). The iron-stimulated ATPase activity was linear with time and required a reducing agent (Fig. 6). Whether copper- and iron-stimulated ATPase activities come from the same enzyme remains unknown.

Acylphosphate Formation

Another characteristic property of P-type ATPase is the intermediate formation of acrylphosphate sensitive to hydroxylamine (Post & Kume, 1973). Acylphosphate formation was assayed by the standard procedure described

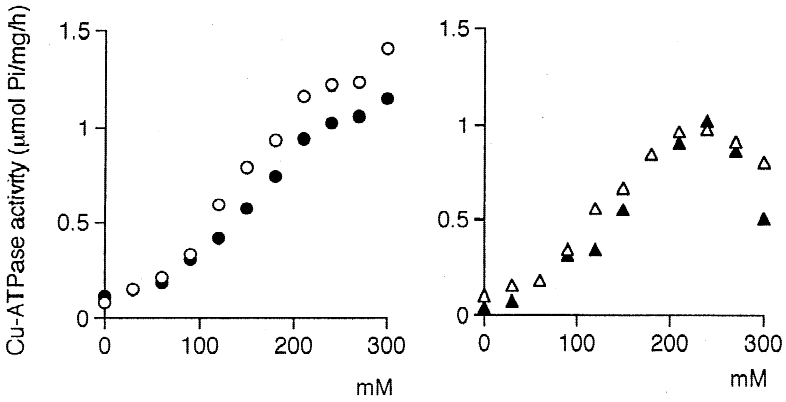

Fig. 2. Effect of salts on copper-stimulated ATPase activity. Copper-stimulated ATPase activity was assayed as described in Materials and Methods, except that the kind (○; KCl, ●; NaCl, △; $NaNO_3$, ▲; NaBr) and concentrations of the salt were varied. The activity was measured with or without 1 mM EDTA at each salt concentration and was plotted after subtracting the activity with EDTA from the activity without EDTA.

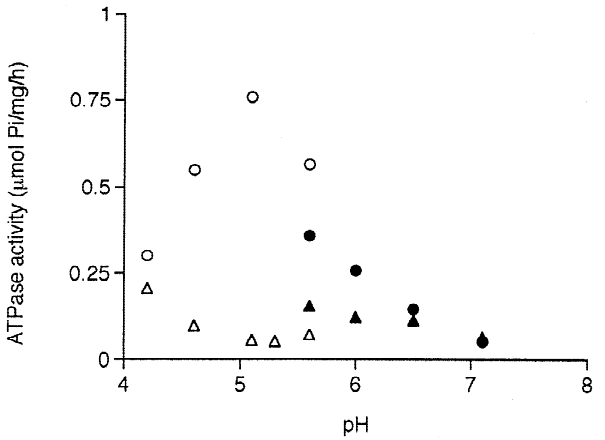

Fig. 3. Optimal pH of copper-stimulated ATPase activity. The ATPase activity was assayed by changing the pH with acetate buffer (○, △) and Mes buffer (●, ▲) either in the presence (○, ●) or absence (△, ▲) of 300 mM KCl.

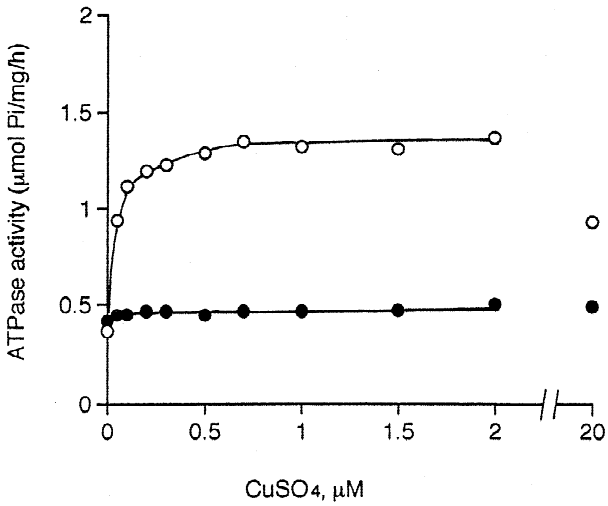

Fig. 4. Copper concentration dependency. The ATPase activity was assayed as described in Materials and Methods, except that the concentration of copper was varied. The concentrations of copper were corrected by atomic spectroscopic assay for copper in the reaction mixtures. The absence of copper was established by adding 1 mM EDTA. The activity was determined in the presence (○) or absence (●) of 300 mM KCl.

Table. Effects of ATPase inhibitors on copper-stimulated ATPase

	Cu-ATPase μmolPi/mg/hr	(%)
Control	0.62	100
NaSCN (10 mM)	0.87	140
Bafilomycin (10 μM)	0.71	114
Ouabain (1 mM)	0.61	98
Vanadate (10 μM)	−0.03	−5

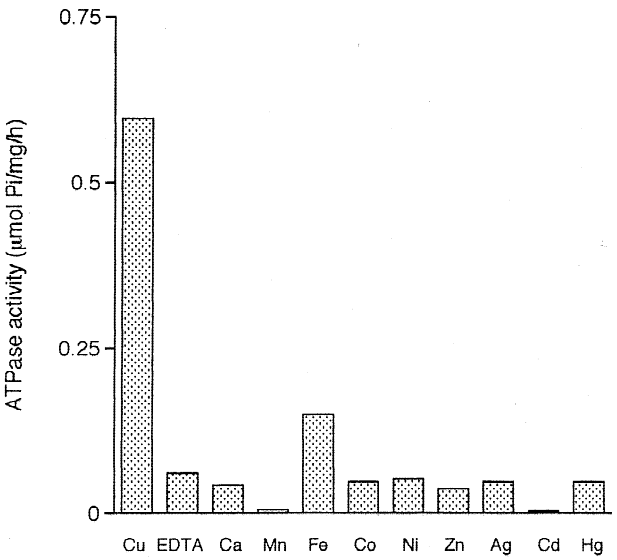

Fig. 5. Effect of metal ions on ATPase activity. ATPase activities in the presence of copper and EDTA were measured as described in the legend for Fig. 1. When copper was replaced with other metal ions, 0.5 mM bathocuporinedisulfonate was added to the reaction mixtures to remove contaminated copper. In this figure metal ion concentrations, including copper, were 1 μM.

in Materials and Methods. Copper-stimulated phosphorylation of OTG-microsome was 0.34 ± 0.15 pmol/mg protein ($n = 3$) and the phosphorylated intermediate was nearly completely destroyed with hydroxylamine, indi-

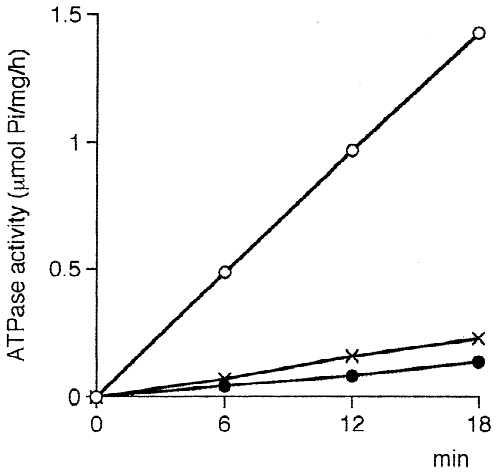

Fig. 6. Time course of iron-stimulated ATPase activity. ATPase activity was measured in a reaction mixture containing 300 mM KCl, 3 mM $MgSO_4$, 3 mM ATP, 0.5 mM bathocuporinedisulfonate and 50 mM acetate buffer at pH5.0 in the presence of 100 μM $FeCl_3$ (○, X) or 1 mM EDTA (●) with (○, ●) or without 10 mM GSH (X). When GSH was added, the reaction mixtures stood for several hours at room temperature before the start of the reaction by adding ATP.

cating copper-stimulated acylphosphate formation by OTG-microsomes. For iron (100 μM)-stimulated phosphorylation, 2.4 ± 0.73 pmol/mg protein ($n = 3$) was obtained. The iron-stimulated phosphorylated intermediate was also hydroxylamine-sensitive.

These results, together with the vanadate sensitivity of copper/iron-stimulated ATPase, strongly suggest that the ATPase is the P-type. Whether the ATPase activity described in this paper is due to Wilson/Menkes ATPase remains to be solved.

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