Domain Organization and Movements in Heavy Metal Ion Pumps

PAPAIN DIGESTION OF CopA, A Cu\(^{2+}\)-TRANSPORTING ATPase\(^\text{a}\)\(^\text{b}\)

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Yuta Hatori\(^1\), Eiji Majima\(^2\), Takeo Tsuda\(^3\), and Chikashi Toyoshima\(^1\)

From the \(^1\)Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032 and \(^2\)ProteNova Co., Ltd., Naruto 772-0051, Japan

To study domain organization and movements in the reaction cycle of heavy metal ion pumps, CopA, a bacterial Cu\(^{2+}\)-ATPase from *Thermotoga maritima* was cloned, overexpressed, and purified, and then subjected to limited proteolysis using papain. Stable analogs of intermediate states were generated using AMPPCP as a nonhydrolyzable ATP analog and AlF\(_3\) as a phosphate analog, following conditions established for Ca\(^{2+}\)-ATPase (SERCA1). Characteristic digestion patterns obtained for different analog intermediates show that CopA undergoes domain rearrangements very similar to those of SERCA1. Digestion sites were identified on the loops connecting the A-domain and the transmembrane helices M2 and M3 as well as on the N-terminal metal binding domain (NMBD) and the first transmembrane helix, Ma. These digestion sites were protected in the E1P-ADP and E2P analogs, whereas the M2–A-domain loop was cleaved specifically in the absence of ions to be transported, just as in SERCA1. ATPase activity was lost when the link between the NMBD and the transmembrane domain was cleaved, indicating that the NMBD plays a critical role in ATP hydrolysis in *T. maritima* CopA. The change in susceptibility of the loop between the NMBD and Ma helix provides evidence that the NMBD is associated to the A-domain and recruited into domain rearrangements and that the Ma helix is the counterpart of the M1 helix in SERCA1 and Mb and Mc are uniquely inserted before M2.

P-type ATPases translocate specific ions against their electrochemical gradient across membranes using free energy liberated by ATP hydrolysis. Based on the primary structures, the P-type ATPase family can be divided into five branches, which are referred to as type I–V (4). PIB-type, a subgroup of PI-type, and PII-type ATPases constitute the major part of the family. PIB-type ATPases transport heavy metal ions, such as Cu\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Pb\(^{2+}\), Cd\(^{2+}\), and Co\(^{2+}\) (5), whereas PII-type ATPases are specific to H\(^+\), Na\(^+\), K\(^+\), and Ca\(^{2+}\) (3, 4).

X-ray crystallography of Ca\(^{2+}\)-ATPase (SERCA1)\(^2\) has revealed how PII-type ATPases work by providing the atomic structures in 6 states (6–11) that cover nearly the entire reaction cycle. The ATPase consists of 3 cytoplasmic domains termed P (phosphorylation), N (nucleotide binding), and A (actuator) and 10 (M1–M10) transmembrane helices, some of which contain acidic residues that constitute high affinity binding sites for ions transported (6). With PIB-type ATPase, atomic structures have been determined for only cytoplasmic domains (12–14). It is now clear that PIB-type ATPases also comprise 3 cytoplasmic domains and their core structures are very similar to those of SERCA1 (13, 14), although there are significant differences in the regions seemingly important for domain interaction (14). In addition, PIB-ATPases are distinguished from other P-type ATPases by the presence of a N-terminal metal binding domain (NMBD), typically consisting of a variable (1 to 6) repeat of a CXxC motif (5). Also the transmembrane domain is clearly different. In contrast to PII-ATPases that have 10 transmembrane helices, PIB-ATPases contain only 8: the 4th to 8th bear similarity to M2 to M6 of the PII-type ATPases (2), whereas the 1st to 3rd are specific (referred to as Ma-Mc here).

Given these differences in structure, the ion-translocation mechanism might also be different. The ion-translocation cycle of PII-ATPases is conventionally explained by the E1/E2 theory (15–17). The transmembrane ion-binding sites have high affinity for transported ions in E1 and low affinity in E2; they face the cytoplasm in E1 and the lumen or extracellular medium in E2.

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To whom correspondence should be addressed. Tel.: 81-3-5841-8492; Fax: 81-3-5841-8491; E-mail: ct@iam.u-tokyo.ac.jp.

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Ion homeostasis is essential in all living organisms. Gradients for sodium and potassium across the cell membrane provide energy for action potentials. Calcium is the most commonly used ion for regulation of protein function. Zinc, ferrous, and copper ions, whereas they are toxic when present in excess, are indispensable cofactors for a variety of proteins, including enzymes involved in the respiratory chain (1). In maintaining the concentrations of these physiologically important ions, P-type ATPases play a principal role (2, 3).

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2 The abbreviations used are: SERCA, sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase; NMBD, N-terminal metal binding domain; DM, n-decyl-β-D-maltoside; PMSF, phenylmethylsulfonyl fluoride; BCS, bathocuproine disulfonate; AMPPCP, adenosine 5′-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DTT, di-thiothreitol; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
Papain Digestion of CopA

Transported ions are occluded in the transmembrane binding sites in a phosphorylated intermediate (E1P) and released into the lumen (or extracellular medium) during the transition to E2P. Thus, it is well established that the reaction cycle of a PII-type ATPase consists of at least four intermediate states, namely, E1P/E2 and their phosphorylated states (E1P/E2P), and is accompanied by large scale domain rearrangements (9, 18). In contrast, our understanding of the mechanism of PIB-ATPases is poor, and we do not know if they undergo similar domain rearrangements, or what role the NMBD provides. We have approached these issues by utilizing limited proteolysis, which has proved very successful with SERCA1 (19–24).

Cu⁺-ATPases are the most abundant members of PIB-ATPases (5) and play central roles in copper homeostasis in diverse organisms from bacteria to human, and their malfunctioning lead to abnormal copper ion levels in cells. For instance, human Cu⁺-ATPases, ATP7A and ATP7B, are implicated in Menkes and Wilson diseases, respectively (25, 26). In the present study, we have expressed and purified a CopA homolog from *Thermotoga maritima* (NCB accession number AE000512) (27), a hyper-thermophile. It is one of the simplest Cu⁺-ATPases, consisting of 726 amino acids with a single CXXC motif in its NMBD.

We performed limited digestions of CopA using various proteases and found papain the most useful. The results show that the cytoplasmic domains of CopA undergo rearrangements quite similar to those observed with SERCA1 and that the NMBD plays an important role in ATP hydrolysis, and participates in domain rearrangements during the reaction cycle.

**EXPERIMENTAL PROCEDURES**

**Subcloning the CopA Gene**—*T. maritima* CopA gene (TM0317) was amplified by PCR using genomic DNA as template and 5′-sense oligonucleotide primer containing a restriction site for Xhol (5′-CACCAGCTCGAGAATGAGCGAT-CAGAAAACG-3′, the restriction site is underlined) and 3′-reverse complement primer with EcoRI site (5′-TTCGAAT-TCTCAGTCTCCTCACC-3′). The PCR product was digested with Xhol and EcoRI, ligated into the vector pBAD/His (Invitrogen) cut with the same restriction enzymes. This vector introduces an N-terminal sequence extension MGGSHHHH-HHGMSMGTGGGQMRDLYDDDCKDPSR. This construct was transformed into *Escherichia coli* DH5α for DNA sequencing and Top10 (Invitrogen) for overexpression.

**Overexpression of CopA in *E. coli***—The transformed Top10 was grown in Luria Bertani medium at 37 °C until the optical density at 600 nm reached 0.5–0.7 and the production of the recombinant protein was then induced by the addition of 1-ara-ginose at a final concentration of 0.01% (w/v). The cells were harvested 6 h later by centrifugation (typically 2.5 g of wet weight per 1 liter of media), washed with a buffer containing 100 mM KCl and 25 mM Tris-HCl, pH 7.0, suspended in a buffer containing 1 mM MgCl₂, 0.5 mM EDTA, and 25 mM Tris-HCl, pH 7.0, and stored at −80 °C until needed.

**Protein Purification**—Frozen cell suspension was thawed, supplemented with 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and “Complete” protease inhibitor mixture (Roche Applied Science) and passed through a Microfluidizer M110-EH (Microfluidics International Corp., Newton, MA) three times at 120 MPa to disrupt cells and centrifuged at 8,000 × g for 30 min. The supernatant was centrifuged at 40,000 × g for 8 h, and the pellet was suspended in a buffer containing 0.5 mM NaCl, 20% (w/v) glycerol, and 50 mM MOPS-NaOH, pH 7.0, and stored at −80 °C.

The microsomal fraction thus obtained was diluted to a protein concentration of 10 mg/ml with the same buffer and incubated at 75 °C for 1 h. After adding 0.05% (w/v) n-decyl-β-D-maltoside (DM), the suspension was incubated at 4 °C for 30 min and centrifuged at 170,000 × g for 30 min. The pellet was suspended in a buffer containing 5 mM imidazole-HCl, 0.5 mM NaCl, 20% (w/v) glycerol, and 50 mM MOPS-NaOH, pH 7.0. DM was added to a final concentration of 1% (w/v), and the suspension was stirred at 4 °C for 1 h. The insoluble fraction was removed by centrifugation at 170,000 × g for 1 h. The DM extract (typically 25 ml) was then mixed with 10 ml of Co²⁺ metal affinity resin (Clontech), pre-equilibrated with a buffer containing 5 mM imidazole-HCl, 0.5 mM NaCl, 20% (w/v) glycerol, 0.1% (w/v) DM, and 50 mM MOPS-NaOH, pH 7.0, and gently stirred for 1 h at 4 °C. The resin was next packed in a column and washed successively with 100 ml of the same buffer. The bound protein was finally eluted with 50 ml of the same buffer including 300 mM imidazole, pH 7.0, and 0.1 mg/ml (w/v) *E. coli* total lipid (Avanti, Alabaster, AL). Fractions containing proteins were immediately supplemented with 2 mM DTT and concentrated by ultrafiltration with a 50,000 cut-off membrane (PALL, East Hills, NY). Imidazole was removed by dialysis against a buffer containing 50 mM NaCl, 20% (w/v) glycerol, and 50 mM MOPS-NaOH, pH 7.0. Purified CopA was frozen and stored in liquid nitrogen. Prior to any use, 2 mg/ml CopA was incubated in a solution supplemented with 2 mg/ml *E. coli* total lipid and 2 mM DTT for 1 h to activate Cu⁺-ATPase activity.

**ATPase Assay**—Activated CopA was diluted in a buffer (ATPase assay medium) containing 3.5 mM MgCl₂, 10 mM cysteine, 30% (w/v) glycerol, 0.5% (w/v) CHAPS, 2.5 mM ATP, 1 mM NaN₃, 20 μM CuCl₂, 0.5 mM DTT, and 50 mM MES-triethanolamine, pH 6.0 (60 °C), and allowed to hydrolyze ATP for 10 min, unless otherwise stated. The pH at 60 °C was calculated based on the measurement at room temperature and pKₐ°C conversion factor of 0.011 for MES (28). Cu⁺-independent ATPase activity was measured in the presence of 0.5 mM bathocuproine disulfonate (BCS) instead of CuCl₂, which was always subtracted to obtain the Cu⁺-dependent ATPase activity. ATP hydrolysis was terminated by lowering the temperature to 4 °C and the released Pi was determined according to Lanzetta et al. (29). V_max and K_m were calculated using the Eadie-Hofstee plot.

For examining effects of AlF₄⁻ on Cu⁺-ATPase activity, purified CopA (1 mg/ml or 6.33 μM) was preincubated in a buffer containing 1 mM MgCl₂, 1 mM AlCl₃, 2 mM potassium fluoride, 20% glycerol, 0.5 mM DTT, and 50 mM MOPS-NaOH, pH 7.0, at 30 °C for 1 h in the presence of various additives. They were 20 μM CuCl₂ (E1-Cu⁺), 20 μM CuCl₂ and 1 mM ADP (E1P-ADP), or 0.5 mM BCS (E2P). This mixture was diluted by ×20 with the ATPase assay medium. ATPase activity was measured for 5 min of reaction time.

**Proteolysis**—Purified CopA (0.5 mg/ml) was digested with 0.5 mg/ml papain (Worthington Biochemical Corp., Lakewood, New Jersey) at pH 7.0, and stored at −80 °C.
NJ) at 30 °C in a buffer containing 1 mM MgCl₂, 0.1% (w/v) DM, 1 mg/ml E. coli total lipid, 0.5 mM DTT, 10 mM cysteine, and 20% (w/v) glycerol, 50 mM MOPS-NaOH, pH 7.0, with additives specific to each intermediate. They were 0.5 mM BCS (likely to be a mixture of E1 and E2; abbreviated as E<typename>), 10 μM CuCl₂ (E₁-Cu⁺), 10 μM CuCl₂, 2.5 mM AMPPCP and 2.5 mM MgCl₂ (E₁-Cu⁺-AMPPCP), 10 μM CuCl₂, 0.1 mM AlCl₃, 2 mM potassium fluoride, and 1 mM ADP (E₁-Cu⁺-AlF₃-ADP) or 0.5 mM BCS, 0.1 mM AlCl₃, and 2 mM KF (E₂-AlF₃). Prior to digestion, CopA was reacted with these ligands by incubation at 30 °C for 1 h; papain was activated by incubation in a buffer containing 5 mM cysteine and 1 mM DTT at 25 °C in a buffer containing 5 mM cysteine and 50 mM MOPS-NaOH, pH 7.0. Proteolysis was terminated by adding 2 mM Edta. The activity of papain in the various conditions was measured with N-α- benzoyl-L-Arg-p-nitroanilide as the substrate and found to be virtually constant. For SDS-PAGE, 2.5% (w/v) trichloroacetic acid was added immediately and then the specimen was diluted three times with a modified Laemmli sample buffer containing 3% (w/v) SDS and 10 mM DTT. Twelve μl of each sample thus obtained was loaded onto a 10–20% gradient gel for SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R. For quantitative analyses, the densities of stained bands were measured using a luminescent image analyzer LAS-3000 (Fuji Photo Film, Tokyo, Japan).

SERCA1 was digested following previous reports (21, 22): 0.3 mg/ml sarcoplasmic reticulum vesicles were treated with 0.03 mg/ml proteinase K or 0.1 mg/ml papain at 25 °C for 1 h; papain was activated by incubation in a buffer containing 5 mM cysteine and 1 mM DTT and 50 mM MOPS-NaOH, pH 7.0. For quantitative analyses, the densities of stained bands were measured using a luminescent image analyzer LAS-3000 (Fuji Photo Film, Tokyo, Japan).

SERCA1 was digested following previous reports (21, 22): 0.3 mg/ml sarcoplasmic reticulum vesicles were treated with 0.03 mg/ml proteinase K or 0.1 mg/ml papain at 25 °C in a buffer containing 1 mM MgCl₂, 0.1 mM KCl, 10 mM cysteine, and 50 mM MOPS-NaOH, pH 7.0, in the presence of various additives specific to each intermediate. They were 0.4 mM EGTA and 0.5 mM CaCl₂ (E₁-2Ca²⁺); 3.5 mM CaCl₂, 2.5 mM MgCl₂, and 2.5 mM AMPPCP (E₁-2Ca²⁺-AMPPCP); 0.4 mM EGTA, 0.5 mM CaCl₂, 0.1 mM AlCl₃, 2 mM potassium fluoride, and 1 mM ADP (E₁-2Ca²⁺-AlF₃-ADP); 1 mM EGTA (E₂), or 1 mM EGTA, 0.1 mM AlCl₃, and 2 mM potassium fluoride (E₂-AlF₃). Sarcoplasmic reticulum vesicles were reacted with these ligands by incubation at 25 °C for 1 h and digestion was initiated by adding proteases. Two mM phenylmethylsulfonyl fluoride was used to terminate proteinase K digestion. All other manipulations were identical to those with CopA.

Mass Spectrometry—Intact CopA and its fragments obtained by papain digestion were analyzed by surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry in a Protein Biology System IIc (Ciphergen Biosystems, Fremont, CA). Samples (0.45 mg/ml protein, 0.5 μl) were applied directly onto each H₅₀ (hydrophobic surface) ProteinChip array spot and dried for 30 min at room temperature. Surfaces on the spot were washed twice with 5 μl of H₂O and dried. One μl of a saturated solution of sinapinic acid (Sigma) in 50% acetonitrile containing 0.5% trifluoroacetic acid was then added on each spot and allowed to air-dry. Mass spectra were collected with 120 laser shots of each spot.

Miscellaneous Methods—Genomic DNA was obtained from ATCC (Manassas, VA). Protein concentrations were determined using the bicinchoninic acid reagent with bovine serum albumin as standard. Sequences were aligned using Clustal W (30).

RESULTS
Expression and Purification of CopA—CopA from T. maritima, a hyper-thermophile, with hexahistidine tag attached to the N terminus was overexpressed in E. coli. To isolate the protein, the microsomal fraction was incubated at 75 °C followed by solubilization with DM. These treatments had no appreciable effect on Cu⁺-ATPase activity. Treatment with n-octyl β-D-glucopyranoside, C₁₂E₉ or N,N-dimethyldodecylamine-N-oxide activatedCopA. DM and n-dodecyl-β-D-maltopyranoside solubilized the ATPase at >90% and kept the ATPase activity unchanged for more than a week at 4 °C. The enzyme was subsequently purified by Cu⁺²-affinity column chromatography, typically at >90% purity (supplemental Fig. S2).

Cu⁺/Ag⁺-ATPase Activity—Cu⁺/Ag⁺-ATPase activity of purified CopA was first measured with various concentrations of cysteine present, because ATPase activities of PIB-ATPases are stimulated by thiolates (31–34). ATPase activity of CopA showed strong dependence on cysteine concentration with a maximum activity at 10 mM (Cu⁺-ATPase) or 5 mM (Ag⁺-ATPase) independent of the metal ion concentration (Fig. 1A). Cysteine of a higher concentration impaired the activities. The dependence of ATPase activity on metal ion concentration was therefore examined in the presence of 10 (Cu⁺) or 5 mM (Ag⁺) cysteine. Cu⁺-independent ATPase activity was measured in the presence of 0.5 mM BCS, a chelator of Cu⁺, and found to be 0.06 – 0.08 μmol min⁻¹ mg⁻¹. This number corresponds to about a half of the activity when no heavy metal is included (filled circles in Fig. 1A). It was always subtracted to obtain the Cu⁺-dependent ATPase activity. Ag⁺-ATPase activity was
examined in the presence of 0.5 mM BCS to eliminate the effects of contaminating copper ions. 

Cu²⁺ dependence of Cu³⁺-ATPase activity was well described by the Michaelis-Menten equation (Kₘ = 3.1 μM; Vₘₐₓ = 1.9 μmol min⁻¹ mg⁻¹) in the presence of 10 mM cysteine at 60 °C. This Vₘₐₓ value corresponds to a turnover rate of 2.6 s⁻¹, which is similar to that reported for A. fulgidus (2.0 s⁻¹; Ref. 35). In the absence of cysteine, the maximum activity of 0.81 μmol min⁻¹ mg⁻¹ was found at 3 mM CuCl₂; at higher concentrations, Cu³⁺-ATPase activity was reduced (Fig. 1B). CopA also showed Ag⁺-dependent ATPase activity with Kₘ and Vₘₐₓ of 2.1 μM, 0.90 μmol min⁻¹ mg⁻¹ (5 mM cysteine) and 0.4 μM, 0.38 μmol min⁻¹ mg⁻¹ (without cysteine), respectively. Higher concentrations of Ag⁺ did not inactivate the ATPase activity even in the absence of cysteine (Fig. 1C). Cu³⁺-ATPase activity of CopA reached the maximum at 60 °C and was 4.4% of the maximum at 30 °C (Fig. 3D). Data at various temperatures from 30 to 60 °C fell on a straight line in the Arrhenius plot (Eₗₐₜ = 21.3 kcal mol⁻¹; inset in Fig. 1D), indicating that the ATP hydrolysis at 30 °C is the same reaction as that at 60 °C. Kinetic properties of the ATPase activity of CopA expressed and purified without His tag were the same, although the purity of the preparation was somewhat inferior (data not shown).

Formation of Stable Fluoroaluminate Complexes of CopA—A key to characterize an enzyme is to find a way to stabilize it in intermediate states. With the members of haloacid dehalogenase superfamily, including P-type ATPases, AlFx is one of the most useful phosphate analogs (36). In fact, AlFx can fix SERCA1 in states analogous to P, P-E₁, and P-E₁-E₂ states (37). We therefore examined Cu³⁺-ATPase activity of CopA to see if AlFx treatment produces a stable complex.

As shown in Table 1, the ATPase activity was almost completely suppressed by incubating CopA (1 mg/ml or 6.33 μM) for 1 h in a solution containing 1 mM MgCl₂, 0.1 mM AlCl₃, 2 mM potassium fluoride in the presence of either 20 μM Cu³⁺, 1 mM ADP, and 20 mM Cu⁺, or 0.5 mM BCS. Under these conditions, CopA is expected to form E₁-Cu³⁺-AlFx, E₁-Cu³⁺-AlFx₂-ADP, and E2-AlFx complexes, respectively, which mimic the E₁ ~ P, E₁ ~ P-ADP, and E₂ ~ P-ADP states. When Mg²⁺ is omitted in forming the E₁-Cu³⁺-AlFx₂-ADP complex, ATPase activity decreased only slightly (~15%), suggesting that the formation of the stable enzyme-AlFx complex requires Mg²⁺. Cu³⁺-independent ATPase activity was not affected by AlFx treatment.

The time course of inhibition by AlFx treatment was measured to estimate the proportion of the complexed ATPase AlFx at any given time. The decay of Cu³⁺-ATPase activity fitted well to first-order kinetics with the rate constants listed in Table 1 ("inhibition"). According to these parameters, for example, more than 99.9% of CopA is expected to form the E₂-AlFx complex in 1 h.

ATPase activity was examined by measuring the amount of liberated phosphate. This requires dilution of the aliquot (usually 20 times) and some reaction time in a buffer devoid of ligands. A problem could arise if the ligands dissociate during the reaction time. In fact, AlFx-treated CopA transferred into the ATPase assay medium gradually regained Cu³⁺-ATPase activity with the rate constants listed in Table 1 ("restoration"). A reaction time of 5 min appeared to be appropriate, as 92% of ATPases would remain complexed and inactive at 5 min after dilution. The Cu³⁺-ATPase activity listed in Table 1 represents, therefore, an average over the reaction time of 5 min.

**Papain Digestion of CopA**

| Conditions | Cu³⁺-ATPase activity | Rate constant |
|------------|----------------------|--------------|
|            | % of untreated CopA  | Inhibition²  |
| 20 μM Cu³⁺, 0.1 mM AlFx, 1 mM MgCl₂ | 4 | 0.22 |
| 20 μM Cu³⁺, 1 mM ADP, 0.1 mM AlFx, 1 mM MgCl₂ | 3 | 0.19 |
| 0.5 mM BCS, 0.1 mM AlFx, 1 mM MgCl₂ | 5 | 0.16 |
| 20 μM Cu³⁺, 1 mM ADP, 1 mM MgCl₂ | 100 | 0.02 |
| 20 μM Cu³⁺, 1 mM ADP, 0.1 mM AlFx | 84 | 0.02 |

*⁴ Untreated CopA has an ATPase activity of 1.88 μmol min⁻¹ mg⁻¹.
*⁵ First-order kinetics was assumed.
*⁶ Inhibition by AlFx treatment.
*⁷ Restoration by dilution of ×20 with the assay medium containing no AlFx.

| Protease | Relative rate constant (%) | E₂ | E₁-2Ca²⁺ | E₁-2Ca²⁺-AMPPCP | E₁-2Ca²⁺-AlFx₂-ADP | E₁-2Ca²⁺-AlFx | E₂-AlFx |
|----------|---------------------------|----|----------|-----------------|---------------------|----------------|---------|
| Proteinase K | 100 | 77 | 13 | 11 | 19 | 6 |
| papain | 100 | 109 | 11 | 13 | 38 | 8 |

*⁸ The rate constants are represented in percentages to those obtained in E₂, which are 1.50 and 1.56 h⁻¹ for proteinase K and papain, respectively.

**TABLE 2**

Papain and proteinase K digests of SERCA1 in various states
domain–M3 helix loop close to the proteinase K site, Thr242 (20). The molecular mass determined by SELDI-TOF mass spectrometry indicated that the 83-kDa fragment retains an intact C terminus. Edman degradation failed with the 26-kDa fragment. As the N terminus of SERCA1 is blocked (38, 39), the 26-kDa fragment is likely to be in an equilibrium between E1 and E2. Proteinase K cleaves SERCA1 at Leu119 in E2 and Ala746 in E1 (20). It is known that, in addition to proteinase K but was more specific to the A-domain–M3 loop. Papain yielded several specific fragments of CopA stable for more than 3 h (Fig. 2), which we designated as p79, p70, and p44, according to their molecular mass. Partial sequencing of the N-terminal end (Table 3) showed that p79 was a 1:1 mixture of polypeptides having Glu15 or Glu18 at the N terminus. The N termini of p70 and p44 were Arg91 and Ser328, respectively. Mass spectrometry showed that these fragments retain the intact C terminus of the full-length polypeptide. Fig. 4 shows the locations of these cleavage sites and the positions of the fragments in the primary structure. Met1 is located near the N terminus, Glu40 on the NMBD-Ma helix loop and Gly327 on the A-domain–M3 loop. Gly327 will be the digestion site in CopA, corresponding to Ala240 (papain) and Thr242 (proteinase K) in SERCA1.

The p79 fragment appeared quickly (rate constant 2.21 min−1) and slowly degraded into p70 and p44 (rate constant 0.01 min−1), indicating that the region near the N terminus is particularly susceptible to papain attack. There was no apparent order for the formation of p70 and p44 (Fig. 5A). Both of them were further degraded, producing low molecular mass fragments of ~10 kDa.

**Papain Digestion of CopA in Other States**—Purified CopA was digested with papain under various conditions in which CopA is expected to be stabilized in Eemp, E1-Cu+, E1-Cu+-AMPPCP, E1-Cu+-AlF4-ADP, and E2-AlF4- (20). Papain yielded several distinct polypeptides stable for more than 3 h and digestion patterns characteristic of the various intermediates (Fig. 3). Some fragments were specific, although most of them appeared identical to those observed in Eemp.

N-terminal sequencing and mass spectrometry confirmed that the 3 polypeptides with apparent masses of 79, 70, and 44 kDa were identical to those observed in Eemp. In E2-AlF4-, there was a specific polypeptide p54 with Ala225 at the N terminus. The corresponding cleavage site Gln224 is located on the M2 helix–A-domain loop and thought to be analogous to Leu119 of SERCA1, a proteinase K cleavage site. A transient accumulation of p35 was observed in E1-Cu+ and E1-Cu+-AMPPCP at 10 min of digestion (Fig. 3). Determination of the N-terminal residue of this fragment was unsuccessful. Nevertheless, given the total

**TABLE 3**

**Locations and masses of cleavage fragments of SERCA1 and CopA**

| Name of fragment | State | N-terminal residues (Edman degradation) | Theoretical mass* kDa | Experimental mass (SELDI-TOF) |
|------------------|-------|-----------------------------------------|-----------------------|-------------------------------|
| **SERCA1**       |       |                                         |                       |                               |
| Full-length      |       |                                         |                       |                               |
| p83              |       | E1-2Ca2+                                | 109                   |                               |
| p26              |       | E1-2Ca2+                                | 83.1                  |                               |
| **CopA**         |       |                                         |                       |                               |
| Full-length      |       |                                         |                       |                               |
| p79              | Eemp | 8EDRAA                                  | 83.4                  | 83.3                          |
|                  |       | 17EEIKK*                                | 78.6                  | 78.6                          |
| p70              | Eemp | 91KKRYE                                 | 69.6                  | 69.8                          |
| p44              | Eemp | 32RSKPI                                 | 43.4                  | 43.6                          |
| p79              | E1-Cu+AMPPCP                          | 8EDRAA                | 78.6                  | 78.5                          |
|                  |       | 17EEIKK*                                | 78.0                  |                               |
| p44              | E1-Cu+AMPPCP                          | 32RSKPI                | 43.4                  | 43.6                          |
| p79              | E2-AlF4-                              | 8EDRAA                | 78.6                  | 78.5                          |
| p54              | E2-AlF4-                              | 235AEKAR               | 54.4                  |                               |

* For the fragment keeping the C terminus of the full-length ATPase (except for p26 of SERCA1).
* Edman degradation failed presumably due to the blocking of the N terminus.
* Peptides with this sequence were detected in equal amounts to those shown in the column above.
* Minor peptide detected with the major one shown in the column above.
Papain Digestion of CopA

![Graph showing papain digestion of CopA in the five principal states.](image)

**FIGURE 3. Papain digestion of CopA in the five principal states.** SDS-PAGE was carried out using 10–20% polyacrylamide gradient gels and stained with Coomassie Brilliant Blue. 0.5 mg/ml purified CopA was digested for the indicated time with 0.5 mg/ml papain under conditions in which the ATPase was expected to be stabilized in E<sup>emp</sup>, E1-Cu<sup>+</sup>, E1-Cu<sup>+</sup>-AMPPCP, E1-Cu<sup>+</sup>-AIF<sub>x</sub>-ADP, and E2-AIF<sub>x</sub>. Papain, CopA, and its proteolytic fragments are identified with dots and in the right margin. Molecular masses are shown in the left.

| State          | E<sup>emp</sup> | E1-Cu<sup>+</sup> | E1-Cu<sup>+</sup>-AMPPCP | E1-Cu<sup>+</sup>-AIF<sub>x</sub>-ADP | E2-AIF<sub>x</sub> |
|---------------|-----------------|-------------------|-----------------------------|-------------------------------------|-------------------|
| Relative rate constants<sup>a</sup> | 100             | 105               | 89                          | 94                                 | 91                |
| Formation of p79 |                  |                   |                             |                                    |                   |
| Degradation of p79 | 100             | 81                | 172                         | 3                                  | 18                |
| Degradation of p44 | s               | wp                | s                           | p                                  | ND<sup>b</sup>   |
| Cleavage sites |                 |                   |                             |                                    |                   |
| Met<sup>a</sup>7/Asn<sup>b</sup>14 | hs              | hs                | hs                          | hs                                 | hs                |
| Glu<sup>a</sup>90 | s               | ND                | ND                          | ND                                 | ND                |
| Gly<sup>a</sup>327 | s               | s                 | hs                          | p                                  | wp                |
| Gln<sup>a</sup>234 | s               | ND                | ND                          | ND                                 | wp                |

**TABLE 4**

Proteolytic characterization of CopA in various states

Susceptibility to papain cleavage is indicated with following symbols: s, susceptible to papain attacks; hs, highly susceptible; p, protected; wp, weakly protected.

<sup>a</sup> Normalized to those in E<sup>emp</sup>, which are 2.21 min<sup>-1</sup> and 0.66 h<sup>-1</sup> for formation and degradation, respectively.

<sup>b</sup> ND, not deducible in this study.

amount of the two major fragments, p79 and p44, p35 is most likely to be the N-terminal fragment generated from p79 by the cleavage at Gly<sup>327</sup>.

In all the conditions, p79 was generated rapidly, as Met<sup>7</sup> was by far the most susceptible site. However, degradation of p79 showed marked differences (Fig. 3). For example, in the E1-Cu<sup>+</sup>-AIF<sub>x</sub>-ADP and E2-AIF<sub>x</sub> states, p79 was very stable. The rate constant of degradation was only 3% (E1-Cu<sup>+</sup>-AIF<sub>x</sub>-ADP) or 18% (E2-AIF<sub>x</sub>) of that in E<sup>emp</sup> (Table 4). This indicates that both digestion sites at Glu<sup>a</sup>90 and Gly<sup>a</sup>327 were protected in these intermediates. In contrast, the susceptibility of Gly<sup>a</sup>327 was higher in E1-Cu<sup>+</sup>-AMPPCP, resulting in an accelerated degradation of p79 (210% of that in E1-Cu<sup>+</sup>, Table 4) and formation of p44. It is noteworthy that p44 was evidently protected from further degradation and stable for more than 12 h. This tendency was also observed with E1-Cu<sup>+</sup> but not with E<sup>emp</sup>, E2-AIF<sub>x</sub>, or E2-AMPPCP (data not shown).

Cu<sup>2+</sup>-ATPase activity of the Fragments—Cu<sup>2+</sup>-ATPase activity was measured for CopA digested with papain for various time in the E<sup>emp</sup>, E1-Cu<sup>+</sup>, or E1-Cu<sup>+</sup>-AMPPCP conditions (Fig. 5). p79, p70, and p44 formed well separated bands on SDS gels and allowed accurate quantitation (supplemental Fig. S3A). The time course of Cu<sup>2+</sup>-ATPase activity exactly followed that of p79 and was independent of p70 and p44 (Fig. 5). The rate constants for decay of Cu<sup>2+</sup>-ATPase activity and that of p79 always coincided under various conditions (i.e. different papain:protein and lipid:detergent:protein ratios) examined (supplemental Fig. S3C). This result indicates that Cu<sup>2+</sup>-ATPase activity of p79 is equal to that of the full-length polypeptide and that p70 and p44 are inactive. Hence, the NMBD or the integrity of the link between the NMBD and the Ma helix is essential for ATPase activity of CopA from T. maritima.

**DISCUSSION**

Our results indicate that CopA from T. maritima, a bacterial Cu<sup>2+</sup> transporting ATPase, contains 3 papain cleavage sites on the C-terminal side of the NMBD. Two of them are located in the loops that connect the A-domain to transmembrane helices. The locations (Fig. 4) and the changes in susceptibility strikingly resemble those of SERCA1, indicating that reorganizations of the cytoplasmic domains accompanying the reaction cycle are very similar despite the differences in topology (Fig. 4). Furthermore, a specific cleavage of the link between the NMBD and Ma helix clearly demonstrates that the NMBD is involved in domain reorganization, as this site is protected against papain attack only in the E1P and E2P analogs (Fig. 3). It is important to note that the A-domain of CopA is smaller than that of SERCA1, comprising only the C-terminal part (A<sub>C</sub>) between the M2 and M3 helices (Fig. 4). Our proteolysis data suggest that the NMBD plays analogous roles to the missing N-terminal part (A<sub>N</sub>) of the A-domain, which directly guides the movements of the M1 helix (8, 9). Accordingly, we propose...
that the Ma helix corresponds to M1 in SERCA1, and that Mb and Mc are insertions prior to M2.

**Similarities in Digestion Pattern between CopA and SERCA1**—It is common to CopA and SERCA1 that the loop connecting the A-domain and M3 helix contains a major cleavage site (Gly^{227} in CopA and Thr^{228} in SERCA1; Fig. 4) and is critical for ATPase activity (Fig. 5) (40). In both pumps, this site is cleaved in E2 (or E_{emp}) and E1-Cu^{+}/E1-2Ca^{2+} but protected in E1-AlF_{4}·ADP and E2-AlF_{4} (21, 22). However, there is a difference in E1-AMPPCP, as SERCA1 is protected (22, 24), but CopA is even more susceptible than in E1-Cu^{+} (Fig. 3). The protection of SERCA1 in E1-AlF_{4}·ADP and E1-AMPPCP is attributed to a strain posed on the loop by a ~20° tilt of the A-domain (8). The protection of SERCA1 in E1-AMPPCP is due to a stabilization of the same closed conformation of the cytoplasmic domains as in E1-AlF_{4}·ADP by the binding of Ca^{2+} to the Mg^{2+} site in the P-domain (8, 10, 24). Thus, the protection of this site by AMPPCP requires millimolar Ca^{2+} (24, 41) and causes occlusion of Ca^{2+} in the transmembrane binding sites. In contrast, if the Ca^{2+} concentration is low (e.g. 0.1 mM), AMPPCP rather enhances exchange of transmembrane-bound Ca^{2+} with those in the cytoplasm (41), presumably because approaches of ATP (analogs) to the P-domain agitate large structural changes in both cytoplasmic and transmembrane domains. Thus, with CopA, where the Cu^{+} concentration is low, the enhanced susceptibility of the A-domain–M3 loop in E1-AMPPCP is not unexpected.

E1-AMPPCP of CopA is characterized by protection of the 44-kDa polypeptide, the C-terminal fragment generated by the cleavage at Gly^{227} in the A-domain–M3 loop (Fig. 4), against proteolytic attack (Fig. 3). It is completely degraded after prolonged digestions in E_{emp} and E_{emp}·AMPPCP. Protection in E1-Cu^{+} is weaker than that in E1-Cu^{+}·AMPPCP but still significant (Figs. 3 and 5).

P54, the C-terminal fragment generated by the cleavage at Gln^{224} in the M2–A domain loop (Fig. 4) accumulated in E_{emp} and E2-AlF_{4} but not in E1-Cu^{+} or E1-Cu^{+}·AMPPCP (Fig. 3). This is again similar to protease K cleavage of SERCA1 at Leu^{119} in the M2–A domain loop specifically in E2 (20).

**The Role and Location of NMBD**—Thus, the digestion data described so far suggest that the cytoplasmic domains of CopA undergo reorganization during the catalytic cycle very similar to those established for SERCA1 (9). The unique digestion site
Papain Digestion of CopA

at Glu\textsuperscript{90} between NMBD and Ma of CopA and its protection only in E1P and E2P, similar to cleavage at Gly\textsuperscript{327} in the A-domain–M3 loop, strongly suggests that NMBD is associated with the A-domain. This would fit very well with the N-terminal end of SERCA1 (A\textsubscript{N} in Fig. 4) also being integral to the A-domain. In SERCA1, the corresponding papain digestion site in the A-domain–M3 link (Ala\textsuperscript{245}) seems to be protected by a strain posed on the loop (8, 9), and this could be the case for the NMBD–Ma loop. It is important to note that the protection is provided by phosphorylation in the P-domain, and not by the binding of metal (to the NMBD) (Fig. 3), and substantiates the involvement of NMBD in domain reorganization. Interestingly, the segments seemingly important in the P-domain–A-domain interactions in SERCA1, namely, P4 helix and the loop including Arg\textsuperscript{998} in the A-domain, are lacking in PIB-ATPases (supplemental Fig. S1, A and B). These segments in SERCA1 make an interface between the A- and P-domains in the E2:Mg\textsuperscript{2+} complex (an E2P, analog) (9), and electrostatically brings the A-domain into the right position. Also Val\textsuperscript{700} in the loop is absolutely critical in E2P-Ca\textsuperscript{2+} → E2 (42), presumably working as a spacer for keeping the two domains at the right distance apart. It is very tempting, therefore, to consider that segments of the NMBD plays a similar critical role, and, in fact, ATPase activity is lost when the link between the NMBD and the transmembrane domain is cleaved (Fig. 5). Also, a deletion mutant of CopA lacking the region from Met\textsuperscript{t} to Glu\textsuperscript{90} showed no Cu\textsuperscript{2+}-dependent but some Cu\textsuperscript{+}-independent ATPase activities.\textsuperscript{3}

In SERCA1, the N-terminal part of the A-domain (A\textsubscript{N}) connected to the M1 helix has a critical role in transmitting the movement of the A-domain to the transmembrane gate, by pulling up the M1 helix toward the cytoplasm. As a result, M1 is largely bent and the amphipathic part (M1\textsuperscript{t}) is laid on the membrane surface (8, 10). If the ion translocation mechanism is similar between PIB- and PII-ATPases, such structural components must exist in CopA. From its topology, only the NMBD and Ma helix could serve as such structural elements. The part around the N-terminal end of the Ma helix consists of residues with long side chains (i.e. Arg, Lys, Glu, and Gln; supplemental Fig. S1C) and, therefore, has an amphipathic nature required for M1\textsuperscript{t}. Ma–M2 are connected by short loops and expected to move together with M1. These considerations lead us to propose a model for domain movements as illustrated in Fig. 6.

The role of the NMBD has been ambiguous because deletion mutants showed, in many reports, impaired but significant ATPase activities (35, 43, 44). This might reflect the stochastic nature of the reaction: it is likely that the NMBD plays a critical role in gating but not in ATP hydrolysis. A critical test for this hypothesis is to measure the transporting activity. In fact, NMBD-deleted CopA from E. coli did not show any Cu\textsuperscript{2+}-transport activity or rescue the CopA-deleted strain of E. coli from toxic amounts of copper (45). A substantial reduction of transport activity was reported for NMBD-deleted CadA (46). A direct interaction between the NMBD and cytoplasmic domains in human Cu\textsuperscript{2+}-ATPase ATP7B was demonstrated by co-purification experiments (47). Bal \textit{et al.} (48) reported that

\textsuperscript{3} Y. Hatori and C. Toyoshima, unpublished result.

FIGURE 6. A schematic depicting the changes in domain organization of CopA during Cu\textsuperscript{2+}-translocation, assuming similar domain reorganizations to SERCA1. Susceptibility to papain attack is represented with arrowheads.

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