Biochemical Characterization of CopA, the *Escherichia coli* Cu(I)-translocating P-type ATPase*

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*Escherichia coli* CopA is a copper ion-translocating P-type ATPase that confers copper resistance. CopA forms a phosphorylated intermediate with [γ-32P]ATP. Phosphorylation was inhibited by vanadate and sensitive to KOH and hydroxylamine, consistent with acylphosphate formation on conserved Asp-523. Phosphorylation required a monovalent cation, either Cu(I) or Ag(I). Divalent cations Cu(II), Zn(II), or Co(II) could not substitute, signifying that the substrate of this copper-translocating P-type ATPase is Cu(I) and not Cu(II). CopA purified from dodecylmaltoside-solubilized membranes similarly exhibited Cu(I)/Ag(I)-stimulated ATPase activity, with a *Kₘ* for ATP of 0.5 mM. CopA has two *N*-terminal Cys-(X)₂-Cys sequences, Gly-Leu-Ser-Cys¹⁴-Gly-His-Cys¹⁷ and Gly-Met-Ser-Cys¹¹₀-Ala-Ser-Cys¹¹₃, which form a Cys³⁷⁹-Pro-Cys⁴⁸¹ motif in a membrane-spanning segment six. The requirement of these cysteine residues was investigated by the effect of mutations and deletions. Mutants with substitutions of the *N*-terminal cysteines or deletion of the first Cys-(X)₂-Cys motif formed acylphosphate intermediates. From the copper dependence of phosphoenzyme formation, the motif formed acylphosphate intermediates. From the function. 

The 834-residue CopA copper pump from *Escherichia coli* (1) is a member of the superfamily of cation-transporting P-type ATPases (2). CopA belongs to a subfamily that transports the cations of soft Lewis acids (or, for simplicity, just soft metal cations) (3). Members of one branch of the subfamily transports monovalent cations such as Cu(I) and Ag(I) (4–6). Divalent cations such as Cu(II) or Zn(II) could not substitute. This clearly demonstrates that the substrate of this cop- 

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Distinctive features of the soft metal ion translocating P-type ATPases are one to six N-terminal Cys-(X)₂-Cys sequences that bind soft metal cations in vitro (11–13), and a Cys-Pro-Cys motif in the sixth transmembrane segment (TM) that may be part of the translocation pathway. Thus far intracellular trafficking in eukaryotes is the only physiological role identified for the Cys-(X)₂-Cys sequences (14–17), but this is unlikely to apply to *E. coli*, which has no intracellular membranes. *E. coli* CopA contains two N-terminal Cys-(X)₂-Cys sequences, Gly-Leu-Ser-Cys¹⁴-Gly-His-Cys¹⁷ and Gly-Met-Ser-Cys¹¹₀-Ala-Ser-Cys¹¹₃. We have previously shown that none of the four cysteine residues in the two sequences is required for either copper resistance or transport (18). However, a deletion of codons 8–150 (ΔNCopA) lost both copper resistance and transport, suggesting that an N terminus is required even though the cysteines are not.

All P-type ATPases have a conserved aspartate residue that is phosphorylated by ATP during the catalytic cycle. In CopA the corresponding residue is Asp⁵²₃. In this report we investigated the ability of CopA to form a phosphorylated intermediate with [γ-32P]ATP. The 32P label on CopA was sensitive to treatment with alkali or hydroxylamine, consistent with acylphosphate formation. Phosphoenzyme formation required a monovalent soft metal cation, either Cu(I) or Ag(I). Divalent Cu(II) or Zn(II) could not substitute. This clearly demonstrates that this copper-translocating P-type ATPase distinguishes between Cu(I) and Cu(II). Paradoxically, mutations of the four cysteine residues in the two N-terminal Cys-(X)₂-Cys sequences increased the apparent affinity for copper, as did deletion of the first Cys-(X)₂-Cys sequence. Mutations in the two cysteine residues in the Cys-Pro-Cys motif in TM6 resulted in loss of copper resistance, transport, and phosphoenzyme formation, indicating that these cysteines play a more critical role in CopA function than the N-terminal cysteines. CopA was solubilized with dodecylmaltoside and purified by metal chelate chromatography. The activity of purified CopA was stimulated by copper or silver ions and inhibited by addition of a Cu(I) chelator.

**EXPERIMENTAL PROCEDURES**

Growth of Cells—Cells of *E. coli* were grown in Luria-Bertani medium (19) at 37 °C. Ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), isopropyl-β-d-thiogalactopyranoside (0.1 mM), 5-bromo-4-chloro-3-indolyl-β-n-galactosidase (80 μg/ml) and L(+)-arabinose (0.0002%) were added as required. To assay inhibition of growth by metal salts, cells were grown overnight in Luria-Bertani medium, diluted 1:100 in the same medium with CuSO₄, and incubated for 6 h at 37 °C with shaking. Growth was monitored from the absorbance at 600 nm. Each strain

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achieved approximately the same final density in the absence of copper. Strain Construction and Plasmide—Standard molecular and genetic techniques were used for strain and plasmid construction (19). The construct of copA deletion strain DC194 and Cys4Cys motif mutants were described previously (18). To introduce mutations in the coding sequence for the cysteines in the Cys-Pro-Cys motif, a sequence corresponding to base pairs 892–1854 of the copA gene was cloned into pGEM-T Easy (Promega) by polymerase chain reaction (PCR) using forward primer 5′-CTCGCCGATATCCTGTAAGGCG-3′ and reverse primer 5′-GTATCCGCCGCGGCTTCCAATG-3′. Site-directed mutagenesis was carried out using a QuickChange method (Stratagene). The insertions were excised by double digestion with NcoI andSacII and ligated into plasmid pGEXCopA2, in which copA is inserted into the pGEX vector (Pharmacia) by polymerase chain reaction (PCR) using forward primer 5′-GGGACGCAGCTCTCAA-3′ (Sac II site) and reverse primer 5′-ATGGCCGCCGGCGGAAAACCATCGTCGGGCCGGAC-3′, with subsequent cloning into plasmid pGEM-T Easy. The insertion was excised by NcoI/SacII double digestion and ligation into pCopA2, generating the plasmid pCopa2ΔN1.Plasmid pCopa2ΔN2, which has a deletion of copA codons 7–342 bp (corresponding to amino acid residues 3–114 and includes both Cys4Cys sequences) was generated by PCR using forward primer 5′-GTCCTCAATGCCAACCCTGCTAAAATGCG-3′ and the same reverse primer as with pCopa2ΔN1. The PCR product was excised by NcoI/SacII double digestion and ligation into plasmid pCopa2 generating plasmid pCopa2ΔN2. All mutations were verified by sequencing the entire insert. In pCopa2 copA is in-frame with the sequence for the Myc epitope and six histidine codons, which are at the 3′-end of copA (1). The wild type and mutant pCopa2 plasmids were introduced into strain DC194.

Preparation of Everted Membrane Vesicles—Cells were grown overnight at 37°C in 5 ml of LB, diluted 100-fold into prewarmed medium and allowed to grow to an optical density of 0.7 at 600 nm. Cells were induced with 0.0002% arabinose for 2.5 h at 30°C. Everted membrane vesicles were prepared as described previously (1). The everted membrane vesicles were suspended in a buffer consisting of 25 mM Tris-HCl, pH 7.0, 10 mM MgCl2, 0.25 M sucrose, 1 mM dithiothreitol (DTT), 0.5 mg of membrane protein. In addition, 0.2 M KNO3 and 0.2 M CuCl2 were added to a final concentration of 50 μM. The mixture was incubated at 37°C for 5 min, and the reaction was initiated by addition of 2.5 μM of [γ-32P]ATP at a final concentration of 50 μM. After incubation on ice (or at room temperature for purified CopA) for the indicated times, 40 μl of ice-cold 50% trichloroacetic acid was added to terminate the reaction. For pulse-chase analysis, 1 mM cold ATP was added 30 s after the start of the reaction, followed by incubation on ice for another 15 to 30 s. Following addition of trichloroacetic acid, the membrane vesicles were kept on ice for an additional 10 min and then harvested by centrifugation at 15,000×g for 10 min. The pellet protein was washed once with 0.5 ml of distilled water and once with 0.5 ml of a solution of 50 mM H2PO4NaOH, pH 2.4. To examine alkaline lability, the pellet material was suspended in 0.2 ml of 0.5 M KOH and kept on ice for 5 min. Sensitivity to hydroxylamine was examined by suspending the pellet material in 0.2 ml of 0.1 mM sodium acetate, pH 5.6, followed by the addition of 0.2 ml of 0.25 M NH4OH, with an additional 10 min incubation at room temperature. The treated samples were again precipitated with trichloroacetic acid and dissolved in 30 μl of 2× concentrated SDS sample buffer diluted with an equal volume of 50 mM H2PO4NaOH, pH 2.4, containing 5% SDS. Samples of 10 μl were loaded on an acidic 8% polyacrylamide gel (21). Following electrophoresis, the gels were stained with Coomassie Brilliant Blue and dehydrated with the DryEase mini-gel drying system (Novex). Radioactivity was analyzed with a PhosphorImager (Molecular Dynamics).

Purification of CopA—Membrane vesicles (5 mg/ml) were solubilized in buffer D, consisting of 25 mM Tris-HCl, pH 7.0, 50 mM KCl, 1 mM CuCl2, 5 mM MgCl2, and 10% glycerol, containing 2% dodecyl maltoside (DDM). The mixture was gently shaken at 4°C for 1 h, and the insoluble material was removed by centrifugation at 25,000×g for 1 h. The soluble fraction was loaded onto a ProBond Ni2+ affinity column (Invitrogen) pre-equilibrated with buffer D containing 0.1% DDM. The column was washed with 5 bed volumes of the same buffer, followed by 5 bed volumes of the same buffer containing 40 mM imidazole. The protein was eluted with the same buffer containing 0.1 mM imidazole. Fractions of 0.5 ml were collected into tubes containing concentrated DTT and EDTA such that the final concentrations became 1 and 0.1 mM, respectively. The samples were analyzed for CopA by SDS-PAGE and immunoblotting. CopA-containing fractions were concentrated 10-fold using Centricon concentrators (Millipore), and the imidazole was removed by gel filtration on a 5-cm column filled with 3 ml of Sephadex G-25 column pre-equilibrated with buffer D containing 0.1% DDM. All buffers were degassed under vacuum or bubbled with Argon before use. All steps were performed at 4°C.

ATPase Assays—ATPase activity was estimated by a coupled spectrometric assay (22). The reaction mixture (0.4 ml) contained 40 mM histidine, pH 6.8, 50 mM KCl, 1% glycerol, 0.1% DDM, 0.4 mg of total E. coli lipids (AVANTI polar-lipids), 0.25 mM Sodium, 1.25 mM phosphoenolpyruvate, 7 units of pyruvate kinase, 10 units of lactate dehydrogenase, 5 mM ATP. Where indicated 1 mM NaN3, 0.25 mM BCS, 10 mM CuCl2 or 10 μM CuI (acetomide copper(I) hexafluoroacetophosphate, Sigma) were added. The reaction mixture was incubated at 37°C for 5 min prior to initiating the assay with 5 mM MgCl2.

RESULTS

CuI-dependent Formation of an Acylphosphate Bond in CopA—A signature property of the P-type ATPases is that the conserved aspartate residue in the DKTG motif accepts the γ-phosphate from ATP during the catalytic cycle, forming a covalent acylphosphate intermediate (23). To investigate phosphoenzyme formation in CopA, everted membrane vesicles were prepared from cells of strain DC194 (ΔcopA) expressing wild type copA on a plasmid. CopA was phosphorylated with [γ-32P]ATP in a time-dependent manner, with maximum labeling within 30 s (Fig. 1A). Vesicles from the copA deletion strain DC194 did not exhibit a radioactive band at the corresponding position (Fig. 1, lane 1). The intermediate was sensitive to basic pH and hydroxylamine (Fig. 1B), which are considered to be reliable criteria for the presence of an acylphosphate bond. The label could be chased by 1 mM unlabeled ATP within 15 s, indicating the transient nature of the intermediate. In the presence of 50 μM sodium orthovanadate, which mimics the transition state when bound in the active site of P-type ATPases, the reaction was entirely inhibited. On the other hand,
CopA Phosphoenzyme Intermediate

**Fig. 1. Properties of CopA acylphosphate intermediate formation.** Membrane vesicles were prepared from cells of *E. coli* strain DC194 (ΔcopA) bearing plasmid pCopA2 or derivatives with copA mutations. The reaction was initiated by addition of 50 μM [γ-32P]ATP (~2.5 μCi) at 4 °C. Samples were analyzed on 8% polyacrylamide gels, as described under “Experimental Procedures.” Radioactivity was imaged and quantified with a phosphorimager. A, time dependence of acylphosphate intermediate formation. Each reaction contained 20 μg of membrane protein from cells expressing wild type CopA and 10 μM CuCl2 with or without 1 mM DTT, as indicated. Reactions were terminated at the indicated times by addition of additional of trichloroacetic acid. **B**, properties of acylphosphate formation in wild type CopA and quadruple cysteine mutant. Wild type and C14A/C17A/C110A/C113A CopAs were reacted with [γ-32P]ATP for 30 s, following which the label was chased with 1 mM nonradioactive ATP for an additional 15 or 30 s. In the other reactions, membranes were incubated with the following inhibitors for 5 min prior to addition of [γ-32P]ATP: 50 μM orthovanadate, 0.25 mM hydroxylamine, 0.5 mM KOH or 1 mM NaN3. C, effect of reductants and metals. Membrane vesicles containing wild type CopA were incubated for 5 min with the indicated additions prior to addition of [γ-32P]ATP for 30 s: 1 mM DTT, 1 mM GSH, 1 mM cysteine, 10 μM CuCl2, 0.2 mM ZnSO4, 0.2 mM CoSO4, 10 or 20 μM AgNO3, 10 μM acetonitrile copper(I) hexafluorophosphate, or 50 μM BCDS.

phosphoenzyme formation was not inhibited by azide, an inhibitor of many ATPases.

Since CopA is a copper ion pump, acylphosphate formation would be expected to be stimulated by copper ion. In the absence of copper ion, no radioactive band was observed (Fig. 1, lane 1). To date there has been no clear determination of the redox state of the substrate of copper ion pumps, that is, whether these ATPases transport Cu(II), Cu(I) or both. Our previous work showed that a reductant, DTT, is required for phosphorylation. Since CopA is a copper ion pump, acylphosphate formation was examined (Fig. 1, lane 1). No phosphoenzyme formation was observed in the presence of Cu(II) and reductant: DTT, GSH, or cysteine (Fig. 1C). DTT and GSH were more effective than cysteine. For those reasons DTT was usually added as reductant in subsequent assays.

The requirement for reductant could be to reduce Cu(II) to Cu(I) or to reduce the cysteines in CopA or both. For that reason the effect of mono- and divalent soft metal ions on acylphosphate formation was examined (Fig. 1C). No phosphoenzyme formation was observed in the presence of the divalent cations Zn(II) or Co(II), whether or not DTT was present. In contrast, either Ag(I) or Cu(I) (added in the form of acetonitrile copper(I) hexafluorophosphate) stimulated phosphoenzyme formation even in the absence of DTT, showing that DTT is not necessary to maintain the enzyme in a reduced state. Moreover, bathocuproindisulfonate (BCDS), which chelates Cu(I) and not Cu(II), prevented labeling. These results demonstrate that CopA is a monovalent (but not a divalent) soft metal ion P-type ATPase.

Requirement of the N Terminus for CopA Activity—One characteristic that differentiates soft metal P-type ATPases from their hard metal homologues is the presence of one or more Cys-X-Cys metal binding domains in the cytosolic N-terminal region. We have previously shown that none of the four cysteine residues in the Gly-Leu-Ser-Cys14-Gly-His-Cys17 and Gly-Met-Ser-Cys110-Ala-Ser-Cys113 sequences of CopA is required for either copper resistance or transport, although deletion of codons 8–150 (∆NCopA) lost both copper resistance and transport (18). To extend this observation, two N-terminally truncated CopAs were constructed: pCopAΔN1, which is truncated immediately after the first Cys-X-Cys motif, and pCopAΔN2, which has both Cys-X-Cys motifs deleted. Cells expressing ∆N1 CopA retained partial resistance to CuSO4 in vivo, while cells expressing ∆N2 CopA were as sensitive as the CopA deletion strain (Fig. 2), even though the truncated proteins were made in amounts comparable to that of the wild type (Fig. 3A). These results suggest that at least a portion of the cytosolic N terminus of the protein is necessary, although whether that is for activity or proper folding cannot be deduced from these results.

CopA mutants with alanine substitutions in the first (C14A/C17A) or second (C110A/C113A) Cys-X-Cys motifs or with all four cysteines substituted (C14A/C17A/C110A/C113A) formed an acylphosphate intermediate equivalent to wild type CopA (Figs. 1B and 3B). Indeed, deletion of the N terminus through the first Cys-X-Cys motif did not appear to affect the ability of the ∆N1 CopA to form a phosphorylated intermediate, just as a strain expressing this mutant copA conferred copper resistance in vivo. Consistent with the loss of resistance in cells bearing pCopAΔN2, the ∆N2 enzyme did not form a phosphorylated intermediate.

Requirement of an Intramembrane CPC Motif for CopA Activity—A distinctive feature of soft metal ATPases is a highly conserved Cys-Pro-Cys motif in TM6, the putative translocation domain (24). The Cys-Pro-Cys motif in CopA includes Cys479 and Cys481. The two cysteines were individually mutated to Cys479A and Cys481A. Since the Enterococcus hirae CopB has a Cys-Pro-His sequence (6), a C481H mutant was also constructed. All three mutant proteins were expressed in amounts comparable to wild type CopA (Fig. 3A). The effect of the mutations on copper resistance in vivo (Fig. 4A) and ATP-driven 64Cu transport in vitro were examined. None of the mutant genes conferred resistance to copper, and everted membrane vesicles from cells expressing those genes were unable to accumulate 64Cu (Fig. 4). No phosphoenzyme intermediates were detected in the C479A, C481A, or C481H proteins (Fig. 3B).
activity in each lane imaged with a phosphorimager.

Lane 5 (C14A/C17A/C110A/C113A), C110A/C113A (lane 4), C14A/C17A (lane 3), C14A/C17A/C110A/C113A (deletion of CopA residues 7–54), or plasmid ΔN1pCopAΔ (deletion of CopA residues 3–113). The bands from three separate 32P-labeled gels were quantified by densitometry. The values were averaged and, for clarity, were normalized to the value at 50 μM (Fig. 5B). A, immunoblotting with antibody against the His6 tag. B, labeling of CopA with [γ-32P]ATP, with radioactivity in each lane imaged with a phosphorimager.

Effect of N-terminal truncations on copper resistance. Copper ion resistance was assayed in strain DC194 (ΔcopA) (○) or DC194 bearing plasmid pCopA2 (wild type) (●), plasmid ΔN1pCopAΔ, or plasmid ΔN2pCopAΔ (deletion of CopA residues 3–113) (□).

Copper resistance and transport by CopA Cys-Pro-Cys mutants. A, copper ion resistance was assayed in strain LMG194 (wild type) (○), DC194 (ΔcopA) (○), or DC194 bearing a plasmid with the gene for wild type CopA (●), C479A (●), C481A (●), or C481H (□). B, uptake of 64Cu in everted membrane vesicles of E. coli strain DC194 (○) or DC194 expressing wild type CopA (●), C479A (●), C481A (●), or C481H (□). Vesicles were prepared as described under “Experimental Procedures.” Cells were induced with 0.0002% arabinose as described under “Experimental Procedures.” Transport was assayed with 10 μM 66CuCl2 reduced with 1 mM DTT.

The apparent Km of ATPase activity of the A. fulgidus strain DC194 bearing plasmid pCopA2 (wild type) (○), plasmid ΔN1pCopAΔ (deletion of CopA residues 7–54) (■), or plasmid ΔN2pCopAΔ (deletion of CopA residues 3–113) (□).

Solubilization and Purification of CopA—Membrane vesicles were prepared from arabinose-induced cells bearing plasmid pCopA2, which carries a copA gene in-frame with the sequence for the Myc epitope and six histidine codons under control of the arabinose promoter. The vesicles were solubilized with 2% dodecylmaltoside, and CopA was purified by metal chelate affinity chromatography (Fig. 6A). The presence of a small amount of copper during solubilization and purification was found to result in more active and stable CopA. A minor band was observed upon immunoblotting (Fig. 6B). Since the slightly smaller protein reacted with antibody to the histidine tag, there was probably some degradation at the N terminus of CopA. Although the purified protein exhibited ATPase activity, hydrolysis was inhibited 80–90% by sodium azide. Since phosphoenzyme formation is not inhibited by sodium azide (Fig. 1B), it is likely that the azide-sensitive ATPase activity is due to minor contamination by a highly active ATPase.

The azide-insensitive ATPase activity of 130 nmol/mg/min was inhibited to 65 nmol/mg/min by BCS, a Cu(I) chelator, that may reflect residual copper remaining from purification. Neither Cu(II) nor Zn(II) stimulated ATPase activity. Compared with the rate in the presence of BCS, there was an ∼4-fold activation by Cu(I) (280 nmol/mg/min) or Cu(II) plus DTT (295 nmol/mg/min). In other experiments, Ag(I)-stimulated activity was 4- to 5-fold higher than the rate with BCS. The Ag(I)-stimulated activity exhibited a relatively broad pH profile, with activity at pH 7 and 8, and about one-half of the activity at pH 6.
Phosphorylation of CopA was assayed as described in the legend to Fig. 1. Radioactivity was imaged and quantified with a phosphoimager. A, each lane of an 8% SDS gel contained 20 μg of membrane protein labeled with [γ-32P]ATP from cells of DC194-bearing plasmids expressing wild type CopA (row 1), C14A/C17A/C110A/C113A (row 2), or ΔN1 (row 3) at the indicated concentrations of CuCl2 without or with 1 mM DTT. B, phosphorylase formation was quantified by densitometry. Values were normalized to the level of phosphorylation in the presence of 50 μM Cu(II). The lines represent best fits of the data to the Michaelis-Menten equation using SigmaPlot and generated half maximal stimulatory concentrations of copper of 1.5 ± 0.5 μM (squares, wild type), 0.45 ± 0.1 μM (inverted triangles, quadruple cysteine mutant), and 0.84 ± 0.2 μM (circles, ΔN1).

third lower at pH 6. These results indicate that the azide-insensitive ATPase activity is catalyzed by CopA in a reaction that requires either Cu(I) or Ag(I). Cu(I)-stimulated ATPase exhibited an apparent kcat for ATP of 0.5 ms (Fig. 6C). The Cu(I)-stimulated Vmax of 0.19 μmol/mg/min is similar to the Vmax for the Ag(I)-stimulated ATPase reported for the CopA homologue from A. fulgidus (25).

**DISCUSSION**

The distinctive feature of P-type ATPases that gave the superfamily its name is the formation of an acylphosphate on a conserved aspartate residue during the catalytic cycle (23). Phosphorylase formation has been shown for several copper-translocating P-type ATPase homologues of CopA (25–28). In this report we demonstrate Cu(I)-dependent phosphorylation of CopA. Phosphorylation was sensitive to alkali and hydroxylamine, consistent with acylphosphate formation on the conserved Asp253.

Whereas it is presumed that copper pumps are specific for Cu(I) and not Cu(II), this has not been proven. Since an N-terminal peptide containing the Cys-X2-Cys motifs of the Wilson disease protein has been shown to bind Cu(I) and other divalent cations (29), it is possible that these proteins could pump both Cu(I) and Cu(II). Phosphorylation of WND and E. hirae CopA were not copper-dependent. This could have been due to copper contamination in the buffers, since copper chelators were found to inhibit. Phosphorylation of MNK and the A. fulgidus CopA showed copper dependence, but it was not clear whether the activity was stimulated by Cu(I), Cu(II), or both. In both cases DTT was present in the phosphorylation assays, which would reduce some or all of the Cu(II) to Cu(I), but DTT might also have been necessary to reduce cysteine thiolates in the proteins. The data reported here clearly show that Cu(I) and not Cu(II) stimulates phosphorylation of CopA, and that DTT is not required. While phosphorylase formation was stimulated by Cu(II) in the presence of DTT, Cu(I) and Ag(I) were equally effective in the absence of DTT. Thus the requirement for DTT appears to be in reduction of copper rather than for maintenance of protein cysteine thiolates. There was no phosphorylation in the presence of Cu(II) or other divalent cations, so CopA is not a divalent cation pump.

The soft metal P-type ATPase are distinguished from their hard metal homologues by the presence of N-terminal metal binding motifs, usually Cys-X2-Cys, and a second cysteine motif, usually Cys-Pro-Cys, in TM6. The physiological role of these motifs and the function of the individual cysteine residues in the biochemical mechanism of the ATPases are open questions. In eukaryotes the N-terminal motifs may interact with metal chaperones or may be involved in copper-regulated trafficking of copper pumps from intracellular compartments to the plasma membrane. Most prokaryotes lack intracellular membranes or compartments, and the function of the Cys-X2-Cys motifs in their copper pumps is less clear. In E. coli there are no intracellular membranes, and no metallochaperones have been identified. In this report we investigated the ability of N-terminal cysteine mutants and deletions to form a phosphorylated intermediate. A quadruple mutant lacking all four cysteine residues of the two N-terminal Cys-X2-Cys motifs was phosphorylated by [γ-32P]ATP. CopA with a deletion of the

**Fig. 6.** Purification and properties of CopA. Membrane vesicles from strain DC194 bearing plasmid pCopA2 were solubilized with 2% DDM and purified on a ProBond Ni2+ column, as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE on two 8% polyacrylamide gels. A, gel was stained with Coomassie Blue. Lane 1, 40 μg of 1% membrane protein from strain DC194 (pCopA); lane 2, 40 μg of membrane protein membranes from strain DC194 pCopA2; lane 3, 40 μg of protein from the DDM extract of membranes from strain DC194 pCopA2; lane 4, flow-through from ProBond column; lane 5, eluate with 40 mM imidazole; lane 6, eluate with 100 mM imidazole; lane 7, 5 μg of pooled and concentrated CopA-containing fractions. The volume of sample in lanes 4–6 was adjusted to add an amount equivalent to that in lane 3. B, immunoblot with antibody to the His6 tag. Each lane of gel B was the same as gel A with one-tenth the amount of protein. The migration of standard proteins is indicated by the left arrows. C, kinetics of ATPase activity of purified CopA. The ATPase activity of purified ATPase was determined in the presence of 10 μM CuCl2, 1 mM DTT, and the indicated concentrations of ATP. Each value is the average of two separate assays. The data fitted to the Michaelis–Menten equation using SigmaPlot yielded a Km of 0.52 ± 0.05 mM and Vmax of 0.19 ± 0.06 μmol/min/mg protein.
Thus, we propose that the Cys-Pro-Cys residues of CopA P-type ATPases, where binding of metal is required for maximal phosphoenzyme formation. Does this apparently counterintuitive result imply that binding of copper to the N-terminal motifs decrease the affinity of the pump for copper, perhaps for a regulatory function? An alternative explanation is that in vitro the Cys-(X)2-Cys motifs reduce access of copper to the translocation pathway or reduce the local concentration of copper in that region. The mutations did not produce altered copper resistance, so it is questionable whether the in vitro results reflect an increased efficiency of the pump in vivo. While none of the four N-terminal cysteine residues are required for CopA activity under the conditions studied, their contributions to CopA function may be apparent only under different physiological conditions. Similar conclusions have been made about the Cys-(X)2-Cys motifs in other copper P-type ATPases. For example, in MNK mutation or deletion of the first four of the six Cys-(X)2-Cys motifs had no apparent effect on copper-induced trafficking, their only identified function (15). Although none of the cysteine residues of the two Cys-(X)2-Cys motifs of CopA are obligatory, deletion of the N terminus to just before the first putative transmembrane region resulted in loss of function, but it is possible that the remainder of the protein does not fold properly.

In three CopA homologues the Cys-Pro-Cys motif that is distinctive in soft metal ion-translocating ATPases has been shown to be located in TM6 (30–32), so it is reasonable to assume that it is in TM6 of CopA as well. The Cys-Pro-Cys motif has been proposed to be part of the translocation domain (24). To examine the requirement of the cysteine residues, Cys479 and Cys481 were changed to alanine residues. Cells expressing either mutant became sensitive to copper. Both mutant proteins were found in the membrane in normal amounts, but neither was phosphorylated by [γ-32P]ATP. Since the CopB homologue from E. hirae has a Cys-Pro-His motif in the corresponding TM6 (6), we constructed a C481H mutant. Cells expressing the mutant CopA were also sensitive to copper, and the protein with a Cys-Pro-His motif did not form an acylphosphate intermediate. A reasonable interpretation of these results is that the Cys-Pro-Cys motif of CopA is essential for Cu(I) binding, and that mutants with Cys-Pro-His, Cys-Pro-Ala, or Ala-Pro-Cys no longer bind metal. This is consistent with the known catalytic cycle for hard metal ion-translocating P-type ATPases, where binding of metal is required for acylphosphate formation at the conserved aspartate residue (33). Thus, we propose that the Cys-Pro-Cys residues of CopA are required for binding of Cu(I) in the ion translocation pathway and hence for Cu(I) transport.

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