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Periplasmic Domain of CusA in an *Escherichia coli* Cu^+/Ag^+ Transporter Has Metal Binding Sites

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The resistance nodulation division (RND)-type efflux systems are utilized in Gram-negative bacteria to export a variety of substrates. The CusCFBA system is the Cu^+ and Ag^+ efflux system in *Escherichia coli*, conferring resistance to lethal concentrations of Cu^+ and Ag^+. The periplasmic component, CusB, which is essential for the assembly of the protein complex, has Cu^+ or Ag^+ binding sites. The twelve-span membrane protein CusA is a homotrimeric transporter, and has a relatively large periplasmic domain. Here, we constructed the periplasmic domain of CusA by joining two DNA segments and then successfully expressed and purified the protein. Isothermal titration calorimetry experiments revealed Ag^+ binding sites with K_ds of 10^{-6}-10^{-5} M. Our findings suggest that the metal binding in the periplasmic domain of CusA might play an important role in the function of the efflux pump.

Keywords: membrane protein, metal efflux pump, Gram-negative bacteria, RND-type transporter

Transition metals such as copper are essential in numerous enzymatic processes due to their redox capability (Nelson, 1999). In particular, copper becomes very toxic because of its ability to reduce or to oxidize when it is present in excess of the required concentrations (Nelson, 1999). This dual nature of copper forces the cells to maintain a concentration (Bagai *et al.*, 2007) balance. In *Escherichia coli*, CopA (Rensing *et al.*, 2000), CueO (Grass and Rensing, 2001), and CusCFBA (Franke *et al.*, 2003) are responsible for the defense from excess copper ion. The P-type ATPase CopA pumps out intracellular copper ions (Rensing *et al.*, 2000), and the multicopper oxidase CueO converts Cu^+ to the less-toxic Cu^{++} in the periplasm (Grass and Rensing, 2001; Outten *et al.*, 2001). The CusCFBA system is a cation efflux pump that pumps periplasmic Cu^+ across the outer membrane (Franke *et al.*, 2001, 2003).

The CusCFBA system consists of the periplasmic adaptor CusB, the inner membrane transporter CusA, the outer membrane protein CusC, and a periplasmic metal chaperone CusF (Franke *et al.*, 2003). Based on the assembly model of the homologous multidrug efflux pump AcrAB-TolC (Bavro *et al.*, 2008; Kim *et al.*, 2010), CusA forms a protein complex with CusB and CusC. The inner membrane transporter CusA is the resistance nodulation division (RND) family proton/substrate antiporter and is also a key player in antibiotic resistance (Poole and Srikumar, 2001). The RND family transporters have been known to homotrimerize with a dome-like large periplasmic domain using the peristaltic mechanism (Murakami *et al.*, 2006; Seeger *et al.*, 2006). The structures in the AcrAB-TolC pump further suggest that CusA uptakes substrates at the bottom of the periplasmic domain and expels substrates at its top, where the exit site is linked directly or indirectly to the CusC central channel across the outer membrane in cooperation with CusB (Murakami *et al.*, 2006; Seeger *et al.*, 2006; Bavro *et al.*, 2008; Yum *et al.*, 2009).

The CusCFBA system has been also shown to confer Ag^+ resistance, which could be explained by the similar coordination chemistries of Ag^+ and Cu^+ (Solioz, 2002). The periplasmic metal chaperone CusF, which contains one metal binding site (Loftin *et al.*, 2005), sequesters periplasmic metal ions and has been proposed to deliver those metal ions to CusB in the CusCBA complex (Bagai *et al.*, 2008). The periplasmic adaptor CusB has one high affinity metal binding site (Bagai *et al.*, 2007); this metal binding site was visualized in the crystal structure (Su *et al.*, 2009) and the functional role was confirmed *in vivo* (Bagai *et al.*, 2007). Since the inner membrane transporter CusA is the energy-consuming machinery responsible for pumping the metal ion, the metal ion should come from CusF, CusB or the periplasmic space into the periplasmic domain of CusA.

In this study, we characterized the role of CusA in the metal binding in the periplasm and showed that CusA has Ag^+ binding sites in its periplasmic domain using recombinant CusA protein.

Materials and Methods

Construction of expression plasmid

To construct the plasmid expressing the CusA periplasmic domain, two fragments were ligated into pPROEXHTA vector in two steps. The DNA encoding the first fragment (residues 37-328) was amplified from the genomic DNA of *E. coli* strain XL1Blue by PCR using

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standard methods and the following oligonucleotides: 5'-gatctctccgatgt ggtgcag-3' and 5'-gctgcg atcgtatgttgtaac-3'. DNA encoding the second fragment (residues 559-860) was amplified using a similar method with the oligonucleotides: 5'-cagcttgttgtatatgccatc-3' and 5'-cccggagaatgcca cgct-3'. The PCR product for the first fragment was digested and ligated into the *Nco*I and *Xho*I sites of pPROEXHTA vector (Invitrogen, USA), and the PCR product for the second fragment were subsequently inserted into the *Xho*I and *Hin*dIII sites of the vector containing the DNA encoding the first fragment. The resulting plasmid (pPROEXHTA-CusAperi) expresses a hexahistidine tag and a TEV protease consensus site at the N-terminus of the CusA periplasmic domain.

Expression and purification of the recombinant protein

The recombinant plasmid pPROEXHTA-CusAperi was transformed into BL21(DE3) to produce the periplasmic domain of CusA. Cells were grown in 1.5 L of LB broth with 50 μg/ml ampicillin at 37°C until reaching an OD_{600} of 0.6-0.8. Protein expression was induced by adding 0.5 mM isopropyl-D-thiogalactoside to the culture at 30°C. Cells were harvested by centrifugation 6 h after induction, and then stored at -80°C until use.

Cells were thawed and disrupted by sonication in 60 ml of 20 mM Tris (pH 8.0) buffer containing 150 mM NaCl and 2 mM β-mercaptoethanol. The soluble lysate was centrifuged at 13,000 rpm for 30 min and the supernatant was mixed with Ni-NTA affinity resin (QIAGEN, Netherlands) that had been pre-incubated with Tris buffer; the mixture was then stirred for 30 min at 4°C. After the slurry was loaded into the column, unbound proteins were washed off with Tris buffer supplemented with 20 mM imidazole. Recombinant CusA periplasmic domain with the hexahistidine tag was eluted with 30 ml of Tris buffer supplemented with 200 mM imidazole. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing CusA were pooled, and the hexahistidine tag was cleaved by the recombinant TEV protease by incubation at room temperature overnight. The CusA periplasmic domain was diluted 3-fold with 20 mM Tris (pH 8.0) buffer and then loaded onto a HiTrapQ column (GE Healthcare, USA). The protein was eluted from the column using a linear gradient of 0-1 M NaCl in 20 mM Tris buffer (pH 8.0). The CusA-containing fractions were concentrated using Centricone (GE Healthcare), and the CusA protein was further purified using HiLoad Superdex 200 (GE Healthcare) pre-equilibrated with 20 mM Tris (pH 8.0) containning 150 mM NaCl and 2 mM β-mercaptoethanol. Total proteins were analyzed by SDS-PAGE on a 15% gel and stained with Coomassie blue. The purified protein was stored at 4°C for use within a week or stored frozen at -80°C until use. The expression and purification of the recombinant CusF and CusB proteins were performed as described previously (Bagai *et al.*, 2007; Xu *et al.*, 2009).

Circular dichroism (CD) spectroscopy

The purified protein was diluted to 0.1 mg/ml in 20 mM sodium phosphate (pH 7.5) buffer containing 100 mM NaCl. The CD spectrum was collected on a JASCO-J810 spectropolarimeter at 25°C. The protein concentration was measured based on UV absorption from Tyr and Trp residues of the protein at 280 nm.

Thermal stability

Thermal stability studies of the monomeric and trimeric forms of CusA periplasmic domain were performed by CD in a JASCO-J750 spectropolarimeter. Samples were prepared in 20 mM Tris, 0.15 M NaCl, pH 8.0, and thermal unfolding experiments were performed by monitoring the CD signal at 220 nm between 25°C and 90°C using a heating rate of 2°C/min at a concentration of 0.25 mg/ml.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) measurements were performed on a Microcal (GE Healthcare) VP-ITC microcalorimeter, typically at 25°C. The monomeric form of the CusA periplasmic domain sample was dialyzed against 20 mM Hepes buffer. The titrant $AgNO_3$ solution was prepared with Hepes buffer by adding 1 mM $AgNO_3$. Both the protein and the titrant $AgNO_3$ solutions were thoroughly degassed in a ThermoVac apparatus (Microcal). For a titration experiment, approximately 1.5 ml of the protein solution was placed in a reaction cell and injected with 300 μl of the titrant $AgNO_3$ solution over the course of 20 sec. The time delay between successive injections was 220 sec, and the reaction cell was continuously stirred at 300 rpm. Data analysis was performed by nonlinear regression fitting to the sequential binding model with 2, 3, or 4 sites using Origin (Microcal). The association constant K_A, the enthalpy change ΔH, and the stoichiometry were obtained from fitting the data (Wiseman *et al*., 1989).

Results

Plasmid construction, expression, and purification of the recombinant periplasmic domain of CusA

CusA is an integral membrane protein, and thus it is difficult to obtain a sufficient amount to characterize structurally and biochemically. AcrB, a structural homologue of CusA in the multidrug efflux pump AcrAB-TolC, largely consists of a periplasmic domain and a transmembrane α-helical domain (Yu *et al*., 2003). The crystal structures of AcrB suggest that the two domains are independently folded because they are linked via flexible loops. Due to the high degree of homology (21% in sequence identity) between *E. coli* AcrB and CusA, we identified the boundaries between the periplasmic and transmembrane domains (Fig. 1A). The periplasmic domains are composed of two discrete regions (residues 37-328; 559-860), while the transmembrane domain includes the N- and C-terminal regions and a central portion between the periplasmic two regions. The distance between the C-terminal residue (residue 328) of the first periplasmic region and the N-terminal residue (residue 559) of the second periplasmic region was estimated to be only 3.5 Å based on the AcrB structure. Since both ends are relatively close and flexible, we expected that a linker was not necessary to replace the central transmembrane region connecting the two periplasmic regions (Fig. 1B). We first inserted the first periplasmic region, and then inserted the second periplasmic region into the vector containing the first periplasmic region. The resulting construct contained the amino acid sequence Leu-Glu between the two periplasmic regions; this sequence originated from the restriction enzyme site (Fig. 1B).

The recombinant periplasmic domain of CusA was successfully expressed at 25°C in the cytosol of *E. coli*. Initial purification of the hexahistidine-tagged CusA periplasmic domain was performed by Ni-NTA affinity chromatography in a detergent-free buffer as described in the 'Materials and Methods.' The protein purity was determined on a Coomassie-stained SDS-polyacrylamide gel (Fig. 2A). Further purification was achieved by size-exclusion chromatography (Fig. 2B).

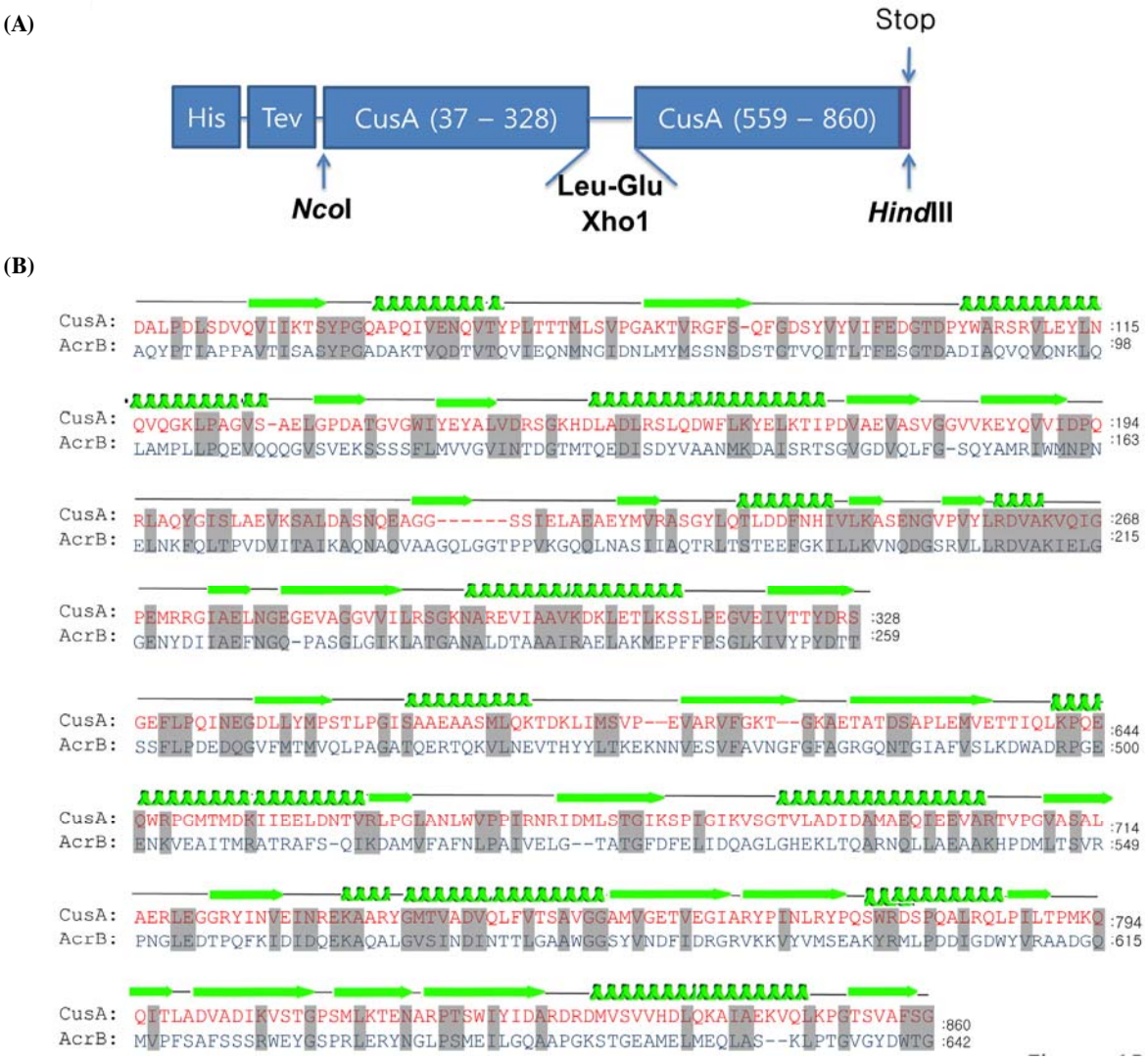

Fig. 1. Construction of the CusA periplasmic domain. (A) A bar diagram of the vector construction. N-terminal hexa-histidine tag (His) and the TEV protease recognition site (Tev) originated from the vector pPROEXHTA (Invitrogen). The first part of CusA (resides 37-328) and the second part (residues 559-860) were directly fused in the vector and linked by the *Xho*I restriction enzyme site encoding Leu-Glu.
(B) Sequence alignment of the periplasmic regions of *E. coli* CusA and *E. coli* AcrB. The alignment was performed using CLUSTAL W (Thompson *et al*., 1997). The secondary structure of AcrB is shown above the sequences. Membrane-embedded regions are omitted in this alignment for clarity. The conserved residues are highlighted.

From this purification (1.5 L culture), 20 mg/ml protein with at least 95% purity was produced (Fig. 2B).

To ascertain the structural integrity of the CusA periplasmic domain, we employed circular dichroism (CD) spectroscopy. The secondary structural characteristics of the CusA periplasmic domain were determined using CD spectroscopy. The results indicate that the periplasmic domain of CusA can properly fold in the absence of the membrane-embedded domain.

Biochemical characterization of the periplasmic domain of CusA

Given the structural and functional similarity between CusA and AcrB, the full-length CusA should be a homotrimer. It is not known which domain plays a major role in the trimerization of CusA. We checked whether the CusA periplasmic domain can form a homotrimer on its own. The size-exclusion chromatography revealed one short and one tall peak. The short peak was measured as ~170 kDa, indicating a trimer, and the tall peak was ~65 kDa, indicating a monomer (Fig. 3A). When a diluted sample was applied to a size exclusion chromatographic column, the trimeric form was significantly decreased. This result suggests that the ability of the periplasmic domain of CusA to trimerize is very low. Furthermore, the trimeric and the monomeric forms of the CusA periplasmic domain showed very similar CD spectra and similar thermal stabilities (Fig. 3). The pattern of the CD spectra suggests that both the proteins are properly folded because the spectra are

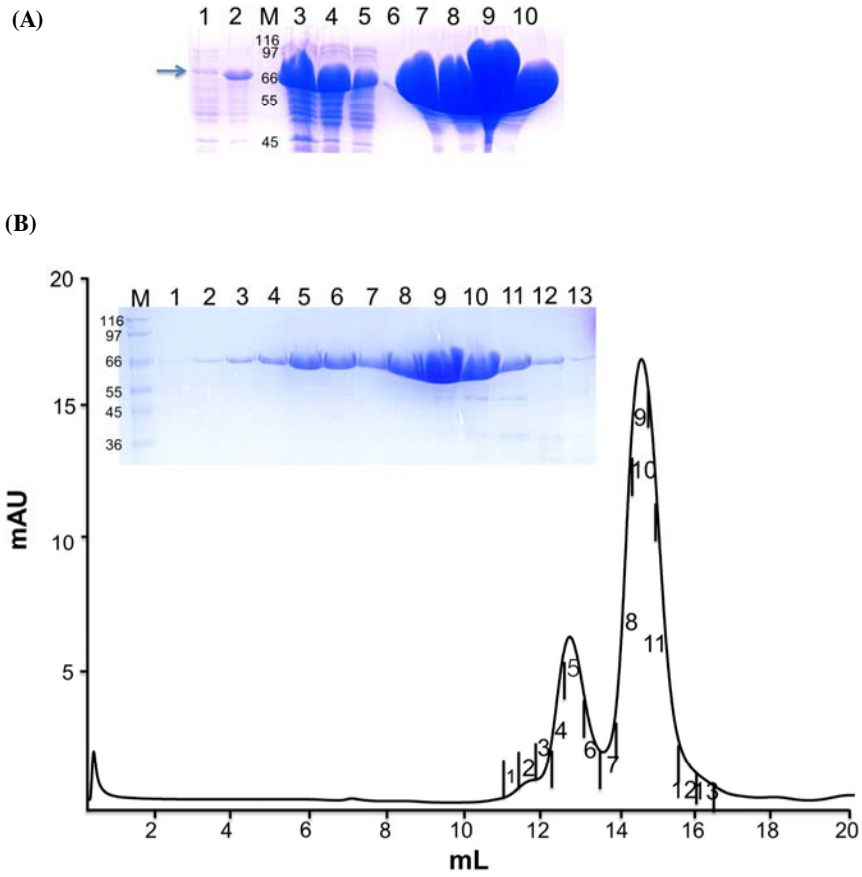

Fig. 2. Expression and purification of the CusA periplasmic domain. (A) SDS-polyacrylamide gel showing the expression of the CusA periplasmic domain. The arrow indicates the CusA periplasmic domain. Whole cell lysates before induction and after induction are shown in *Lanes* 1 and 2, respectively. After sonication, the cell lysate and the soluble fraction are shown in *lanes* 3 and 4, respectively. During the Ni-NTA chromatography, the unbound and the washing fractions are shown in *lanes* 5 and 6, respectively. The elution fractions are shown in *lanes* 7, 8, 9, and 10. The gel was stained with Coomassie blue. The numbers in *lane* M indicate relative molecular mass expressed in kilodaltons.
(B) The elution profile of the CusA periplasmic domain protein from the size exclusion chromatographic column. SDS-PAGE analyses of the fractions are shown with the numbers corresponding to the fractions on the chromatogram.

matched to the expected structure of CusA periplasmic domain. Taken together, the trimeric and the monomeric forms of the CusA periplasmic region are in dynamic equilibrium, and the transmembrane domain of CusA might be required for the stable trimerization of CusA.

The periplasmic domain of CusA has Ag^+ binding sites

To test the metal binding ability of the CusA periplasmic domain, ITC experiments were carried out using $AgNO_3$ as titrant at pH 7.0. Cu^+ is predominantly present only under anaerobic conditions, while Ag^+ can be present under both aerobic and anaerobic conditions (Fenwick, 1926). Thus, the ITC experiment was conducted only for Ag^+ in this study. The binding isotherm was not fitted to a single binding site, 1:1 model; instead, the isotherm was best fitted when a sequential binding model with 4 sites was applied. The results showed that there are four Ag^+-binding sites in the periplasmic domain of CusA, two with high affinities (K_d=6.6 and 10 μM, respectively), one with moderate affinity (K_d=70 μM), and one with low affinity (K_d=230 μM). However, the binding strengths of even the high affinity sites are much weaker than those of CusF and CusB to Ag^+ (K_d=38 and 35 nM) (Kittleson *et al.*, 2006; Bagai *et al.*, 2008). The detailed thermodynamic parameters are listed in Table 1. In this ITC experiment, only monomeric form of the protein was able to be applied in this experiment because of the limited quantity of the trimeric form of the protein sample. However, we believe that the protein sample of the trimeric form would show the same binding constant. In fact, the trimeric form would dissociate into the monomeric forms due to the dynamic equilibrium between the monomeric and trimeric forms in such a time-consuming method as the ITC experiment.

Discussion

Although expression of the full-length version of membrane proteins can be the best choice for characterization because it reflects the native state of the proteins, the expression yields of membrane proteins are usually too low for structural and biochemical studies. In this study, we studied the multi-span

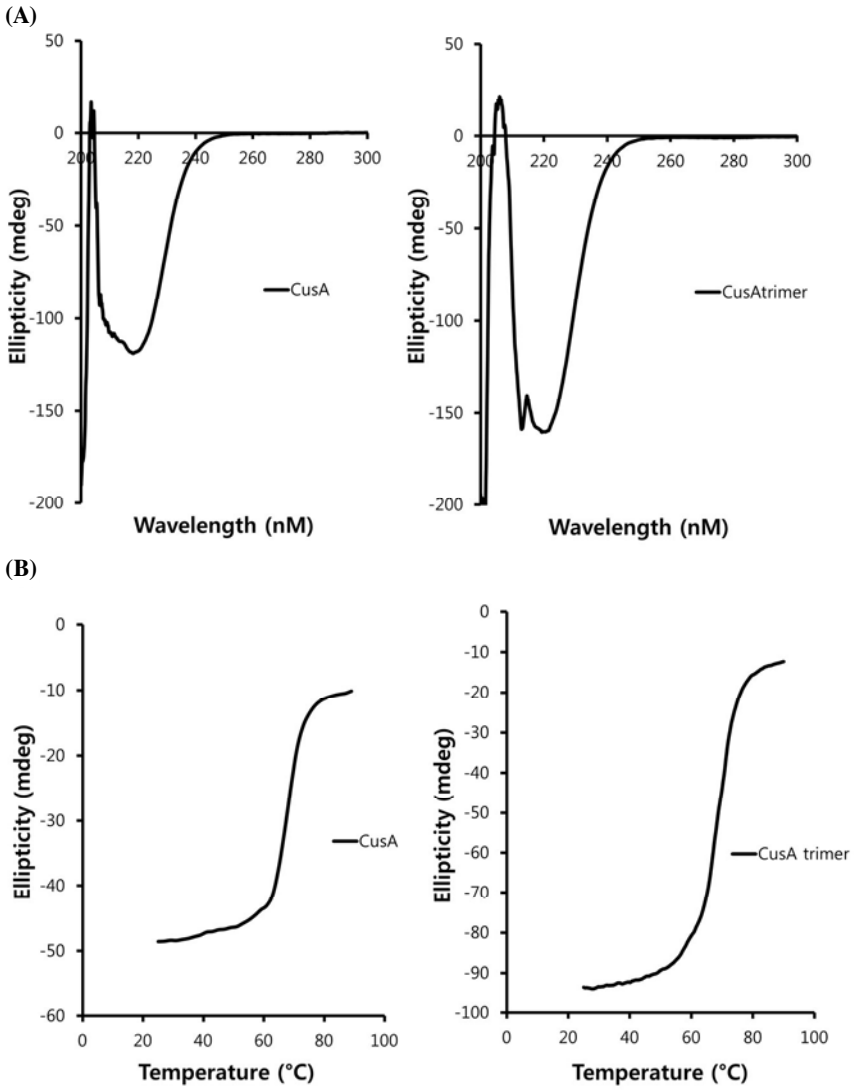

Fig. 3. Analysis of trimeric and monomeric forms of CusA periplasmic domain. (A) CD spectra of the CusA periplasmic domain proteins. The CD spectrum of the monomeric form is shown in the left panel, while that of the trimeric form is in the right panel. (B) Comparison of the melting temperatures (Tm) of the trimeric and monomeric forms of CusA periplasmic domain. The Tm values were measured using CD spectroscopy. The arrows indicate the Tm value of each protein.

membrane protein CusA by truncating the transmembrane domain to facilitate further characterization by soluble expression. We constructed the periplasmic domain of CusA by joining two DNA segments and then successfully expressed and purified the protein; this was similar to the method used in our previous report on outer membrane porin TdeA from *Actinobacillus actinomycetemcomitans*, which is a TolC homologue (Kim *et al*., 2008). The periplasmic domain of CusA behaved as a well-folded protein, as evaluated by the CD spectroscopy. The size exclusion chromatography further suggested that the transmembrane domain is essential for the trimerization of CusA since a majority of the CusA periplasmic domain was eluted as a monomer in solution. We were also able to perform ITC experiments, revealing that the periplasmic domain of CusA has Ag^+ binding sites. Of the four binding sites from the fitted equation, the highest binding affinities had K_d of 7 and 10 μM. Because Ag^+ is one of the substrates of the CusCFAB pump, these metal binding sites are likely the substrate entry or exit sites. Moreover, the differential metal binding affinities suggest that the metal ions might transfer within the metal binding sites. It should be noted that the metal binding affinities could be modulated in the functional state by proton movement, facilitating the expulsion of metal ions.

Table 1. ITC measurements of Ag^+ binding to CusA periplasmic domain

	K_d μM	ΔH kcal/mol	TΔS kcal/mol	ΔG kcal/mol
site 1	6.6	163	170	-6.6
site 2	10	-121	-114	-7.0
site 3	70	-115	-109	-6
site 4	230	-19.8	-14.8	-5

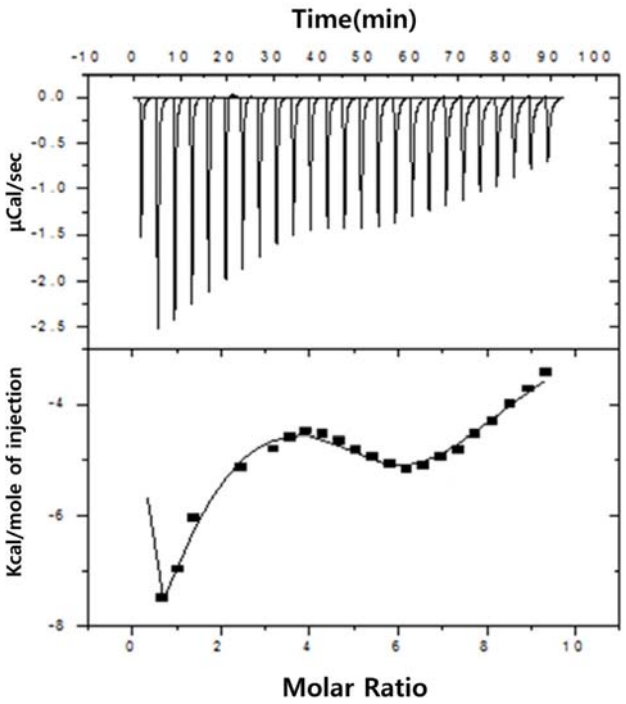

Fig. 4. Representative isotherm for the binding of Ag^+ to CusA periplasmic domain.
Top: raw data output of power (heat released) for each of 25 consecutive injections of $AgNO_3$ (1 mM) into the protein (0.4 μM). *Bottom*: heat exchange at each injection obtained by integration of each injection, normalized to kcal/mol of $AgNO_3$. The computer-generated titration curve is best fit to a model of sequential binding with four sites (solid line).

It was reported that the periplasmic adaptor CusB and the periplasmic chaperone CusF also have Cu^+ or Ag^+ binding sites. Since the binding affinities are similar (both ~30 nM K_d values), the metal ion was able to be transferred from CusF to CusB (Franke *et al*., 2003). Furthermore, its binding to CusB induced a conformational change, suggesting that CusB could have a direct role in substrate efflux and that it does not simply serve as a passive anchor linking CusA and CusC. Based on these results, it was proposed that that the metal ion taken up by CusB is transferred to CusA or that metal binding induces a conformational change to open the outer membrane protein. However, our results may refute the hypothesis of metal ion transfer from CusB to CusA, because the affinity of CusA is much lower than that of CusB (Bagai *et al*., 2007). Thus, the metal ion binding to CusB might be involved in the conformational change of CusB to activate the pump, which is in agreement with the fact that this efflux pump is inactivate when copper ions are not present in excess.

In this study, we successfully expressed the periplasmic region of the multi-span membrane protein CusA, which enabled us to perform the biochemical analysis. From our studies, we conclude that the periplasmic region of CusA has metal ion binding sites that might be linked to substrate entry or exit. Combined with the previous study, our findings aid the understanding of the mechanism of action and of regulation in the tripartite CusCFBA pump.

During the reviewing process of this manuscript, Long *et al.* (2010) published the crystal structures of the full-length CusA in the metal-free and Cu^+ or Ag^+ bound forms. The authors revealed that the metal binding site is located in the periplasmic region of each protomer, as shown in our result. They further demonstrated that the metal binding site is functionally important.

Acknowledgements

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