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Crystal structure of the membrane fusion protein CusB from *Escherichia coli*

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Abstract

Gram-negative bacteria, such as *Escherichia coli*, frequently utilize tripartite efflux complexes belonging to the resistance-nodulation-division family to expel diverse toxic compounds from the cell. These systems contain a periplasmic membrane fusion protein that is critical for substrate transport. We here present the x-ray structures of the CusB membrane fusion protein from the copper/silver efflux system of *E. coli*. This is the first structure of any membrane fusion proteins associated with heavy-metal efflux transporters. CusB bridges the inner membrane efflux pump CusA and outer membrane channel CusC to mediate resistance to Cu⁺ and Ag⁺ ions. Two distinct structures of the elongated molecules of CusB were found in the asymmetric unit of a single crystal, which suggests the flexible nature of this protein. Each protomer of CusB can be divided into four different domains, whereby the first three domains are mostly β-strands and the last domain adopts an entirely helical architecture. Unlike other known structures of membrane fusion proteins, the α-helical domain of CusB is folded into a three-helix bundle. This three-helix bundle presumably interacts with the periplasmic domain of CusC. The N and C-termini of CusB form the first β-strand domain, which is found to interact with the periplasmic domain of the CusA efflux pump. Atomic details of how this efflux protein binds Cu⁺ and Ag⁺ were revealed by the crystals of the CusB-Cu(I) and CusB-Ag(I) complexes. The structures indicate that CusB consists of multiple binding sites for these metal ions.

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Accession Numbers

Coordinates and structural factors have been deposited in the Protein Data Bank with accession numbers 3H9I (apo-CusB), 3H9T (CusB-Cu(I)) and 3H94 (CusB-Ag(I)).

These findings reveal novel structural features of a membrane fusion protein in the resistance-nodulation-division efflux system, and provide evidence that this protein specifically interacts with transported substrates.

Introduction

Silver is a heavy metal with relatively high toxicity to prokaryotes. Ionic silver exhibits antimicrobial activity against a broad range of microorganisms, and is used widely as an effective antimicrobial agent to combat pathogens.^{1,2} Copper, although required in trace amounts for bacterial growth, is highly toxic even at low concentrations.³ Thus, both silver and copper are well-known bactericides, and their biocidal effects have been used for centuries. It has been shown that silver and copper ions effectively eliminate *Legionella* in drinking water pipelines.⁴ Silver and copper ions are capable of penetrating biofilms that build up in hospital plumbing, destroying entrenched *Legionella* and other pathogenic organisms.^{4,5} In addition, silver cations are commonly employed in treating patients with burns, wounds, eye infections and ulcers.¹ To date, the antimicrobial uses of ionic copper have been expanded to include fungicides, antifouling paints, antimicrobial medicines and antiseptics.⁶ Because of the widespread use of silver and copper as antimicrobial agents, the presence of silver and copper resistant bacterial strains appear to be on the rise.^{1,2,7-9}

Bacteria, such as *Escherichia coli*, have developed various mechanisms to overcome toxic environments that are unfavorable to their survival. One important strategy that bacteria use to subvert toxic compounds, including toxic metal ions such as Ag^+ and Cu^+ , is the expression of membrane efflux transporters that recognize and actively export these compounds out of bacterial cells, thereby allowing them to survive in extremely toxic conditions. In Gram-negative bacteria, efflux systems of the resistance-nodulation-division (RND) family play major roles in the intrinsic and acquired tolerance of antibiotics and toxic compounds.^{10,11} As a Gram-negative bacterium, *E. coli* contains seven different RND efflux transporters. Six of these transporters, including AcrB, AcrD, AcrF, MdtB, MdtC and YhiV, are multidrug efflux pumps. They belong to the hydrophobic and amphiphilic efflux RND (HAE-RND) protein family.¹⁰ *E. coli* consists of only one heavy-metal efflux RND (HME-RND) transporter, CusA, which specifically recognizes and confers resistance to Ag(I) and Cu(I) ions.^{12,13}

Typically, an RND transporter works in conjunction with a periplasmic component, belonging to the membrane fusion protein (MFP) family,^{14,15} and an outer membrane channel to form a functional protein complex.¹⁶ The resulting tripartite efflux system spans the inner and outer membranes of Gram-negative bacterium to export substrates directly out of the cell.¹⁶ For the CusA inner membrane transporter, it interacts with the periplasmic membrane fusion protein CusB and the outer membrane channel CusC to form the CusABC tripartite efflux complex.^{12,13} Heavy-metal efflux by CusABC is driven by proton import. This process is catalyzed through the inner membrane transporter CusA.

Among all known RND family of transporters, the *E. coli* AcrB¹⁷⁻²⁰ and *Pseudomonas aeruginosa* MexB²¹ HAE-RND pumps are the only two membrane proteins that have been crystallized. These proteins span the entire width of the inner membrane and protrude approximately 70-Å into the periplasm. The crystal structures of the outer membrane channels, *E. coli* TolC and *P. aeruginosa* OprM, have also been determined.^{22,23} TolC is anchored in the outer membrane and forms a 100-Å-long periplasmic α -helical tunnel.²² The *P. aeruginosa* OprM channel possesses a similar elongated α -helical tunnel that projects into the periplasmic space.²³ Recently, two structures of the periplasmic membrane fusion proteins, *E. coli* AcrA²⁴ and *P. aeruginosa* MexA,²⁵⁻²⁷ associated with the HAE-RND transporters have been solved. The structures suggest that these two periplasmic proteins are folded into

elongated secondary structures that consist of ~47-Å-long α -hairpin domain, presumably interacting with the α -helical tunnels of their corresponding outer membrane channels. Further, the N and C-terminal ends of these membrane fusion proteins are thought to contact their respective inner membrane transporters, creating a functional complex that spans both membranes.

Currently, no structural information is available for any components of the HME-RND tripartite efflux complex. Presumably, the three components of the HME-RND system form a tripartite complex that resembles the AcrAB-TolC complex. Different from the HAE-RND family, members of the HME-RND family are highly substrate specific, with the ability to differentiate between monovalent and divalent ions. As an initial step to examine the mechanisms used by the CusABC efflux system to facilitate Ag(I) and Cu(I) ions recognition and extrusion, we here describe the crystal structures of the periplasmic membrane fusion protein CusB in the absence and presence of these metal ions.

Results

Overall structure of CusB

We cloned, expressed and purified the full-length CusB protein containing a 6xHis tag at the C-terminus. We obtained crystals of the *E. coli* CusB efflux protein in detergent following an extensive screening for crystallization conditions. We then used the multiple-wavelength anomalous dispersion method to solve the selenomethionyl-substituted (SeMet) CusB crystal structure. The resulting experimental electron density maps shown in Figure 1 revealed that the asymmetric unit of the CusB crystal consists of two protomers (labeled A and B). These two molecules are related to each other by an approximately twofold symmetry. The native crystal structure of CusB was then determined to a resolution of 3.4 Å (Table 1), revealing that the A molecule of CusB is folded into an elongated polypeptide of ~121 Å long and ~37 Å wide; whereas the dimensions of the B molecule are ~116 Å long and ~40 Å wide. The mature protein of CusB consists of 379 amino acids (residues 29 through 407). Currently, 78.1% of the residues (residues 89–385) are included in our final model.

Intriguingly, the crystal structure suggested that each elongated molecule of CusB can be divided into four different domains (Figure 2). The first three domains of the protein are mostly β -strands. However, the fourth domain forms an all α -helical domain, which is folded into a three-helix bundle secondary structure. Alignment of amino acid sequences indicates that CusB shares 13% identity and 52% similarity to that of MexA. The alignment also shows an overall identity and homology of 16% and 54% to AcrA, respectively. Because of the relatively low sequence identity, it is not surprising that the crystal structure of CusB is quite different from the known structures of MexA^{25–27} and AcrA.²⁴

The β -strand domains

As mentioned earlier, each CusB molecule consists of three different β -domains. The first β -domain (Domain 1) is formed by the N and C-terminal ends of the polypeptide (residues 89–102 and 324–385). Presumably, this domain is located directly above the outer-leaflet of the inner membrane and interacts with the CusA efflux pump. Overall, Domain 1 in molecule A is a β -barrel domain. It is composed of six β -strands, with the N-terminal end forming one of the β -strands while the C-terminus of the protein constitutes the remaining five strands (Figure 2). Domain 1 in molecule B of CusB exhibits an almost identical structure, which is also folded into a six β -barrel domain.

The second β -domain (Domain 2) of CusB is formed by residues 105–115 and 243–320. In molecule A, this domain consists of six β -strands and one short α -helix. Again, the N-terminal

residues form one of the β -strands that is incorporated into this domain. The C-terminal residues contribute a β -strand, an α -helix and four anti-parallel β -sheets. In molecule B, this domain differs by assembling into six β -strands and two short α -helices. In an asymmetric unit of the crystal, Domains 1 and 2 of molecule A interact closely with Domains 2 and 1 of molecule B, respectively (Figure 1a).

Domain 3 is another globular β -domain adjacent to the second domain of molecule A. This domain consists of residues 121–154 and 207–239, with a majority of these residues folding into eight β -strands. Similar to that of molecule A, the corresponding domain in molecule B is also an eight β -barrel structure.

The α -helical domain

Perhaps the most interesting motif appears to be in the fourth domain (Domain 4) of CusB. This region forms an all-helical domain. In molecule A, this α -domain comprises residues 156–205. Surprisingly, this domain is folded into an anti-parallel, three-helix bundle. This structural feature, not found in other known protein structures in the MFP family, highlights the uniqueness of the CusB protein. The helix bundle creates an ~ 27 Å long helix-turn-helix-turn-helix secondary structure, making it at least 20 Å shorter than the two-helical hairpin domains of MexA^{25–27} and AcrA.²⁴ Domain 4 of molecule B exhibits a similar three-helix bundle motif when compared to the secondary structure of molecule A. To date, CusB is the only periplasmic protein in the MFP family that possesses this three-helical domain instead of a two-helical hairpin motif. The overall structure of CusB is quite distinct from the known structures of other membrane fusion proteins.

It is interesting to note that both crystals of CusB and MexA²⁷ contain two copies of the molecules, each of which consists of four different domains. We superimposed these protein structures and observed that the pair containing molecule A of CusB and the “unrotated” molecule of MexA displays the closest topological similarity (Figure 3). A pairwise alignment of these two structures cannot be calculated because their detailed secondary structures are quite different. We tried to superimpose the α -domains (Domain 4 of CusB and the α -hairpin domain of MexA) of these two structures together and found that these two α -domains cannot be aligned. However, their individual β -domains can be superimposed separately. For example, superposition of Domain 1 of CusB with the membrane proximal domain of MexA gives an overall rmsd of 2.4 Å calculated over the C $^{\alpha}$ atoms. Domain 2 of CusB and the β -barrel domain of MexA can also be superimposed, resulting in an overall rmsd of 2.0 Å. Likewise, Domain 3 of CusB can be paired up with the lipoyl domain of MexA, and this superimposition results in an overall rmsd of 1.8 Å.

Conformational flexibility of CusB

Two distinct conformations of CusB were captured in the single crystal, suggesting that the membrane fusion protein is quite flexible in nature. A comparison of the A and B molecules of CusB indicates that these two molecules are quite different, presumably representing two transient states of the membrane fusion protein. However, the two conformations are related, whereby a small hinge motion is attributed to the differences. Superimposition of these two molecules gives an overall rmsd of 2.6 Å calculated over the C $^{\alpha}$ atoms. Comparison of these two structures reveals that molecule A adopts a more open conformation, while molecule B exhibits a relatively compact form of the structure (Figure 4). Thus, these two molecules may correspond to the open and closed states of the membrane fusion protein.

It appears that Domains 1 + 2 of molecules A and B can easily be superimposed with high structural similarity, giving an overall rmsd of 0.8 Å (168 C $^{\alpha}$ atoms). Superposition of Domains 3 + 4 alone of these two molecules can also be calculated, showing an overall rmsd of 0.8 Å

(118 C α atoms). Using Domain 1 + 2 as references, the α -helical domains of molecules A and B are found to differ by $\sim 21^\circ$ overall (Figure 4a). When Domains 3 + 4 are superimposed, the orientation of the β -barrels of Domain 1 in molecules A and B display an overall shift of $\sim 23^\circ$ (Figure 4b). Taken together, these superimpositions suggest that the linker region, which is composed of two loops (residues 116–120 and 240–242) between Domains 2 and 3, forms a flexible hinge in the membrane fusion protein. This hinge region effectively permits the protein to shift from one conformation to another simply by performing a rigid-body movement at Domains 1 + 2 with respect to Domains 3 + 4. The two structures of CusB found in a single crystal indeed underscore the conformational flexibility of this membrane fusion protein.

Structures of the CusA-Cu(I) and CusA-Ag(I) complexes

To identify the metal binding sites of CusB, we prepared the CusB-Cu(I) and CusB-Ag(I) crystal complexes by soaking these metal ions into the apo-CusB crystals. Table 1 also demonstrates the crystallographic data of these metal complexes. The overall structures of these complexes are very similar to those of apo-CusB. For example, superimposition of molecule A of CusB-Cu(I) to that of apo-CusB shows an overall rmsd of 0.8 Å. The structure of molecule B of CusB-Cu(I) can also be easily superimposed to molecule B of apo-CusB, giving a 0.8 Å overall rmsd.

Four strong peaks at the copper edge were observed in the CusB-Cu(I) crystal, indicating the presence of four Cu $^{+}$ binding sites in the asymmetric unit. Two of these copper-binding sites (designated as C1 and C2) were found in molecule A (Figure 5). Cu $^{+}$ in site C1 of molecule A is located in the first domain formed by the N and C-termini of the protein. Coordinating with the bound Cu $^{+}$ ion at this site are M324, F358 and R368. Site C1 is located near the bottom of the elongated CusB molecule. Presumably, this region may interact directly with the periplasmic domain of the CusA efflux pump. The binding of Cu $^{+}$ in site C2 of molecule A is located close to the center of the three-helix bundle in Domain 4. This α -helical domain may make a direct contact with the outer membrane channel CusC. Cu $^{+}$ in this location is bound by M190, W158 and Q162. The remaining two copper-binding sites (designated as C1' and C2') were identified within molecule B of CusB (not shown). The binding of Cu $^{+}$ ions at sites C1' and C2' are the same as those in C1 and C2 of molecule A, respectively.

For the CusB-Ag(I) complex crystal, we have found two anomalous difference Fourier peaks in the asymmetric unit. One of the Ag $^{+}$ binding sites, designated as A1, is found right next to M324 of molecule A (Figure 5). It appears that the location of this Ag $^{+}$ -binding site is the same as that of site C1 for Cu $^{+}$ -binding. Thus, the bound Ag $^{+}$ at site A1 is coordinated with M324, F358 and R368. Ag $^{+}$ at the other site, designated as A1', is nearby M324 of molecule B (not shown). The binding mode for Ag $^{+}$ ion at site A1' in molecule B is the same as that for site A1 in molecule A. Thus, Ag $^{+}$ is bound by M324, F358 and R368.

Discussion

In this study, we presented the crystal structure of the membrane fusion protein CusB, an essential component in the CusABC efflux system which extrudes silver and copper ions from *E. coli*. This is the first structure of any membrane fusion protein that is associated with an HME-RND-type transporter. Currently, CusB and MexA²⁷ exhibit the most complete three-dimensional structures among those resolved for membrane fusion proteins, including AcrA²⁴ and MacA.²⁸ The overall structures of MexA, AcrA and MacA are very similar to each other. For example, a superposition of 183 C α atoms of AcrA with their corresponding residues in the MexA structure gives an overall rmsd of 0.8 Å.²⁴ CusB, however, is folded into a distinct secondary structure when compared with the current crystal structures of other membrane fusion proteins. Like MexA, the structure of CusB revealed that this membrane fusion protein consists of four major domains, including three β -strand domains and one α -helical domain.

Strikingly, the α -helical domain of CusB features a three-helix bundle which contrasts other structures of membrane fusion proteins. This structural feature, not found in other members of the MFP family, may underscore the unique functionality of CusB. The distinct secondary structure of CusB may also imply that its tripartite partners, the inner membrane transporter CusA and the outer membrane channel CusC, may also possess unique secondary structural features that distinguish them from the existing structures of their homologous proteins. Exactly how these individual heavy-metal efflux components assemble into a functional complex must await the elucidation of the CusA and CusC structures.

To determine how CusB interacts with the CusA efflux pump and the relative orientation of CusB in the efflux complex, we cloned, expressed and purified the full-length *E. coli* CusA membrane protein that contains a 6xHis tag at the N-terminus. We then cross-linked the purified CusA and CusB proteins using the lysine-lysine cross-linker disuccinimidyl suberate (DSS). The resulting product was digested with trypsin and examined using LC-MS/MS. Analysis of the mass spectral data suggests that the lysine residue of the polypeptide β (IDPTQTQNLGVKTATVTR) originating from the N-terminal residues (84–101) of CusB directly interacts with the lysine residue of peptide α (SGKHDLADLR) which belongs to the periplasmic domain (residues 148–157) of the CusA efflux pump (see Suppl. Fig. S1 and Table S1). Although the CusA and AcrB efflux pumps only share 19% protein sequence identity, we generated a structural model of the CusA transporter based on the crystal structure of AcrB and alignment of protein sequences of these two transporters (Figure 6). The model indicates that polypeptide α (residues 148–157 of CusA) is located directly above the vestibule region of CusA, facing the periplasm. This location should correspond to the PN2 region of AcrB.¹⁷ If this is the case, the C-terminus of CusB should interact with CusA at a position corresponding to the PC1 region in AcrB (Figure 6). According to the most recently determined MexA structure,²⁷ it is suggested that both the N and C-terminal ends of MexA are located close to the MexB transporter. In addition, *in vivo* cross-linking studies also demonstrated that the N and C-termini of AcrA directly interact with PN2 and PC1 of the periplasmic domain of AcrB, respectively.²⁷ Thus, together with the crystal structure of CusB and the mass spectrometric data, we suggest that Domain 1, formed by the N and C-terminal ends, of CusB should interact with the periplasmic domain of the CusA transporter.

The crystal structure of CusB demonstrated that this protein exists in two distinct conformations, one of which presents a more open form while the other exhibits a more compact structure. In the crystal lattice, these two molecules (molecules A and B) interact with one another to form a dimer. It should be noted that this dimeric arrangement might not be biologically relevant because, similar to the other isolated membrane fusion proteins associated with the HAE-RND family, *in vitro* study indicated that CusB exists as a monomer in solution.²⁹ The fact that two copies of CusB have been found in a single crystal highlights the conformational flexibility of this membrane fusion protein. The flexible nature of these membrane fusion proteins has also been observed with MexA, AcrA and MacA. There is a good chance that conformational flexibility is a common feature among members of the MFP family. Indeed, four different conformations of AcrA have been identified within a single crystal.²⁴ It is interesting to note that the linker region between the membrane proximal and β -barrel domains of MexA seemingly forms a flexible hinge to allow this protein to flip from the “unrotated” form to the “rotated” state. This flexible linker should be related to the linker region between Domain 1 and Domain 2 in the CusB structure. In the case of AcrA, the flexible hinge between the α -helical hairpin and lipoyl domain (corresponds to the linker region between Domains 3 and 4 of CusB) is attributed to the freedom of this protein. For CusB, the crystal structure indicates that the flexible hinge is located between Domains 2 and 3 of the protein. Thus, it is likely that all of these linker regions located between different domains of the membrane fusion protein potentially can provide flexibility for the protein to perform its biological function.

The full-length CusB membrane fusion protein containing a 6xHis tag at the C-terminus was overproduced in *E. coli* BL21(DE3) cells possessing pET15b Ω cusB. Briefly, cells were grown in 12 L of Luria Bertani (LB) medium containing 100 μ g/ml ampicillin at 37°C. The culture was induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) at OD₆₀₀ value of approximately 0.5. Cells were harvested within 4 h of induction. The collected bacteria were resuspended in ice-cold buffer containing 20 mM Na-HEPES (pH 7.2) and 100 mM NaCl. The cells were then lysed in a French pressure cell. Cell debris was removed by centrifugation for 45 min at 4°C and 20,000 rev/min. The crude lysate was collected and 0.5% *n*-dodecyl- β -D-maltoside (DDM) was added into the protein solution. The protein solution was then purified with Ni²⁺-affinity and G-200 sizing columns. The purity of the protein (> 95%) was judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. The N-terminal sequence of the CusB protein was confirmed by sequencing. The purified CusB protein was then concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.04% DDM for crystallization.

For 6xHis SeMet-CusB protein expression, a 10 ml LB broth overnight culture containing *E. coli* B834/ pET15b Ω cusB cells was transferred into 120 ml of LB broth containing 100 μ g/ml ampicillin and grown at 37°C. When the OD₆₀₀ value reached 1.2, cells were harvested by centrifugation at 6000 rev/min for 10 min, and then washed two times with 20 ml of M9 minimal salts solution. The cells were re-suspended in 120 ml of M9 media and then transferred into a 12 L pre-warmed M9 solution containing 100 μ g/ml ampicillin. The cell culture was incubated at 37°C with shaking. When the OD₆₀₀ reached 0.4, 60 mg/l of L-selenomethionine were added. The culture was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) after 15 min. Cells were then harvested within 4 h after induction. The procedures for purifying the 6xHis SeMet-CusB were identical to those of the native protein.

Crystallization, data collection, structural determination and refinement

Crystals of the 6xHis CusB were obtained using hanging-drop vapor diffusion. The CusB crystals were grown at room temperature in 24-well plates with the following procedures. A 2 μ l protein solution containing 20 mg/ml CusB protein in 20 mM Na-HEPES (pH 7.5) and 0.04% (w/v) DDM was mixed with a 2 μ l of reservoir solution containing 15% PEG 1000, 0.1 M Na-citrate (pH 5.6), 0.36 M Na-citrate, 5% isopropanol and 5% glycerol. The resultant mixture was equilibrated against 500 μ l of the reservoir solution. The crystallization conditions for SeMet-CusB were the same as those for the native CusB protein. Crystals of CusB grew to a full size in the drops within a month. Typically, the dimensions of the crystals were 0.4 mm $\times$ 0.4 mm $\times$ 0.1 mm. Cryoprotection was achieved by raising the PEG 1000 concentration stepwise to 30% with a 5% increment in each step.

The CusB-Cu(I) complex crystals were prepared by incubating crystals of apo-CusB in solution containing 15% PEG1000, 0.1 M Na-citrate (pH 5.6), 0.36 M Na-citrate, 5% isopropanol, 5% glycerol, 2 mM [Cu(CH₃CN)₄]PF₆, 2 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.04% (w/v) DDM for 6 hours at 25°C. Crystals of the CusB-Ag(I) complex were prepared using the same method in solution containing 15% PEG1000, 0.1 M Na-citrate (pH 5.6), 0.36 M Na-citrate, 5% isopropanol, 5% glycerol, 1 mM Ag(NH₃)₂⁺ and 0.04% (w/v) DDM for 5 hours at 25°C.

The diffraction data sets of both apo native and SeMet-CusB were collected at 100 K in the Advanced Photon Source (beamline 24-ID-C) using an ADSC Quantum 315 CCD-based detector. Diffraction data were processed with DENZO and scaled with SCALEPACK.³⁷ Both the native and SeMet crystals took the space group of *I*222, with unit cell parameters very similar to each other. The structure of CusB was determined by the multiple-wavelength anomalous dispersion (MAD) method. The three wavelengths of the MAD data were cross-scaled, and 10 selenium sites were identified in the asymmetric unit using SHELXD³⁸ in the

HKL2MAP package.³⁹ Phase refinement was carried out using the program AutoSHARP.⁴⁰ The electron density map was then subjected to density modification using the program RESOLVE.⁴¹ After tracing the initial model manually in the program Coot,⁴² the model was then refined against the native data at 3.4 Å resolution using CNS⁴³ and PHENIX.⁴⁴

Diffraction data sets for both CusB-Cu(I) and CusB-Ag(I) were collected at 100K in the Advanced Light Source (beamline 5.0.2) using an ADSC Quantum 315 CCD-based detector. The structures of both the CusB-Cu(I) and CusB-Ag(I) complexes were determined using molecular replacement (MR). The initial phases were calculated using the structure of apo-CusB. Model refinements were achieved using CNS⁴³ and PHENIX.⁴⁴

Cloning, expression and purification of CusA

The ORF of *cusA* from *E. coli* K12 chromosomal DNA was amplified by PCR using the primers 5'-AAACATATGATTGAATGGATTATTCGTCGCTCGGTGG-3' and 5'-AAACTCGAGTTATTTCCGTACCCGATGTCGGTGCAGC-3' to generate a product that would encode a CusA recombinant protein with a 6xHis tag at the N-terminus. Engineered restriction sites and the 6xHis-encoding sequence were designed in the primers. The 3,146 bp PCR fragment of the *cusA* gene with flanking sequences was extracted from the agarose gel using a gel-extraction kit (Qiagen) and then digested with *NdeI* and *XhoI* (New England Biolabs). The digested products were ligated into pET15b (Novagen) to form the expression vector, pET15b Ω *cusA*. The recombinant plasmid was transformed into DH5 α cells and transformants were selected on LB agar plates containing 100 µg/ml ampicillin. The presence of the correct *cusA* sequence in the plasmid construct was verified by DNA sequencing.

Briefly, the full-length CusA membrane protein containing a 6xHis tag at the N-terminus was overproduced in *E. coli* BL21(DE3) Δ *acrB* cells, which harbors a deletion in the chromosomal *acrB* gene, possessing pET15b Ω *cusA*. Cells were grown in 12 L of LB medium with 100 µg/ml ampicillin at 37°C. When the OD₆₀₀ reached 0.5, the culture was treated with 1 mM IPTG to induce *cusA* expression, and harvested within 3 h. The collected bacteria were resuspended in low salt buffer containing 100 mM sodium phosphate (pH 7.2), 10 % glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethanesulfonyl fluoride (PMSF), and then disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10 % glycerol, 1 mM EDTA and 1 mM PMSF, and once with 20 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM PMSF.¹⁸ The membrane protein was then solubilized in 1 % (w/v) DDM. Insoluble material was removed by ultracentrifugation at 370,000 × g. The extracted protein was purified with Ni²⁺-affinity and G-200 sizing columns. The purity of the CusA protein (>95%) was judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. The purified protein was then concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.05% DDM.

Chemical cross-linking and mass spectrometry

A 50 µl protein mixture consisting of 10 pmol/µl the purified CusA and CusB (1:1 molar ratio) in buffer containing 20 mM Na-HEPES pH 7.5 and 0.05% (w/v) DDM was cross-linked by the lysine-lysine cross-linker, disuccinimidyl suberate (DSS), with a final protein-to-DSS molar ratio of 1:50 at 35°C for 30 min and then quenched with ammonium bicarbonate at a final concentration of 50 mM. The cross-linked product was loaded into an 8% SDS-PAGE. Thereafter, gel-bands at molecular weights ≥ 500 kDa were selected and digested with trypsin overnight at 37°C. The resulting mixture was loaded into LC-MS/MS. Mass spectrometric data were acquired using a high-resolution mass spectrometer (LTQ-FT; Thermo Scientific, San Jose, CA) with 120 min gradient reverse phase LC separation (UPLC; Waters, Milford, MA). Analysis of the data was performed using the program X!Link.⁴⁵ We identified a total of five

cross-linked peptide pairs. Three of these lysine-lysine cross-linked peptides were identified as CusA-CusA cross-links, and one of these peptides was found to be a CusB-CusB cross-link. We have identified one CusA-CusB lysine-lysine cross-linked peptide that involves a cross-link between residue K95 of the polypeptide β , IDPTQTQNLGVKTATVTR (from CusB), and residue K150 of the polypeptide α , SGKHDLADLR (from CusA).

Supplementary Material

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Acknowledgments

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References

1. Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol. Rev* 2003;27:341–353. [PubMed: 12829274]
2. Percival SL, Bowler PG, Russell D. Bacterial resistance to silver in wound care. *J. Hosp. Infect* 2005;60:1–7. [PubMed: 15823649]
3. Ercal N, Gurer-Orhan H, Aykin-Burns N. Toxic metals and oxidative stress part I: mechanisms involved in metal induced oxidative damage. *Curr. Topics Med. Chem* 2001;1:529–539.
4. Stout JE, Yu VL. Experiences of the first 16 hospitals using copper-silver ionization for *Legionella* control: implications for the evaluation of other disinfection modalities. *Infect. Control Hosp. Epidemiol* 2003;24:563–568.
5. Rohr U, Senger M, Selenka F, Turley R, Wilhelm M. Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system. *Clin. Infect. Dis* 1999;29:1507–1511. [PubMed: 10585804]
6. <http://www.cda.org.uk/antimicrobial/>
7. Silver S, Phung LT, Silver G. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *J. Ind. Microbiol. Biotechnol* 2006;33:627–634. [PubMed: 16761169]
8. Chopra I. The increase use of silver-based products as antimicrobial agents: a useful development or a cause for concern? *J. Antimicrob. Chemother* 2007;59:587–590. [PubMed: 17307768]
9. Nies DH. Microbial heavy metal resistance. *Appl. Microbiol. Biotechnol* 1999;51:730–750. [PubMed: 10422221]
10. Tseng TT, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier MH Jr. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development protein. *J. Mol. Microbiol. Biotechnol* 1999;1:107–125. [PubMed: 10941792]
11. Nies DH. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol. Rev* 2003;27:313–339. [PubMed: 12829273]
12. Franke S, Grass G, Nies DH. The product of the *ybdE* gene of the *Escherichia coli* chromosome is involved in detoxification of silver ions. *Microbiol* 2001;147:965–972.
13. Franke S, Grass G, Rensing C, Nies DH. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J. Bacteriol* 2003;185:3804–3812. [PubMed: 12813074]
14. Saier MH Jr, Tam R, Reizer A, Reizer J. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol. Microbiol* 1994;11:841–847. [PubMed: 8022262]

15. Dinh T, Paulsen IT, Saier MH Jr. A family of extra-cytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. *J. Bacteriol* 1994;176:3825–3831. [PubMed: 8021163]
16. Thanassi DG, Suh GS, Nikaido H. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. *J. Bacteriol* 1995;177:998–1007. [PubMed: 7860612]
17. Murakami S, Nakashima R, Yamashita E, Yamaguchi A. Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* 2002;419:587–593. [PubMed: 12374972]
18. Yu EW, McDermott G, Zgruskaya HI, Nikaido H, Koshland DE Jr. Structural basis of multiple drug binding capacity of the AcrB multidrug efflux pump. *Science* 2003;300:976–980. [PubMed: 12738864]
19. Murakami S, Nakashima R, Yamashita E, Matsumoto T, Yamaguchi A. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature* 2006;443:173–179. [PubMed: 16915237]
20. Seeger MA, Schiefner A, Eicher T, Verrey F, Dietrichs K, Pos KM. Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. *Science* 2006;313:1295–1298. [PubMed: 16946072]
21. Sennhauser G, Bukowska MA, Briand C, Grütter MG. Crystal structure of the multidrug exporter MexB from *Pseudomonas aeruginosa*. *J. Mol. Biol* 2009;389:134–145. [PubMed: 19361527]
22. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* 2000;405:914–919. [PubMed: 10879525]
23. Akama H, Kanemaki M, Yoshimura M, Tsukihara T, Kashiwag T, Yoneyama H, Narita S, Nakagawa A, Nakae T. Crystal structure of the drug discharge outer membrane protein, OprM, of *Pseudomonas aeruginosa*. *J. Biol. Chem* 2004;279:52816–52819. [PubMed: 15507433]
24. Mikolosko J, Bobyk K, Zgurskaya HI, Ghosh P. Conformational flexibility in the multidrug efflux system protein AcrA. *Structure* 2006;14:577–587. [PubMed: 16531241]
25. Higgins MK, Bokma E, Koronakis E, Hughes C, Koronakis V. Structure of the periplasmic component of a bacterial drug efflux pump. *Proc. Natl. Acad. Sci. USA* 2004;101:9994–9999. [PubMed: 15226509]
26. Akama H, Matsuura T, Kashiwag S, Yoneyama H, Narita S, Tsukihara T, Nakagawa A, Nakae T. Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. *J. Biol. Chem* 2004;279:25939–25942. [PubMed: 15117957]
27. Symmons M, Bokma E, Koronakis E, Hughes C, Koronakis V. The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proc. Natl. Acad. Sci. USA* 2009;106:7173–7178. [PubMed: 19342493]
28. Yum S, Xu Y, Piao S, Sim S-H, Kim H-M, Jo W-S, Kim K-J, Kweon H-S, Jeong M-H, Lee K, Ha N-C. Crystal structure of the periplasmic component of a tripartite macrolide-specific efflux pump. *J. Mol. Biol* 2009;387:1286–1297. [PubMed: 19254725]
29. Bagai I, Liu W, Rensing C, Blackburn NJ, McEvoy MM. Substrate-linked conformational change in the periplasmic component of a Cu(I)/Ag(I) efflux system. *J. Biol. Chem* 2007;282:35695–35702. [PubMed: 17893146]
30. Xue Y, Davis AV, Balakrishnan G, Stasser JP, Staehlin BM, Focia P, Spiro TG, Penner-Hahn JE, O'Halloran TV. Cu(I) recognition via cation- π and methionine interactions in CusF. *Nature Chem. Biol* 2008;4:107–109. [PubMed: 18157124]
31. Loftin IR, Franke S, Blackburn NJ, McEvoy MM. Unusual Cu(I)/Ag(I) coordination of *Escherichia coli* CusF as revealed by atomic resolution crystallography and x-ray absorption spectroscopy. *Protein Sci* 2007;16:2287–2293. [PubMed: 17893365]
32. Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran TV, Mondragón A. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* 2003;301:1383–1387. [PubMed: 12958362]
33. Arnesano F, Banci L, Bertini I, Huffman DL, O'Halloran TV. Solution structure of the Cu(I) and apo-forms of the yeast metallochaperone, Atx1. *Biochemistry* 2001;40:1528–1539. [PubMed: 11327811]
34. Prigge ST, Kolhekar AS, Eipper BA, Mains RE, Amzel LM. Substrate-mediated electron transfer in peptidylglycine α -hydroxylating monooxygenase. *Nature Struct. Biol* 1999;6:976–983. [PubMed: 10504734]

35. Evans JP, Ahn K, Klinman JP. Evidence that dioxygen and substrate activation are tightly coupled in dopamine β -monooxygenase. *J. Biol. Chem* 2003;278:49691–49698. [PubMed: 12966104]
36. Borges-Walmsley MI, Beauchamp J, Kelly SM, Jumel K, Candlish D, Harding SE, Price NC, Walmsley AR. Identification of oligomerization and drug-binding domains of the membrane fusion protein EmrA. *J. Biol. Chem* 2003;278:12903–12912. [PubMed: 12482849]
37. Otwinowski Z, Minor M. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
38. Schneider TR, Sheldrick GM. Substructure solution with SHELXD. *Acta Crystallogr* 2002;D58:1772–1779.
39. Pape T, Schneider TR. HKL2MAP: a graphical user interface for macromolecular phasing with SHELX programs. *J Appl Crystallogr* 2004;37:843–844.
40. Terwilliger TC, Berendzen J. Automated MAD and MIR structure solution. *Acta Crystallogr* 1999;D55:849–861.
41. Terwilliger TC. Maximum-likelihood density modification using pattern recognition of structural motifs. *Acta Cryst* 2001;D57:1755–1762.
42. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr* 2004;D60:2126.
43. Brünger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Cryst* 1998;D54:905–921.
44. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCroy AJ, Moriarty NW, et al. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr* 2002;58:1948–1954.
45. Lee YJ, Lachner L, Nunnari J, Phinney BS. Shotgun cross-linking analysis for studying quaternary and tertiary protein structures. *J. Proteome. Res* 2007;6:3908–3917. [PubMed: 17854217]

Figure 1a

Figure 1b

Figure 1. Stereo view of the experimental electron density map at a resolution of 3.8 Å. (a) The electron density map contoured at 1.2σ is in gray. The C α traces of molecules A and B of CusB are in orange and green, respectively. (b) Representative section of the electron density in the second domain of CusB. The electron density (colored blue) is contoured at the 1.2σ level and superimposed with the final refined model (orange, carbon; red, oxygen; blue, nitrogen).

Figure 2.

Crystal structure of the CusB membrane fusion protein. The structure can be divided into four distinct domains. Domain 1 is formed by the N and C-termini and is located above the inner membrane. The loops between Domains 2 and 3 appear to form an effective hinge to allow the molecule to shift from an open conformation to a more compact structure. Domain 4 is folded into an anti-parallel, three-helix bundle, which is thought to be located near the outer membrane.

Figure 3a

Figure 3b

Figure 3c

Figure 3d

Figure 3.

Structural comparison of the membrane fusion proteins. (a) Superimposition of the crystal structures of CusB (orange) and MexA (purple). (b) Superimposition of Domain 1 of CusB (orange) with the membrane proximal domain of MexA (purple). (c) Superimposition of Domain 2 of CusB (orange) with the β -barrel domain of MexA (purple). (d) Superimposition of Domain 3 of CusB (orange) with the lipoyl domain of MexA (purple).

Figure 4a

Figure 4b

Figure 4.

Comparison of the two conformations of CusB observed in the crystal. (a) Superposition of Domains 1 + 2 of molecule A onto Domains 1 + 2 of molecule B, displaying an $\sim 21^\circ$ overall shift of the three-helix bundle of Domain 4. (b) Superposition of Domains 3 + 4 of molecule A onto Domains 3 + 4 of molecule B, displaying an overall shift of the β -strands of Domain 1 by $\sim 23^\circ$.

Figure 5.

Cu⁺ and Ag⁺ binding sites of molecule A of CusB. Cu⁺ and Ag⁺ ions are represented by purple and green spheres, respectively. The overall locations of sites C1, C2 and A1 are circled. Anomalous difference Fourier maps are contoured at 4.6 σ , 4.0 σ and 4.2 σ for sites C1, C2 and A1, respectively. Anomalous peak heights for sites C1', C2' and A1' in molecule B of CusB (not shown) were found to be 4.6 σ , 4.6 σ and 5.4 σ , respectively.

Figure 6.

Specific interaction between CusA and CusB. The model of CusA (gray) was created based on protein sequence alignment and the crystal structure of AcrB. Mass spectral data suggest that the periplasmic domain of CusA specifically interacts with the N-terminus of CusB (light brown). Polypeptides α and β were in red and blue, respectively.

Table 1

Data collection, phasing and structural refinement statistics.

Data Collection		Native	SeMet remote	SeMet peak	SeMet infect. pt.	CusB-Cu(I)	CusB-Ag(I)
Detector type/source		ADSQ Q315/APS-24IDC	ADSQ Q315/APS-24IDC	ADSQ Q315/APS-24IDC	ADSQ Q315/APS-24IDC	ADSQ Q315/ALS-5.0.2	ADSQ Q315/ALS-5.0.2
Wavelength (Å)		1.008	0.9636	0.9792	0.9793	1.3779	1.7000
Space group		I222	I222	I222	I222	I222	I222
Resolution (Å)		3.40	3.85	3.83	3.98	3.78	3.84
Total reflections		49,228	68,943	142,012	77,254	459,914	363,533
Unique reflections		17,168	12,449	12,596	11,322	12,925	12,345
Completeness (%)		94.6 (92.2)	95.8 (85.0)	99.6 (99.3)	96.4 (88.7)	99.2 (98.5)	99.4 (95.2)
R _{sym} (%)		4.6 (38.8)	8.4 (49.1)	8.9 (42.8)	9.4 (45.2)	6.2 (40.6)	7.5 (41.2)
Average I/σ(I)		19.7 (1.7)	16.5 (2.2)	27.5 (4.5)	21.3 (2.9)	20.6 (2.4)	25.3 (2.3)
Phasing							
Phasing power				1.93			
R _{collis}				0.89			
Figure of merit (acentric/centric)				0.40/0.26			
Figure of merit after density modification				0.66/0.62			
Refinement		CusB				CusB-Cu(I)	CusB-Ag(I)
Resolution (Å)		50-3.40				50-3.78	50-3.84
R _{work} (%)		26.5				29.0	28.0
R _{free} (%)		31.7				32.9	30.0
Protein residues built		592				592	592
rms deviation from ideal							
Bond lengths (Å)		0.008				0.010	0.010
Bond angles (°)		1.6				1.8	1.9