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Periplasmic disulfide isomerase DsbC is involved in the reduction of copper binding protein CueP from *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular pathogen with the ability to survive and replicate in macrophages. Periplasmic copper binding protein CueP is known to confer copper resistance to *S.* Typhimurium, and has been implicated in ROS scavenge activity by transferring the copper ion to a periplasmic superoxide dismutase or by directly reducing the copper ion. Structural and biochemical studies on CueP showed that its copper binding site is surrounded by conserved cysteine residues. Here, we present evidence that periplasmic disulfide isomerase DsbC plays a key role in maintaining CueP protein in the reduced state. We observed purified DsbC protein efficiently reduced the oxidized form of CueP, and that it acted on two (Cys104 and Cys172) of the three conserved cysteine residues. Furthermore, we found that a surface-exposed conserved phenylalanine residue in CueP was important for this process, which suggests that DsbC specifically recognizes the residue of CueP. An experiment using an *Escherichia coli* system confirmed the critical role played by DsbC in the ROS scavenge activity of CueP. Taken together, we propose a molecular insight into how CueP collaborates with the periplasmic disulfide reduction system in the pathogenesis of the bacteria.

1. Introduction

The host defense system produces hydrogen peroxide and the superoxide anion via NADPH oxidase in the phagosomes of macrophages [1,2]. The superoxide anion can be efficiently scavenged by superoxide dismutases in both the periplasm and the cytosol of the some Gram-negative bacteria, but hydrogen peroxide can diffuse rapidly across bacterial membranes and form the toxic hydroxyl radical in the presence of iron or copper ions via the Fenton reaction [3]. Recent studies have highlighted the importance of copper ions in the killing of invading pathogens in the phagosomes of activated macrophages and neutrophils [4–6]. Macrophage phagosomes accumulate the copper ion during bacterial infections, and this mediates the conversion of hydrogen peroxide to the hydroxyl radical [7]. However, some intracellular bacteria can counteract this copper-mediated immune response by up-regulating copper export and detoxifying genes. Furthermore, these activities have been suggested to be important determinants of pathogen virulence [6,8].

Gram negative *Salmonella enterica* is an important human pathogen associated with worldwide food-associated diseases, such as, gastroenteritis, septicemia, and typhoid fever [1], and can survive and replicate in macrophage phagosomes. CueP was initially identified as a copper resistance gene in *S. enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium), and can act as a substitute Cu^{+}-specific efflux pump in Gram negative bacteria [9]. In a previous study, a *cueP*-deleted strain of *S.* Typhimurium was found to be highly susceptible to copper, particularly in terms of anaerobiosis [9]. Moreover, the survival of a *cueP*-deleted *S.* Typhiumurim strain has been reported to be significantly reduced in macrophages capable of the copper ion-mediated Fenton reaction [10].

CueP has also been characterized as a periplasmic copper binding protein [11], and can supply the copper ion to periplasmic Cu,Zn-superoxide dismutase (SodCII), which requires the copper ion as an essential cofactor. The crystal structure of CueP suggests

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that its copper binding site involves three conserved cysteine residues [12]. Considering the enzymatic activity of SodCII, CueP appears to protect bacteria from the ROS produced by the host defense system [13]. Furthermore, $CuCl_2$ has been reported to cause disulfide bonds in CueP (as determined by SDS–PAGE) under non-reducing conditions [9]. Furthermore, it has been suggested CueP could inhibit copper-mediated hydroxyl radical formation from hydrogen peroxide under experimental conditions lacking SodCII, and shown that the reduced form of CueP reduces Cu^{2+} *in vitro*, and thus, prevents the Fenton reaction [10]. These findings suggest that CueP needs to be maintained in its reduced state because the sulfhydryl form of its cysteine residues can coordinate

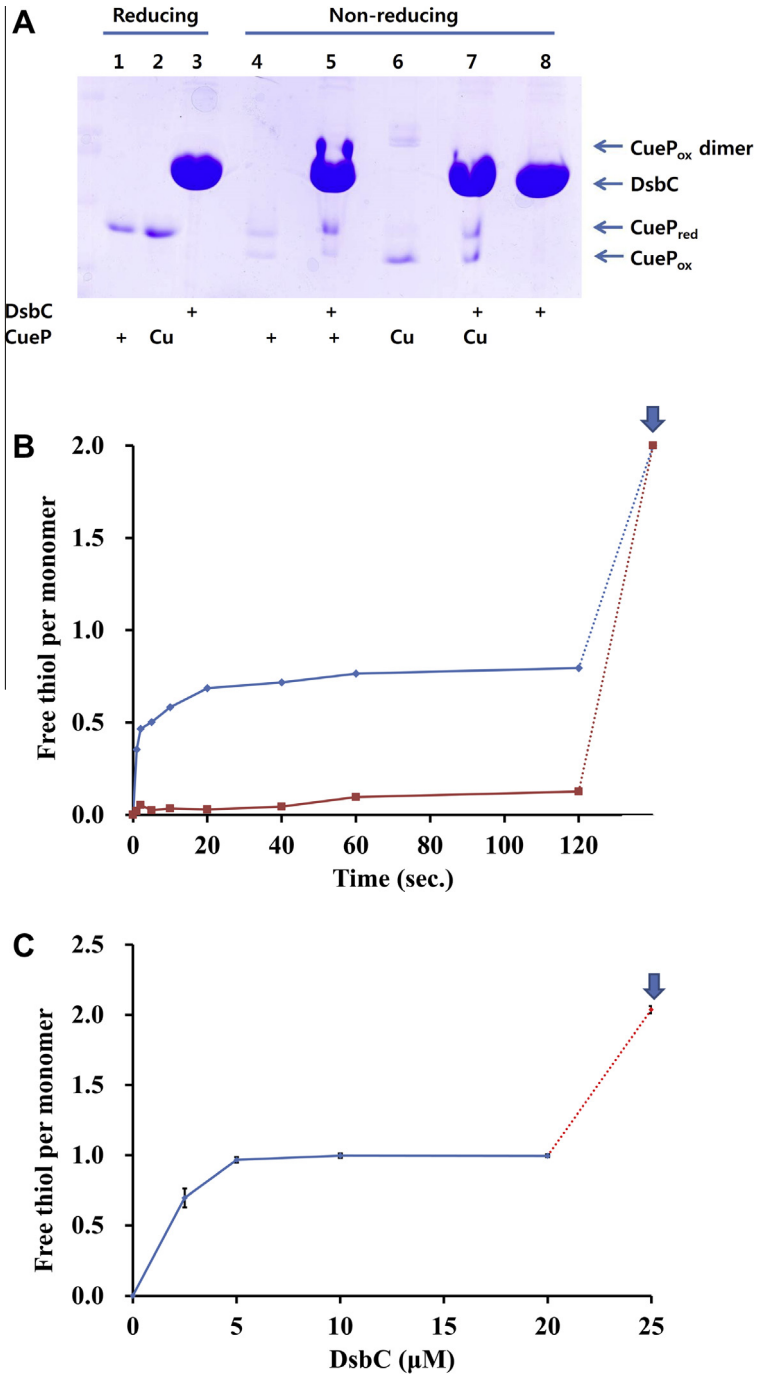

Fig. 1. Reductase activity of DsbC on CueP. (A) Purified DsbC protein reduced the disulfide bond of CueP *in vitro*, as observed by SDS–polyacrylamide gel electrophoresis. Cu^{2+}-oxidized CueP protein was used in lanes 2, 5, and 7, indicated by the presence of Cu. To determine the oxidation state of CueP ($CueP^{ox}$), reaction mixtures were subjected to SDS–PAGE under reducing and non-reducing conditions. (B) Reductase assay of DsbC using NTB-labeled CueP protein (CueP-NTB) as a substrate. Fully reduced DsbC (20 μM) and CueP-NTB (5 μM) were reacted at room temperature for the times shown. After reaction, 0.1 mM DTT was added to the reaction mixture, as indicated by the arrow to measure the NTB labeling efficiencies of CueP proteins. Amounts of NTB^{2-} liberated from CueP-NTB protein were estimated by measuring absorbance at 412 nm. The error bars indicate the standard errors of three independent experiments. (C) Dose-dependent reduction by DsbC. DsbC reductase activity was measured at different DsbC protein concentrations. NTB-labeled CueP protein (WT; 5 μM) were incubated with the fully reduced DsbC (2.5, 5, 10, and 20 μM) at room temperature for 5 min. After reaction, 0.1 mM DTT was added to reaction mixtures, indicated by the arrows. Reductase activities were estimated by measuring absorbances at 412 nm. The error bars indicate the standard errors of three independent experiments.

with or bind to the copper ion and only the reduced form of CueP can scavenge ROS.

To determine how CueP is maintained in the reduced state in the periplasm of *S.* Typhimurium, we sought to determine whether the disulfide isomerizing reductive pathway (composed of DsbC, DsbD, and cytoplasmic thioredoxin [14]) is involved in the reduction of CueP.

2. Materials and methods

2.1. Bacterial strains and plasmids

DNA construction, protein purification, and determination of protein expression in *S.* Typhimurium CueP (residues 22–178 in the numbering of the precursor CueP) have been previously described [10,12,23]. The substitution variants of CueP were generated using the QuikChange method (Invitrogen).

2.2. Preparation of CueP and DsbC proteins

Wild-type and mutant CueP proteins and the wild-type DsbC of *S.* Typhimurium were purified as described previously [10,15]. To prepare the reduced forms of CueP and DsbC in a reducing agent-free buffer, purified protein samples were dialyzed against degassed 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl twice for 2.5 h, and then immediately concentrated using a Centricon device (GE Healthcare). All biochemical experiments were carried out within 3 h of this concentration step. To prepare Cu^{2+}-oxidized CueP protein, purified CueP protein (0.2 mM) was treated with 0.5 mM $CuCl_2$ at room temperature for 15 min. Excess $CuCl_2$ was removed using a HiPrep 26/10 desalting column (GE Healthcare) previously equilibrated with degassed 20 mM Tris–HCl (pH 8.0) and 150 mM NaCl. The protein was then concentrated and stored at −80 °C until use. Protein concentrations were estimated using extinction coefficients at 280 nm.

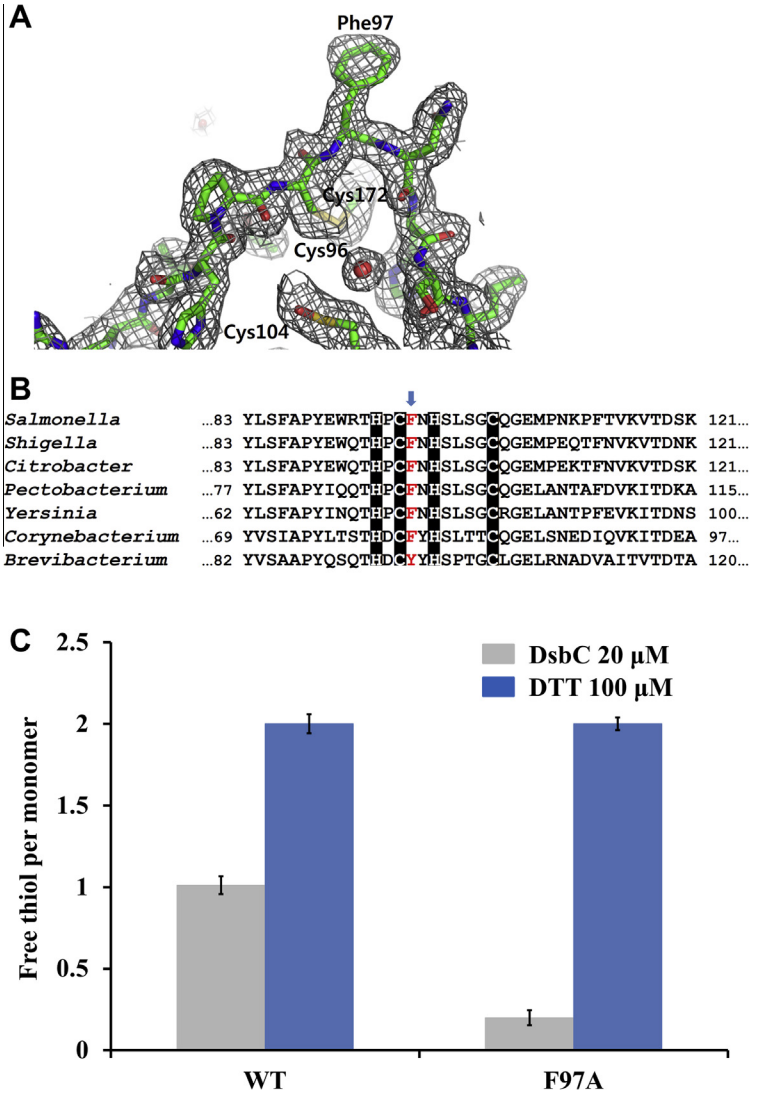

Fig. 2. CueP Phe97 plays an important role in the reduction of CueP by DsbC. (A) CueP crystal structure showing surface-exposed Phe97 [12]. The stick representation was drawn in gray and 2Fo-Fc map contoured at 1.0σ. (B) Amino acid sequence alignment of the conserved region of CueP and CueP-like proteins identified by BLATP from *S.* Typhimurium (NP_462551.1), *Shigella flexneri* (EIQ56183), *Citrobacter youngae* (WP_006687548.1), *Pectobacterium atrosepticum* (YP_049057), *Yersinia pseudotuberculosis* (YP_001399664), *Corynebacterium diphtheriae* (YP_005156771), and *Brevibacterium sp.* JC43 (WP_019157937). The conserved Phe97 is indicated by an arrow. The key conserved residues of CueP, suggested to be involved in copper binding, are highlighted. (C) Comparison of DsbC reductase activities on F97A mutant CueP and on wild type CueP. CueP proteins were labeled with NTB using DTNB. Fully reduced DsbC (20 µM) and NTB-labeled CueP proteins (5 µM) were reacted at room temperature for 5 min, and then the amounts of NTB^{2-} liberated were measured. The error bars indicate the standard errors of three independent experiments.

2.3. DsbC reductase SDS–PAGE assay

Freshly prepared DsbC protein (50 μg), Cu^{2+}-oxidized CueP, or reduced CueP protein (5 μg) were incubated in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl for 15 min at 37 °C. To determine the oxidation state of CueP, reaction mixtures were subjected to SDS–PAGE under reducing and non-reducing conditions.

2.4. Reductase assay of DsbC using NTB-labeled CueP

To prepare NTB-labeled CueP proteins (wild-type, C104S/C172S, C96S/C172S, C96S/C172S, and F97A), proteins (20 μM, 5 mL) were first incubated with a 100-fold excess (2 mM) of DTT for 1 h at room temperature. Excess DTT was then removed using a HiPrep 26/10 desalting column (GE Healthcare) previously equilibrated with 20 mM Tris–HCl (pH 8.0) and 150 mM NaCl. Next, proteins were incubated with 4 mM of DTNB for 2 h at room temperature to produce 2-nitro-5-thiobenzoic acid (NTB)-labeled CueP proteins. Unreacted DNTB and free NTB^{2-} were removed using HiPrep 26/10 desalting columns using the 20 mM Tris–HCl (pH 8.0) and 150 mM NaCl. Upon reduction of the mixed disulfide by DsbC or DTT, NTB^{2-} was released. In the reductase assay, NTB-labeled CueP proteins (5.0 μM) were reacted with reduced DsbC protein or DTT at room temperature. Absorbance of the liberated NTB^{2-} was measure at 412 nm.

2.5. Sensitivity of Escherichia coli harboring S. Typhimurium CueP to the Fenton reaction

The *E. coli* BW25113 strains (wild-type and ΔdsbC) were obtained from the KEIO collection [24]. The wild-type *S.* Typhimurium *cueP* gene was inserted into pACYC184 vector, as previously described [10], and then transformed into each *E. coli* strain. *E. coli* was grown in LB at 37 °C to an OD_{600} range of 0.7–0.9. Cells were then immediately washed twice with pre-warmed 20 mM Hepes buffer (pH 7.5) and incubated with 0.1 mM or 0 mM $CuCl_2$ in 20 mM Hepes buffer for 15 min at room temperature. After washing cells twice with 20 mM Hepes buffer (pH 7.5), 30 mM or 0 mM H_2O_2 was added to the cells for 5 min. To measure survival rates, cells were spread on agar plates.

3. Results

3.1. Purified DsbC protein reduced CueP protein

The periplasmic disulfide bond isomerase DsbC, which has a V-shaped dimeric structure, catalyzes the isomerization or reduction of disulfide in misfolded proteins with the conserved CXXC motif [15,16]. Since DsbC is maintained in the reduced state by the disulfide isomerizing reductive pathway, DsbC can repetitively reduce the disulfide bonds of substrate proteins in periplasm [17]. Thus, we asked whether DsbC reduces the oxidized form of CueP. To test this possibility, we first examined whether pure DsbC reduces pure CueP by adding oxidized CueP protein (induced by Cu^{2+}) to freshly prepared DsbC. It was found that DsbC converted some of the oxidized CueP protein to its reduced form, as evidence by a mobility shift in SDS polyacrylamide gel under non-reducing conditions (Fig. 1A).

To confirm this result, we performed a reductase assay using 2-nitro-5-thiobenzoic acid (NTB)-labeled CueP protein as a substrate for DsbC. To label CueP protein with NTB on its cysteine residues, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to the reduced form of CueP protein. DTNB forms a disulfide bond with the free cysteines of CueP to produce an NTB-adduct, and thus, reduction and cleavage of the disulfide bond(s) of the NTB–protein adduct by DsbC would liberate yellow-colored NTB^{2-} [18]. We found that DsbC exhibited strong reductase activity in only 1–2 s (Fig. 1B). Furthermore, the control, glutathione S-transferase (GST), which possesses surface cysteine residues, was not reduced by DsbC under the same experimental condition (Fig. 1B). We also observed that the reducing effect of DsbC on CueP increased in proportion to the concentration of DsbC in the range 0–5 μM in the presence of 5 μM of CueP (Fig. 1C). These results demonstrate that DsbC efficiently and specifically reduces the oxidized form of CueP.

3.2. The surface-exposed Phe97 of CueP

We found that a hydrophobic residue Phe97 located on the surface region of CueP [12], which is an unusual observation especially in globular proteins. Furthermore, the conservation of this residue among CueP homologues and its proximity to the conserved cysteine residues, suggests that they are functionally related (Fig. 2A and B). Moreover, given that DsbC has affinity for the hydrophobic residues of unfolded substrate proteins [19,20], we hypothesized DsbC recognizes Phe97 of CueP with its hydrophobic cleft and that this leads to the specific reduction of adjacent cysteine residues. To test this hypothesis, we performed the reductase assay with a mutant CueP protein harboring an alanine substitution at Phe97. As shown in Fig. 2C, the reductase activity of the mutant CueP protein was dramatically lower than that of the wild type. However, dithiothreitol (DTT) showed similar reductase activity on both wild type and mutant CueP proteins (Fig. 2C). These observations suggest that DsbC efficiently reduces the oxidized forms of CueP, and that CueP Phe97-mediated interaction may be important.

3.3. DsbC mainly attacks Cys104 of CueP

CueP has three conserved cysteine residues at its putative copper binding site [12]. Accordingly, we sought to determine which cysteine residues are attacked by DsbC. Three double mutant CueP proteins each containing one of the three cysteine residues were constructed by double serine substitution. The reductase assay was performed with reduced DsbC protein using the wild type or the mutant CueP proteins, which were labeled with NTB. DsbC protein efficiently reduced mutant CueP (C96S/C172S) and CueP (C96S/C104S) protein with intact Cys104 and C172 residues,

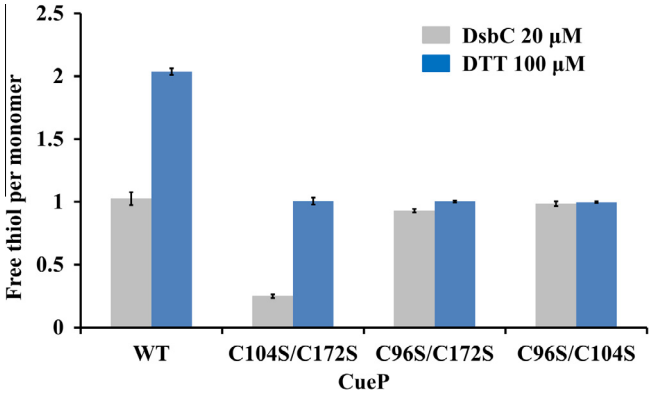

Fig. 3. DsbC acts on Cys104 and Cys172 of CueP. A reductase assay was performed using 3 double mutants of CueP possessing only one of the three conserved cysteine residues. NTB-labeled CueP variants (WT, C104S/C172S, C96S/C172S, or C96S/C172S; 5 μM) were incubated with fully reduced DsbC protein (20 μM) or 0.1 mM DTT at room temperature for 5 min. The reducatase activities were determined by measuring at 412 nm. The error bars indicate the standard errors of three independent experiments.

respectively. However, the other CueP variant (C104S/C172S) did not react with DsbC as efficiently as wild type CueP or the other two CueP variants (Fig. 3B). These results indicate that Cys104 and Cys172 are the main targets of DsbC. Our findings are consistent with expectations based on the crystal structure, as Cys96 is partly buried whereas the other two cysteine residues are fully exposed on the surface [12].

3.4. DsbC is required for CueP

In a previous study, it was reported that expression of the wild type CueP protects *E. coli* cells against an exogenously induced copper ion-mediated Fenton reaction [10]. To examine the functional association between *dsbC* and *cueP* with respect to the survival of *E. coli* against the copper ion-mediated Fenton reaction, a pACYC plasmid or the plasmid containing the wild-type *cueP* gene were transformed into a wild-type *E. coli* strain and a *dsbC*-deleted *E. coli* strain. As shown in Fig. 4A, the introduction of *cueP* increased the survival of the *E. coli* upon the subsequent exposure to $CuCl_2$ and H_2O_2 under the *dsbC*-containing *E. coli* strain. However, *cueP* failed to increase the survival of the *E. coli* under the *dsbC*-deleted background, indicating that DsbC plays an important role in the function of CueP (Fig. 4) Since *E. coli* DsbC is highly homologous with *S.* Typhimurium DsbC (sequence identity: 84%), it is likely that *E. coli* DsbC functions as a surrogate of *S.* Typhimurium DsbC. This suggests the reduced form of CueP is maintained by the action of DsbC in the periplasmic disulfide bond isomerase pathway, and a novel role for DsbC in the regeneration of free CueP sulfhydryl.

4. Discussion

A high concentration of copper ion can catalyze the formation of non-specific intermolecular disulfide bonds in periplasm under

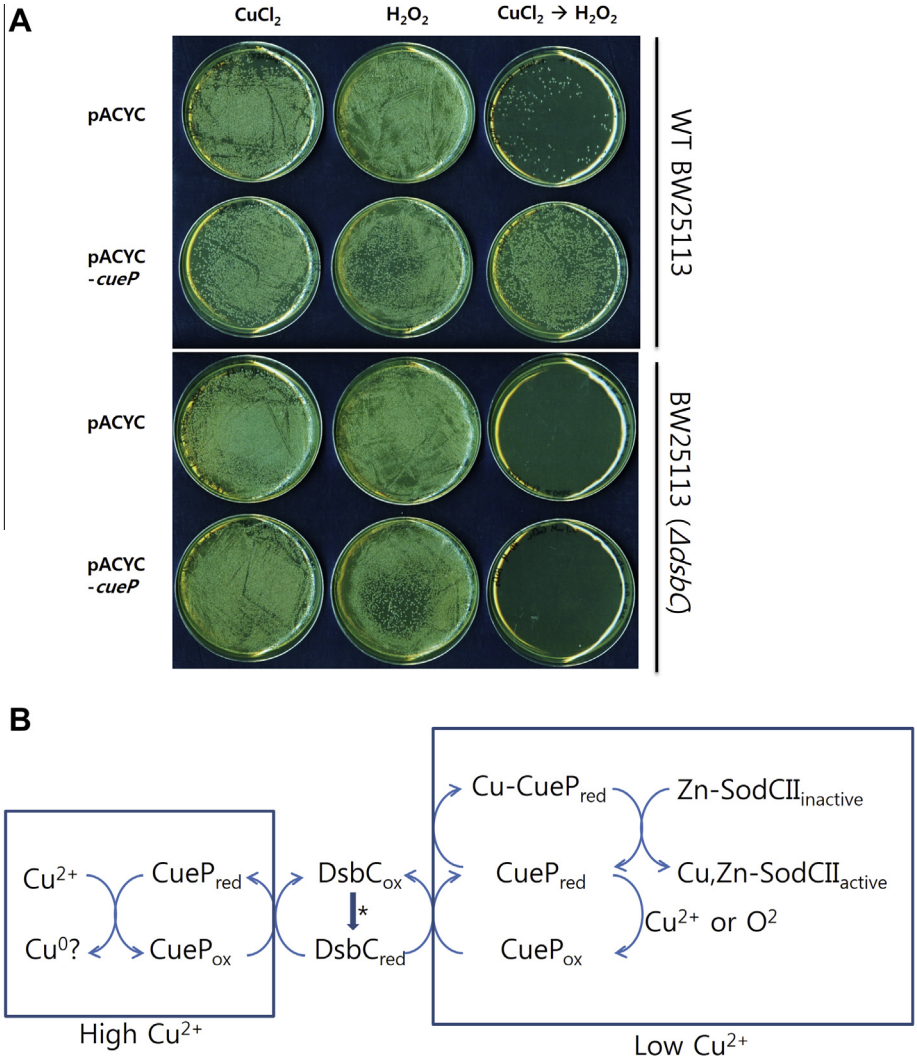

Fig. 4. The functional relationship between DsbC and CueP. (A) The requirement of *dsbC* for the ROS scavenging activity of *cueP* in *E. coli*. The empty plasmid (pACYC) or *S.* Typhimurium CueP-containing plasmid (pACYC-*cueP*) was transformed into wild type (WT) or *dsbC*-deleted (*ΔdsbC*) *E. coli* BW25113 strain. Each *E. coli* strain was exponentially grown in LB and then immediately washed with 20 mM Hepes buffer (pH 7.5) at room temperature. Cells were incubated with 0.1 mM $CuCl_2$ in 20 mM Hepes buffer (pH 7.5) and then washed with copper-free buffer ($CuCl_2$ and $CuCl_2 \rightarrow H_2O_2$). Cells were then treated with H_2O_2 (30 mM) for 5 min to induce intracellular Fenton reactions (H_2O_2 and $CuCl_2 \rightarrow H_2O_2$). Cell viabilities were determined by plating on LB agar. (B) Proposed mechanism for the functions of CueP and DsbC at high or low Cu^{2+} concentrations. The left box describes the copper reducing activity of the reduced form of CueP ($CueP_{red}$) at a high Cu^{2+} concentration for the direct scavenge of the copper-mediated Fenton reaction. $CueP_{red}$ turns to the oxidized form of CueP ($CueP_{ox}$) after the reduction of Cu^{2+} [10]. $CueP_{ox}$ is converted to $CueP_{red}$ by the enzymatic action of the reduced form of DsbC ($DsbC_{red}$). The asterisk indicates the conversion of the oxidized form of $DsbC_{ox}$ into $DsbC_{red}$ by the disulfide isomerizing reductive pathway in periplasm. The right box describes the copper supply activity of $CueP_{red}$ to inactive Zn-SodCII at a low Cu^{2+} concentration, and the subsequent formation of active Cu,Zn-SodCII [13]. The reduced form of CueP could be oxidized by Cu^{2+} or by molecular oxygen, in which case, DsbC maintains CueP in the reduced state using the disulfide isomerizing reductive pathway.

aerobic conditions [21], and disulfide-bonded oligomers can be resolved by the periplasmic disulfide isomerase DsbC [21]. The periplasmic copper binding protein CueP is known to form a intramolecular disulfide bond in the presence of Cu^{2+} or molecular oxygen [10,12]. The present study provides evidence that DsbC maintains the reduced state of the periplasmic copper-binding protein CueP in *S.* Typhimurium. Furthermore, an *E. coli*-based experiment showed that the gene expression of *dsbC* was required for the *cueP* scavenging of hydroxyl radicals, generated from hydrogen peroxide under copper-mediation. Since only reduced the form of CueP could directly scavenge generated ROS [10], our findings suggest collaboration between CueP and DsbC. It was previously reported Cu^{2+} generates a disulfide bond between Cys96 and Cys104 of CueP [10], whereas molecular oxygen forms a disulfide bond between Cys96 and Cys172 [12], whereas the present study reveals that DsbC targets the Cys104 and Cys172 residues of CueP. Thus, our results indicate that DsbC can cleave disulfide bonds generated by the copper ion or molecular oxygen and suggest that DsbC can efficiently maintain CueP protein in the reduced state.

In addition to the direct ROS scavenging activity of CueP, this protein exhibits ROS scavenging activity by supplying the copper ion to periplasmic Cu,Zn-superoxide dismutase [13]. In a previous study, a low concentration of $CuCl_2$ (5 μM) was used to determine the copper supply activity of CueP to SodCII [13]. However, in another study, Yoon et al. used 100 μM of $CuCl_2$ to investigate the direct copper reduction activity of CueP [10]. In macrophage phagosomes, copper concentrations are increased to in excess of 400 μM during *Mycobacterium tuberculosis* infections [22], and thus it is likely that direct copper reduction activity could contribute to the survival of *S.* Typhimurium in macrophages. Furthermore, it is noteworthy that CueP or CueP homologues are almost exclusively found in intracellular pathogens, such as, *S.* Typhimurium, *Yersinia pestis*, and *Corynebacterium diphtheriae*.

If the reducing effect of DsbC on CueP is combined with the Cu^{2+} reducing activity of CueP, the reducing function of CueP would be hugely increased due to continual and repetitive reactions between CueP and DsbC. CueP. In addition, the reductase activity of DsbC on CueP might be important for the supply of the copper ion to SodCII when the copper ion concentration is low. The free cysteine residues of CueP are required to bind Cu^{2+} and are eventually oxidized by Cu^{2+} or by molecular oxygen in periplasm under aerobic conditions [12]. Taken together, we propose a new ROS scavenge mechanism in *S.* Typhimurium, whereby CueP and DsbC cooperate and contribute to ROS scavenging using different mechanisms that are dependent on the concentration of Cu^{2+} (Fig. 4B). CueP directly reduces and removes copper ion from the intracellular milieu to prevent the formation of hydroxyl radicals when the concentration of Cu^{2+} is study, and plays a more important role in the transfer of Cu^{2+} to SodCII when Cu^{2+} concentrations are low. In both cases, disulfide isomerization reductive pathways including the DsbC pathway maintain CueP in a reduced state, which is an essential prerequisite for the function of CueP. Both of these mechanisms would contribute to the survival of the bacteria in harsh environments, such as, those encountered in macrophage phagosomes.

In conclusion, our study reveals CueP as a novel substrate of DsbC, and suggests cooperation between CueP and DsbC in the contexts of scavenging ROS and copper homeostasis. Furthermore, our results expand understanding of the host-pathogen interaction in terms of the copper tolerance of invading pathogens, and provide a molecular insight into how the copper tolerance collaborates with the periplasmic disulfide reduction system in the pathogenesis of the bacteria.

Acknowledgments

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