A Primary Role for Disulfide Formation in the Productive Folding of Prokaryotic Cu,Zn-superoxide Dismutase*

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**Background:** Prokaryotic Cu,Zn-superoxide dismutase (SodC) forms an intramolecular disulfide bond.

**Results:** Disulfide formation is essential for folding and enzymatic activation of SodC.

**Conclusion:** The thiol-disulfide status controls the intracellular stability of SodC.

**Significance:** The oxidizing environment of the periplasm is required for antioxidant activity of SodC.

Enzymatic activation of Cu,Zn-superoxide dismutase (SOD1) requires not only binding of a catalytic copper ion but also formation of an intramolecular disulfide bond. Indeed, the disulfide bond is completely conserved among all species possessing SOD1; however, it remains obscure how disulfide formation controls the enzymatic activity of SOD1. Here, we show that disulfide formation is a primary event in the folding process of prokaryotic SOD1 (SodC) localized to the periplasmic space. *Escherichia coli* SodC was found to attain β-sheet structure upon formation of the disulfide bond, whereas disulfide-reduced SodC assumed little secondary structure even in the presence of copper and zinc ions. Moreover, reduction of the disulfide bond made SodC highly susceptible to proteolytic degradation. We thus propose that the thiol-disulfide status in SodC controls the intracellular stability of this antioxidant enzyme and that the oxidizing environment of the periplasm is required for the enzymatic activation of SodC.

In eukaryotic SOD1, the overall structural fold is not significantly affected by the thiol-disulfide status (5), but formation of the disulfide bond is essential for enzymatic activity by controlling orientation of the arginine residue that optimally guides superoxide anion to the catalytic copper site (6). Also, an enzymatically active form of eukaryotic SOD1 exists as a non-covalent homodimer, but reduction of the intramolecular disulfide bond facilitates monomerization and eventually misfolding of SOD1, which has been observed as a pathological change in a subset of amyotrophic lateral sclerosis (2). Formation of the intramolecular disulfide bond is, therefore, a critical factor for SOD1 to assume its enzymatically competent conformation.

Notably, the activation process of eukaryotic SOD1, including its metal binding and disulfide formation, is well regulated in the cell. Binding of a zinc ion has been proposed to first occur in SOD1 (2), although the mechanism of zinc ion acquisition remains totally unknown. Next, this zinc-bound form of SOD1 is specifically recognized by a copper chaperone protein, CCS, and a copper ion is then supplied from CCS to SOD1 (7). Simultaneously, a disulfide bond is introduced in SOD1 by CCS, which facilitates the homodimerization of SOD1 and finalizes the enzymatic activation of SOD1 (6). Given that eukaryotic SOD1 is mainly localized in the highly reducing cytoplasm (8), additional care should be taken to retain the disulfide bond. Indeed, x-ray structural analysis of SOD1 has shown that the disulfide bond is buried at the dimer interface (9); therefore, dimerization of SOD1 presumably protects the disulfide bond from its reductive cleavage in the cytoplasm.

In addition to the CCS-dependent activation of SOD1, a CCS-independent pathway(s) has been proposed for the maturation of SOD1 in several eukaryotes (10), and interestingly, prokaryotes do not possess a gene corresponding to CCS. A thiol-disulfide oxidoreductase, DsbA, has been suggested to form the disulfide bond in prokaryotic SOD1 (often called SodC) (11); however, the activation process of SodC remains unknown. Unlike eukaryotic SOD1, SodC is localized in the periplasmic space, where free copper/zinc ions would be available for activation of SodC through a simple diffusion process (12). Furthermore, the periplasmic space provides a more oxidizing environment than the cytoplasm (13), implying no need for metallochaperones to introduce the disulfide bond as well as metal ions and also no need to protect the disulfide bond from reduction.

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Cu,Zn-superoxide dismutase (SOD1)

Cu,Zn-superoxide dismutase (SOD1) is an enzyme that converts superoxide anion into oxygen and hydrogen peroxide at a catalytically active site of a bound copper ion (1). In addition to the copper-binding site, a typical form of SOD1 is also equipped with a zinc-binding site and an intramolecular disulfide bond (2). It is, however, important to note that the copper- and zinc-binding sites are not always conserved among SOD1 proteins. For instance, a zinc-binding site is missing in SOD1 from *Mycobacterium tuberculosis* (3), and an SOD1-like protein from *Bacillus subtilis* does not even bind a catalytic copper ion (4). On the other hand, the intramolecular disulfide bond is completely conserved in all species possessing SOD1, suggesting its crucial role in the structure and function of SOD1.

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2 The abbreviations used are: SOD1, Cu,Zn-superoxide dismutase; SodC, prokaryotic SOD1; CCS, copper chaperone for SOD1.
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Indeed, not all SodC proteins form homodimers; notable examples include the monomeric SodC from Escherichia coli (14), Salmonella enterica (15), and B. subtilis (4), in which the disulfide bond is highly solvent-exposed. Although SodC activity has been considered to play important roles in the pathogenicity of bacteria (16), it remains unknown how enzymatic activation of SodC is regulated, and the role of the disulfide bond in the physiological function of SodC is obscure.

In this study we have used E. coli SodC (17) as a model for a monomeric SOD1 of prokaryotes and found that the intramolecular disulfide bond is essential for the folding and intracellular stability of SodC. Unlike eukaryotic SOD1, E. coli SodC without the disulfide bond has little secondary structure, albeit with ability to bind a copper ion. Reductive cleavage of the disulfide bond was, furthermore, found to make SodC highly susceptible to proteolytic degradation 

Based upon these results, we propose that the intracellular activity of prokaryotic SodC is regulated by its thiol-disulfide status.

EXPERIMENTAL PROCEDURES

Preparation of SodC Proteins—A cDNA of E. coli SodC without the N-terminal periplasmic signal sequence was cloned into a pET-15b plasmid (Novagen) where the thrombin cleavage recognition sequence was replaced with a HRV3C cleavage recognition sequence. The C74A/C169A mutations (the numbering is based on the entire sodC gene including the N-terminal signal peptide) were inserted by the inverse PCR method using a KOD-Plus-Neo DNA polymerase (TOYOBO). All constructs in this study were confirmed by DNA sequencing. E. coli BL21 (DE3) (New England Biolabs) was transformed with the respective plasmids, and expression of SodC proteins was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 6 h in LB medium with ampicillin. Cells were lysed with a cycle of freeze-thaw, resuspended, and ultrasonicated in PBS with 2% Triton X-100, 5 mM MgSO4, and 8 mg/liter DNase I. The soluble supernatant after centrifugation at 20,000 × g for 20 min was filtered through a 0.22-μm syringe filter and loaded onto a Profinity IMAC Ni2+ resin to remove metal ion contamination. Furthermore, to prevent contamination of metal ions in samples (Zn2+ ions), the resin was washed with 50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8, and then the bound SodC proteins were eluted with 50 mM Tris, 100 mM NaCl, 250 mM imidazole, pH 7. For introduction of the disulfide bond in SodC in vitro, the eluted SodC proteins were further incubated with 100 μM CuSO4 at 4 °C for 1 h.

To remove the N-terminal His tag, the SodC proteins were then incubated with 1 unit of HRV3C protease (Novagen) per 100 μg of SodC at 4 °C for 20 h. Samples were further purified by size exclusion chromatography using a gel filtration column (TSKgel G2000SW, TOSOH) equilibrated with 50 mM Tris, 100 mM NaCl, pH 7.0. After confirming successful removal of the His tag from SodC by SDS-PAGE, the purified tag-free SodC was then demetallated by precipitation with 20% trichloroacetic acid (TCA) followed by washes with cold acetone. The dried protein pellets were redissolved in an appropriate metal-free buffer that was treated with Chelex 100 resin (Bio-Rad). The concentration of SodC was spectrophotometrically determined from the absorbance at 225 and 215 nm (18).

SOD Activity Assays—3.2 μg of SodC proteins in 50 mM HEPES, 100 mM NaCl, pH 8.0, were assayed for SOD activity using the SOD assay kit WST (DOJINDO) in a 96-well plate, and the absorbance at 450 nm was measured using a plate reader (Epoch, BioTek). For the metallated forms, an equimolar amount of CuSO4/ZnSO4 was added before the assay.

Cir2+ and Co2+ Ion Titration Experiments—E. coli SodC with- out the disulfide bond was treated with Chelex 100 resin. Cells were centrifuged at 7,500 rpm for 1 min to remove any insoluble materials and then used for the measurement of visible absorption spectra with a spectrophotometer (UV-1800, Shimadzu). All buffer solutions were treated with Chelex 100 resin to remove metal ion contamination. Furthermore, to avoid contamination of metal ions in samples (Zn2+ ions, in particular), we used a plastic disposable cuvette in place of a glass cell.

Quantitation of Copper and Zinc Ions by Colorimetric Assay—Concentrations of copper and zinc ions in samples were measured using Metallo Assay Copper LS (Metallogenics) and Metallo Assay Zinc LS (Metallogenics), respectively. These kits are based upon the colorimetric assay of the specific chelation of copper and zinc ions with 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-(3-sulfopropyl)aniline and 2-[(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol, respectively.

Spectroscopic Analysis—Far-UV CD spectra were measured with a J-720WI spectropolarimeter (Jasco). Concentration of protein samples was set to 10 μM in 20 mM sodium phosphate, 50 mM NaCl, pH 8.0, treated with Chelex 100 resin. For the analysis of metallated SodC proteins, an equimolar amount of CuSO4/ZnSO4 was added to the samples before measurements.

Fourier transform infrared spectroscopy (FTIR) spectra were measured using an IRAffinity-1S spectrophotometer (Shimadzu) attached with an attenuated total reflection accessory (DuraSampIR II, nine reflections). SodC proteins were demetallated by TCA precipitation and redissolved in a deuterated buffer containing 100 mM Tris and 100 mM NaCl at pH 8.4. The protein concentration was set to 1 mM. The second derivative of the absorption spectrum was obtained using the LabSolutions IR software (Shimadzu). Peaks were assigned based on the work of Byler and Susi (21) and of assignments on bovine SOD1 (22).

Size Exclusion Chromatography—50 μl of 10 μM SodC was loaded onto a gel filtration column (TSKgel G2000SW, TOSOH), and the absorbance change at 215 nm was monitored. To avoid contamination of divalent metal ions from the HPLC system, 50 μM EDTA was included in the running buffer, containing 50 mM Tris and 100 mM NaCl, pH 7.0. For the analysis of metallated SodC, a 1.2-fold molar excess amount of CuSO4 and/or ZnSO4 was added to SodC proteins before loading onto the column. For the estimation of molecular weight, the column was calibrated with bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), equine heart myoglobin (17.7 kDa), α-lactoalbumin (14.2 kDa), and insulin (5.7 kDa).

Western Blotting Analysis of SodC in E. coli—E. coli BW25113 and the sodC/disA/degP/pcr-knock-out cells from the Keio collection (23) were provided by National BioResource Project.
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(Ohio State University); E. coli were cultured in LB medium to stationary phase at 37 °C, and the periplasmic fraction was obtained by a cold osmotic shock method (24) that was slightly modified. Cells were first resuspended in 50 mM Tris, 20% (w/v) sucrose, 2.5 mM EDTA, 2 mM CaCl₂, pH 8.0, together with 100 mM iodoacetamide for protecting free thiols and then incubated on ice for 10 min. After centrifugation at 20,000 g for 10 min, the pellets were resuspended in 0.5 mM MgSO₄ with 100 mM iodoacetamide and incubated on ice for 10 min. The supernatant obtained by centrifugation at 20,000 g for 10 min was collected as the periplasmic fraction. The total amount of proteins in the periplasmic fraction was measured by using a Micro BCA Protein Assay kit (Thermo Scientific) using bovine serum albumin as a standard.

To examine the effects of reductants on endogenous SodC, dithiothreitol (DTT, 0–2.5 mM) was added to the E. coli BW25113 cultures in the stationary phase. 0.15 g/liter chloramphenicol was also added to inhibit protein synthesis (25). The sodC-knock-out E. coli cells transformed with a pGEX-4T-1 plasmid in which the glutathione S-transferase gene was replaced by SodC with an N-terminal periplasmic signal sequence were also used for these experiments. After the addition of DTT and chloramphenicol, E. coli cells were cultured for 1 h at 37 °C, and the periplasmic fraction was then isolated as described above.

Periplasmic proteins were analyzed by non-reducing SDS-PAGE using a 12.5% polyacrylamide gel. After electrophoresis, the gel was immersed in a 1% PAGE using a 12.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualize protein bands.

**RESULTS**

**Formation of the Disulfide Bond Is Essential for Enzymatic Activity of SodC**—To test if formation of the intramolecular disulfide bond was essential for enzymatic activity of SodC, recombinant E. coli SodC with the disulfide bond (SodC<sup>S-S</sup>) and a disulfide-null mutant protein in which Cys<sup>74</sup> and Cys<sup>169</sup> were replaced with Ala (SodC<sup>noCys</sup>) were prepared. The disulfide-reduced form of SodC (SodC<sup>H</sup>) was also prepared by incubation of SodC with DTT. As described under “Experimental Procedures,” the two thiols of Cys<sup>74</sup> and Cys<sup>169</sup> in SodC were oxidized to form an intramolecular disulfide bond by the addition of CuSO₄, which was then removed by TCA precipitation. SodC<sup>noCys</sup> proteins were also treated with TCA precipitation; treatment of SodC proteins with TCA precipitation assures the preparation of demetallated SodC (E,E-SodC<sup>S-S</sup> and E,E-SodC<sup>noCys</sup>). As shown in Fig. 1A, the electrophoretic mobility of SodC<sup>S-S</sup> analyzed by SDS-PAGE was retarded in the presence of a reductant, DTT, whereas SodC<sup>H</sup> and SodC<sup>noCys</sup> did not change their mobility upon the addition of DTT. These results confirmed successful introduction of the disulfide bond in SodC<sup>S-S</sup>.

SodC catalyzes the disproportionation of superoxide anion, which can be spectrometrically assayed based upon the formation of formazan by superoxide anion (see “Experimental Procedures”). As shown in Fig. 1B, both E,E-SodC<sup>S-S</sup> and E,E-SodC<sup>noCys</sup> showed no activity, consistent with a catalytic role of a bound copper ion. Indeed, significant activity became evident after the addition of a copper ion to SodC<sup>S-S</sup>; however, SodC<sup>noCys</sup> remained inactive even in the presence of a copper ion. Given that the further addition of a zinc ion did not activate SodC<sup>noCys</sup>, formation of the intramolecular disulfide bond is essential for the enzymatic activity of SodC.

**Proteolytic Digestion of SodC**—0.3 g/liter E,E-SodC<sup>S-S/noCys</sup> was incubated with 0.3 × 10⁻³ – 0.3 × 10⁻¹ g/liter Pronase (Calbiochem) at 37 °C for 30 min in 50 mM Tris, 100 mM NaCl, pH 7.0. The digestion reaction was quenched by adding a protease inhibitor mixture (cOmplete, EDTA-free, Roche Applied Science) and a Laemml sample buffer with β-mercaptoethanol. The samples were boiled and then loaded onto a 12.5% polyacrylamide gel and analyzed by SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualize protein bands.
SodC Binds a Copper Ion in the Absence of the Disulfide Bond but Remains Inactive—Given that SodCnoCys was inactive even in the presence of copper ions, it is possible that formation of the disulfide bond is essential for binding of the copper ion in SodC. To examine this possibility, the titration of SodC proteins with Cu$^{2+}$/H$^{+}$ ions was monitored by visible absorption spectroscopy.

When an equimolar amount of Cu$^{2+}$ ion was added to E,E-SodCS-S, an absorption peak was observed at 655 nm (Fig. 2A), consistent with the binding of a copper ion at the copper-binding site (27). A new peak at 830 nm then emerged after the addition of a 2-fold molar excess of Cu$^{2+}$ ions to SodCS-S (Fig. 2A), suggesting that a Cu$^{2+}$ ion was also bound in the zinc-binding site (19). Further addition of Cu$^{2+}$ ions to SodCS-S increased the intensity of an absorption peak at 760 nm (Fig. 2A), which corresponds to that of free Cu$^{2+}$ ions in the acetate buffer (data not shown). Based upon these results, SodCS-S preferentially binds a Cu$^{2+}$ ion at its copper-binding site; therefore, SodCS-S becomes enzymatically active in the presence of copper ions.

Unexpectedly, E,E-SodCnoCys was also found to bind a Cu$^{2+}$ ion in the copper-binding site; a characteristic absorption peak at 650 nm appeared after the addition of an equimolar Cu$^{2+}$ ion to E,E-SodCnoCys (Fig. 2B). It is, therefore, unlikely that the lack of enzymatic activity in SodCnoCys (Fig. 1B) is due to an inability to bind a copper ion. Notably, however, further addition of Cu$^{2+}$ ions to SodCnoCys did not produce the absorption peak around 830 nm corresponding to a Cu$^{2+}$ ion bound at the zinc-binding site; instead, an absorption peak at 730 nm due to free copper ions increased in intensity (Fig. 2B). In addition, when
200 μM copper-bound SodC^noCys/S-S was mixed with a copper chelator, EDTA (500 μM), the dissociation of the copper ion was found to be slightly more rapid from SodC^noCys than that from SodC^S-S, although the dissociation completed within 5 min in both SodC^noCys and SodC^S-S (data not shown). This implies roles of the disulfide bond in increasing the affinity, albeit slight, of the bound copper ion in SodC. Taken together, formation of a disulfide bond is not necessary for binding of a copper ion in SodC but appears to be critical to attain a native conformation for enzymatic activity.

A Disulfide Bond Is Required for a Native Conformation around the Zinc-binding Site—It should be noted that, in the absence of the disulfide bond, the zinc-binding site in SodC was not able to bind an additional copper ion; therefore, the disulfide bond may be required for establishing the native conformation of the zinc-binding site. As described previously (19, 28), a Co^{2+} ion has been used as a substitute for a spectroscopically silent Zn^{2+} ion and was titrated to E,E-SodC^S-S and E,E-SodC^noCys. The addition of an equimolar amount of Co^{2+} ions to E,E-SodC^S-S resulted in a characteristic absorption peak centered at 563 nm (Fig. 2C), indicating binding of a Co^{2+} ion at the zinc-binding site (19). Upon further addition of Co^{2+} ions, an additional absorption peak at 600 nm emerged, consistent with binding of a Co^{2+} ion at the copper-binding site (Fig. 2C) (29, 30). In SodC^S-S, therefore, the zinc-binding site is considered to be available for binding of a Zn^{2+} ion.

In contrast to SodC^S-S, however, SodC^noCys was unable to bind a Co^{2+} ion; the addition of up to a 10-fold molar excess of Co^{2+} ions to E,E-SodC^noCys produced an absorption peak at 520 nm (Fig. 2D), which corresponds to that of free Co^{2+} ions in the HEPES buffer (data not shown). We also tested the binding of Co^{2+} ion to SodC proteins in the presence of Cu^{2+} ion. In SodC, Cu^{2+} binding to the copper site (Fig. 2, A and B) and Co^{2+} binding to the zinc site (Fig. 2C) produced distinct absorption spectra, and the sum of those spectra was observed when both Cu^{2+} and Co^{2+} ions were added to E,E-SodC^S-S (Fig. 2E). In contrast, the absorption peaks characteristic to the binding of Co^{2+} ions at the zinc-binding site were not observed when both Cu^{2+} and Co^{2+} ions were added to E,E-SodC^noCys (Fig. 2E); instead, only the absorption peak at 650 nm due to the binding of Cu^{2+} ion to the copper-binding site was confirmed. Accordingly, SodC^noCys was considered to have limited ability to bind metal ions at the zinc-binding site even in its copper-bound form.

We have also confirmed no binding of a Zn^{2+} ion in SodC^noCys by direct quantitation of Zn^{2+} ions. E,E-SodC^S-S and E,E-SodC^noCys were first incubated with 1.2-fold molar excess of Zn^{2+} ions, and unbound Zn^{2+} ions were then removed by ultrafiltration. Approximately an equimolar amount of Zn^{2+} ions (133 ± 16%) remained in SodC^S-S, whereas SodC^noCys was unable to bind Zn^{2+} ions (28 ± 10%). These results thus show that the zinc-binding site was not properly formed in SodC without the disulfide bond. Therefore, even though the disulfide bond is not required to bind a copper ion in SodC, the conformation of SodC in the absence of the disulfide bond is not optimized for catalyzing the disproportionation of a superoxide anion.

SodC Folds into a β-Sheet Structure upon Formation of the Disulfide Bond—To investigate the effects of the disulfide bond on the folding of SodC, the content of secondary structure in SodC was examined by circular dichroism (CD) spectroscopy. The CD spectrum of E,E-SodC^noCys had a large negative peak at 199 nm (Fig. 3A), and its spectral shape is characteristic of a random coil structure (31). E,E-SodC^SH, in which both Cys residues (Cys^{74} and Cys^{169}) are in the thiol state, also exhibited a similar CD spectrum to that of E,E-SodC^noCys with a large negative peak at 199 nm (Fig. 3A), confirming little artificial effects of the Cys-to-Ala mutations on the conformation of SodC lacking the disulfide bond. Given that disulfide-reduced human SOD1 has been shown to fold into a structure rich in β-sheets (5), almost no secondary structure in SodC without the disulfide bond was unexpected.

When the disulfide bond was introduced into SodC (E,E-SodC^S-S), the CD spectrum exhibited remarkable changes (Fig. 3A); the intensity of the negative peak at 199 nm largely decreased, and the spectral shape also changed. We thus suppose that these spectral changes highlight important roles of the disulfide bond in the secondary structure formation of SodC.

As shown in Fig. 3B, the addition of either Cu^{2+} ion, Zn^{2+} ion, or both to E,E-SodC^noCys also slightly decreased the intensity at 199 nm of its CD spectrum, but the spectral shape was not markedly affected. It should also be noted that the addition of metal ions to E,E-SodC^S-S further changed the CD spectrum (Fig. 3C); in particular, the spectral shape of SodC^S-S was significantly changed in the presence of both copper and zinc ions. Binding of metal ions in SodC^S-S is hence supposed to have effects on the structural maturation, but how these metal ions affect the conformation of SodC^S-S is difficult to speculate from the spectral changes of SodC upon binding of metal ions.

On the other hand, a relatively large difference in the CD spectral shape between SodC^S-S and SodC^SH (or SodC^noCys) allows us to elucidate the conformational changes of SodC caused by the disulfide formation, and indeed, the difference spectrum between E,E-SodC^S-S and E,E-SodC^SH (or E,E-SodC^noCys) is consistent with the formation of a β-sheet structure with a characteristic negative peak at 218 nm and a positive peak at 200 nm (Fig. 3D) (31). These results thus support an important role for the disulfide bond in realizing β-sheet structures in SodC.

Formation of a β-sheet structure in SodC by introduction of the disulfide bond was also confirmed by FTIR. Fig. 4A shows the second derivative FTIR spectrum of E,E-SodC^noCys, and peaks were observed at 1,641 and 1,665 cm^{-1}, which were assigned to a random coil and a β-turn structure, respectively (21). These results are consistent with the random coil nature of E,E-SodC^noCys suggested by CD spectroscopy (Fig. 3A). A peak was also observed at 1,617 cm^{-1}, but its assignment remains obscure. Notably, the spectrum of E,E-SodC^noCys remained unchanged by the addition of both copper and zinc ions but was significantly different from that of SodC with the disulfide bond; the second derivative FTIR spectrum of E,E-SodC^S-S detected peaks at 1,634 and 1,677 cm^{-1} due to a β-sheet structure (Fig. 4A). A peak was also observed at 1,658 cm^{-1} (Fig. 4A), which could be assigned to either a random coil or an α-helical
Further addition of both copper and zinc ions to E,E-SodCS-S did not significantly change the spectrum (Fig. 4A), again consistent with a primary role of the disulfide bond in the formation of secondary structures. Moreover, a difference FTIR spectrum between E,E-SodCnoCys (dotted curve in A) and E,E-SodCS-S (thin solid curve in A) is shown as a thin curve, whereas a thick curve represents a difference CD spectrum between E,E-SodCSH (thin solid curve in A) and E,E-SodCS-S (thin solid curve in A).

Based upon these results, a $\beta$-sheet structure was formed in SodC after introduction of the disulfide bond; therefore, SodC without the disulfide bond was not able to become enzymatically active even in the presence of copper ions.

**Formation of a Disulfide Bond Makes the Conformation of SodC More Compact**—The random coil nature of SodC without the disulfide bond was also inferred by size exclusion chromatography. As shown in Fig. 5A, E,E-SodCnoCys eluted at 7.3
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In *E. coli*, SodC is equipped with an N-terminal signal peptide for its transport to the periplasmic space (32), where the signal peptide is cleaved from SodC. To examine the effects of the disulfide bond on the intracellular stability of SodC, sodC-knock-out *E. coli* cells were transformed with plasmids encoding SodC/SodC\(^{\text{noCys}}\) with an N-terminal signal peptide under the control of a tac promoter. As shown in Fig. 6B, SodC with a disulfide bond, in which the N-terminal signal peptide was cleaved, was detected in a periplasmic fraction extracted from *E. coli* cells grown to the stationary phase. In contrast, almost no SodC\(^{\text{noCys}}\) was confirmed in the periplasmic fraction (Fig. 6B) or cytoplasmic fractions (data not shown), supporting the idea that formation of the disulfide bond increases the intracellular stability of SodC proteins. Intracellular protein levels are, however, also affected by factors other than the susceptibility to proteolysis; therefore, we attempted to directly reduce the disulfide bond in SodC under conditions in which protein synthesis was inhibited.

The sodC-knock-out *E. coli* cells, in which SodC with an N-terminal signal peptide was overexpressed, were first cultured to the stationary phase. DTT, an efficient reductant permeable to the outer membrane, was then added to the culture together with chloramphenicol to halt new protein synthesis. Periplasmic extracts were prepared by cold osmotic shock in the presence of a thiol-specific modifier, iodoacetamide, and the thiol-disulfide status of SodC was investigated by Western blotting. As shown in Fig. 6C, all of overexpressed SodC proteins were found to possess the disulfide bond in the absence of DTT, and the disulfide-reduced form of SodC appeared only after the addition of 1.0 mM DTT. These results well reproduce a previous study (11) and show that the disulfide bond in existing SodC proteins is reduced by DTT. Furthermore, we have noted that the total amounts of SodC were significantly decreased by the addition of DTT (Fig. 6C). Based upon SDS-PAGE analysis of the periplasmic fraction followed by Coomassie Brilliant Blue staining, we confirmed that total protein levels and composition of proteins in the periplasmic fraction were not drastically affected by the addition of DTT (data not shown); therefore, the disappearance of SodC by the addition of increasing amounts of DTT is not considered to be due to DTT-induced disruption of the cell wall and leakage of periplasmic proteins. In addition, endogenous SodC proteins in *E. coli* BW25113 cells were found to possess the disulfide bond but disappeared when treated with increasing amounts of DTT (Fig. 7A). Because the expression level of endogenous SodC was not as high compared with that of SodC expressed from plasmid-borne cDNA (Fig. 6C), the remaining disulfide-reduced form of endogenous SodC would be below the detection limit of
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**FIGURE 6.** *SodC* without the disulfide bond is highly susceptible to proteolytic degradation. A, 0.3 g/liter *SodC* in 50 mM Tris/100 mM NaCl, pH 7.0, was incubated with varying amounts of Pronase (0.3 × 10⁻³–0.3 × 10⁻¹ g/liter) at 37°C for 30 min and then analyzed by reducing SDS-PAGE. *SodC* and *SodC*noCys in the absence (E,E-SodC) and presence (Cu,Zn-SodC) of equimolar CuSO₄ and ZnSO₄ were examined. B, periplasmic fractions (5 μg) of the sodC knock-out *E. coli* (BW25113 ΔsodC) cells transformed with a plasmid encoding cDNA of *SodC*/*SodC*noCys with an N-terminal signal peptide were analyzed by Western blotting using anti-*SodC* sera. C, *E. coli* (BW25113 ΔsodC) cells transformed with a plasmid encoding cDNA of *SodC* with an N-terminal signal peptide were first cultured to the stationary phase, and then the indicated concentration of DTT was added together with chloramphenicol. After 1 h, periplasmic fractions (5 μg) were prepared and analyzed by Western blotting with anti-*SodC* sera.

Western blot (Fig. 7A). Given that protein synthesis is largely suppressed in the stationary phase and was further inhibited by chloramphenicol, these data support the idea that reduction of the disulfide bond facilitates the degradation of existing SodC proteins.

A thiol-disulfide oxidoreductase, DsbA, has been proposed to introduce a disulfide bond in SodC at the periplasmic space of *E. coli* (11); therefore, we assumed that knock-out of DsbA retards disulfide formation and thereby promotes the degradation of SodC. Unexpectedly, in *dsbA*-knock-out *E. coli* cells, all endogenous SodC were found to form the disulfide bond (Fig. 7A), suggesting that a DsbA-independent pathway(s) is available for disulfide formation in SodC. Despite this, the band intensity of SodC in *dsbA*-knock-out *E. coli* was found to be significantly lower than that of the parent strain, BW25113 (Fig. 7A). Also, SodC expression was found to be more sensitive to DTT when DsbA was absent (Fig. 7A). Quite notably, throughout the experiments, no bands corresponding to the disulfide-reduced form of endogenous SodC were observed, implying the immediate degradation of SodC upon reduction of the disulfide bond.

We suspected that disulfide-reduced SodC could accumulate in the absence of an endogenous protease responsible for degradation of SodC, but we failed to detect disulfide-reduced SodC when a periplasmic protease, DegP or Prc, was deleted from *E. coli* cells (Fig. 7B). Although further studies will be required to test several other periplasmic proteases for their involvement in the degradation of disulfide-reduced SodC, it is also possible that not one but several proteases are involved. Despite this, disulfide-reduced SodC was considered to assume a random coil-like conformation that was highly susceptible to proteolytic degradation. Therefore, based upon the results here, we propose a mechanism whereby the intracellular stability of SodC is regulated by its thiol-disulfide status.

**DISCUSSION**

*SOD1* is a ubiquitous antioxidant enzyme that has been identified in all aerobic organisms from bacteria to humans, where dysfunction of *SOD1* is often detrimental. Indeed, deletion of *sod1* gene in yeast (33), flies (34), and mice (35) results in decreased lifespan, and furthermore, dominant mutations in human SOD1 have been identified to cause a familial form of amyotrophic lateral sclerosis (36). Also, in infectious bacteria, periplasmic SodC proteins combat against the respiratory burst of phagocytes, assuring successful survival in the host (16). Therefore, the enzymatic activation of SOD1/SodC controls various physiological processes, and we have found that formation of the disulfide bond plays a primary role in the folding and activation of SodC.

**Folding of SodC Is Regulated Primarily by Formation of the Disulfide Bond**—As shown in this study, prokaryotic *E. coli* SodC has little secondary structure in the absence of the disulfide bond. This is in sharp contrast to previous findings on eukaryotic SOD1; for example, a β-barrel-like folding pattern of human SOD1 is maintained even in the disulfide-reduced state.
(5), whereas its quaternary structure has been shown to be affected by the thiol-disulfide status (6, 37). Notably, furthermore, formation of the disulfide bond is absolutely necessary for SodC to bind metal ions at the zinc-binding site (Fig. 2); however, human SOD1 has been shown to tightly bind a cobalt ion (orange), and the disulfide bond (yellow) are also shown. B, our proposed mechanism of SodC activation regulated by disulfide formation. SodC with an N-terminal signal peptide is translated in the cytoplasm and transported to periplasm. In the periplasm, disulfide-reduced SodC remains unfolded and highly susceptible to proteolytic degradation; therefore, the disulfide formation is required to occur as the first step of SodC maturation (a disulfide-first mechanism). Then SodC with the disulfide bond binds copper/zinc ions to become enzymatically active. Alternatively, an as-yet unidentified copper chaperone may activate SodC by simultaneously supplying a disulfide bond as well as a catalytic copper ion.

Although further investigations are necessary to determine why prokaryotic SodC but not eukaryotic SOD1 is almost unfolded in the absence of the disulfide bond, we suspect that the disulfide-dependent folding of SodC is an important aspect for describing the intracellular localization of SodC (Fig. 8B).

**Activation Mechanism of SodC in Prokaryotic Cells**—After being transported to the periplasm, SodC needs to acquire copper and zinc ions and also the disulfide bond for enzymatic activation. Almost no free copper/zinc ion has been shown to exist in the cytoplasm, and the periplasm is considered to contain significantly higher copper/zinc concentrations than the cytoplasm (41). Although it is still unclear how much freely available copper/zinc ions exist in the periplasmic space, SodC...
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will acquire its metal cofactors after being transported to the periplasm. In eukaryotic SOD1, the first step of the activation appears to be the binding of a zinc ion in the disulfide-reduced apo state (6). Notably, however, our results show that disulfide-reduced SodC is unable to bind a cobalt/zinc ion (Fig. 2). Also, even in the presence of metal ions, the disulfide-null SodC is considered to remain in the extended conformation and exhibited high susceptibility to proteolysis (Fig. 6A). Taken together, formation of the disulfide bond is considered to first occur rapidly in SodC just after translocation to the periplasm, which is then followed by the binding of metal ions; otherwise, SodC would become degraded without being enzymatically activated (the “disulfide-first” mechanism in Fig. 8B).

In many eukaryotic cells the disulfide bond is introduced into SOD1 by the copper chaperone, CCS (6); however, prokaryotes including E. coli do not have CCS. Instead, E. coli is equipped with the disulfide bond formation (Dsb) system in the periplasm that catalyzes the correct introduction of disulfide bonds into periplasmic proteins (42). The disulfide bond in exogenously overexpressed SodC in E. coli has been shown to become more susceptible to reduction with a reducing agent upon deletion of DsbA, a periplasmic protein that serves as a disulfide bond donor to proteins (11). Even in dsbA-knock-out E. coli cells, however, the disulfide bond was introduced efficiently into all endogenous SodC proteins (Fig. 7A), suggesting a DsbA-independent mechanism for disulfide formation in SodC. Furthermore, no accumulation of the disulfide-reduced endogenous SodC was observed in E. coli cells where DsbA, DsbB, DsbC, DsbD, or DsbG was deleted.3 Nonetheless, there is a notable caveat about no apparent roles of the Dsb system on the disulfide formation in SodC; namely, it may be difficult to detect disulfide-reduced SodC simply because of its high susceptibility to proteolytic degradation in cells. It thus remains an open question whether formation of the disulfide bond in SodC is performed solely by the Dsb system, and further investigations will be required to identify a DsbA-independent pathway(s) in the SodC activation mechanism. Also, a cupric ion itself is able to function as an oxidant to form a disulfide bond. Because the disulfide-null SodC can bind a copper ion (Fig. 2B), the interaction between SodC and a cupric ion might play roles in introducing the disulfide bond into SodC under oxidizing environment of the periplasm.

As proposed in the CCS-dependent activation of eukaryotic SOD1, it is also likely that disulfide formation in SodC occurs simultaneously with the copper supply step by an as-yet unidentified copper chaperone protein in E. coli. Interestingly, in S. enterica, a periplasmic cupro-protein, CueP, has been recently proposed to supply a copper ion to SodC (43), whereas it remains to be tested if CueP is able to form the disulfide bond in SodC. E. coli lacks CueP, but its functional counterpart, CysF, has been shown to bind a copper ion in the periplasm and have a copper chaperoning activity to the membrane protein CusB (44). Although all SodC proteins in cysF-knock-out E. coli cells were again found to possess the disulfide bond,3 the ability of CysF to introduce a disulfide bond as well as a copper ion into SodC is currently under investigation in our group.

In conclusion, we propose here that disulfide formation is a primary event for the folding of prokaryotic SodC. This is consistent with the fact that the bacterial periplasm, where SodC is localized, generally provides an oxidizing environment for stabilizing a disulfide bond. When the disulfide bond is lacking, however, SodC is rapidly degraded and loses its enzymatic activity. Although it remains obscure how much the periplasm can change its redox potential during the life cycle of bacteria, the disulfide bond in SodC could function as a sensor to modulate the enzymatic activity in response to the redox environment of the periplasm. Also, SodC activity is an important countermeasure of pathogenic bacteria against the respiratory burst of macrophages; therefore, reduction of the disulfide bond in SodC would be a promising strategy to mitigate the virulence of such bacteria.

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