Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS

Yoshiaki Furukawa¹, Andrew S Torres¹
and Thomas V O'Halloran^{1,2,*}

¹Department of Chemistry, Northwestern University, Evanston, IL, USA
and ²Department of Biochemistry, Molecular Biology and Cell Biology,
Northwestern University, Evanston, IL, USA

The antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1) has the distinction of being one of the most abundant disulfide-containing protein known in the eukaryotic cytosol; however, neither catalytic nor physiological roles for the conserved disulfide are known. Here we show that the disulfide status of *Saccharomyces cerevisiae* SOD1 significantly affects the monomer–dimer equilibrium, the interaction with the copper chaperone CCS, and the activity of the enzyme itself. Disulfide formation in SOD1 by O₂ is slow but is greatly accelerated by the Cu-bound form of CCS (Cu-CCS) *in vivo* and *in vitro* even in the presence of excess reductants; once formed, this disulfide is kinetically stable. Biochemical assays reveal that Cu-CCS facilitates Cys oxidation and disulfide isomerization in the stepwise conversion of the immature form of the enzyme to the active state. The immature form of SOD1 is most susceptible to oxidative insult and to aggregation reminiscent of that observed in amyotrophic lateral sclerosis. Thus Cu-CCS mediation of correct disulfide formation in SOD1 is important for regulation of enzyme activity and for prevention of misfolding or aggregation.

The EMBO Journal (2004) 23, 2872–2881. doi:10.1038/sj.emboj.7600276; Published online 24 June 2004

Subject Categories: proteins; molecular biology of disease

Keywords: copper chaperone; disulfide bond; fALS; oxidative stress; superoxide dismutase

Introduction

Disulfide bonds are required for the stability and function of a large number of secreted proteins (Thornton, 1981), and recent biochemical studies on thiol-disulfide oxidoreductases describe pathways for disulfide formation within cellular compartments (Bader *et al*, 1999; Sevier and Kaiser, 2002). In contrast, few intracellular proteins are known to maintain persistent disulfides in the cytosol, where a 30- to 100-fold excess of the reduced glutathione over the oxidized form supports a reducing environment (Hwang *et al*, 1992). One

apparent exception is the housekeeping enzyme Cu,Zn-superoxide dismutase (SOD1), which catalyzes removal of superoxide anion radical (McCord and Fridovich, 1969). This dimeric protein has a highly conserved pair of cysteines, which are found in the disulfide state in all isolated forms of the protein. Given the high copy number of SOD1 proteins (ca. 10 μ M in *Saccharomyces cerevisiae*; Rae *et al*, 1999) and the predominately cytosolic localization in eukaryotes (Bordo *et al*, 1994), it is not clear how the disulfide is formed in this reducing environment. While the endoplasmic reticulum (ER) has specialized machinery for oxidative folding (Bader *et al*, 1999), most evidence indicates that there is no SOD1 localization to the ER (Lindenau *et al*, 2000). Crystallographic studies of SOD1 suggest a structural role for the disulfide in guiding substrate into the active site (Fisher *et al*, 1994); however, little else is known about the function of the disulfide or the mechanism of its formation in eukaryotes. Furthermore, the effect of the disulfide on dismutase activity and quaternary structure is not clear, even though altered or aggregated forms of the protein are thought to be at the center of a familial form of amyotrophic lateral sclerosis (fALS) (Cleveland and Rothstein, 2001).

In addition to disulfide formation, the nascent SOD1 polypeptide must undergo three other modifications: copper and zinc acquisition and dimerization. Each of these factors ultimately contributes to the unusual stability of the mature enzyme, which is active in stringent denaturing conditions such as 8 M urea (Malinowski and Fridovich, 1979). Earlier studies show that the essential copper cofactor is typically acquired by SOD1 in yeast with the assistance of the CCS1 gene product, known as the copper chaperone for SOD1, CCS (Culotta *et al*, 1997). The *S. cerevisiae* and the human CCS proteins have been shown to insert directly the essential copper cofactor (Rae *et al*, 1999, 2001) after docking in a specific manner with SOD1 *in vitro* and *in vivo* both in the cytosol (Schmidt *et al*, 2000) and in the inner membrane space (IMS) of the mitochondria (Field *et al*, 2003). The crystal structure of an inactive SOD1 mutant complexed with CCS (Lamb *et al*, 2001) unexpectedly exhibits an intermolecular disulfide bond; however, it is not clear whether this crosslink is an artifact of crystallization conditions. The recent demonstration of a key role for oxygen in the post-translational activation of SOD1 (Brown *et al*, 2004) led us to test directly the physiological and mechanistic relevance of the disulfide.

Here we show that SOD1 disulfide formation is essential for the SOD1 activity and provide data supporting a stepwise mechanism for the formation of the conserved disulfide bond in a Cu-bound form in a CCS- and O₂-dependent manner. If the SOD1 disulfide is formed before copper insertion, the enzyme cannot be activated by Cu-CCS. The reduced apo-form of SOD1 has recently been shown to be the best substrate for uptake into the mitochondria (Field *et al*,

*Corresponding author. Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA. Tel.: +1 847 491 5060; Fax: +1 847 491 7713; E-mail: t-ohalloran@northwestern.edu

Received: 25 March 2004; accepted: 21 May 2004; published online: 24 June 2004

2003), but here we show that it is also most susceptible to potentially deleterious aggregation by oxidative stress. The regulation of correct disulfide formation by CCS is thus important in controlling both SOD1 activity and preventing misfolding or aggregation. These results underscore the biochemical complexity of the emerging SOD1 pathway and provide a molecular basis for the fact that the cell can rapidly upregulate this antioxidant activity in response to oxidative stress (Brown *et al*, 2004) by converting a pool of inactive protein into active enzyme via a post-translational modification, namely disulfide formation.

Results

The intramolecular disulfide plays a critical role in SOD1 activity

Prior studies have revealed a biochemical role for CCS in copper insertion into SOD1; however, the SOD1 disulfide status was not evaluated in those studies. Biochemical assays on wild-type (WT) and Cys-mutant proteins used here test the effect of the disulfide bond on SOD1 activity. For these *in vitro* experiments, metal cofactors were removed, and Cys residues were reduced with excess dithiothreitol (DTT) in an anaerobic chamber to give the totally demetallated and reduced protein, E,E-ySOD1^{SH}. The superscript indicates the status of the conserved Cys residues and E represents an empty metal-binding site; that is, E,E-ySOD1^{S-S} is the disulfide form with no metal ion bound. The Zn-only or Cu- and Zn-bound forms of ySOD1 with the disulfide intact were made in the anaerobic chamber and are designated as E,Zn- and Cu,Zn-ySOD1^{S-S}, respectively.

Native gel electrophoresis followed by in-gel nitro blue tetrazolium (NBT) assay for SOD1 activity (Beauchamp and Fridovich, 1971) reveals that E,Zn-ySOD1^{SH} reacts with the Cu-bound form of yeast CCS (Cu-yCCS) or CuSO₄ (lanes 1–3,

Figure 1A); after 1 h aerobic incubation, both reactants restore SOD1 activity although CuSO₄ is less efficient. A spectrometric assay of SOD1 activity (Ukeda *et al*, 1997) indicates that the protein reconstituted with CuSO₄ is half as active as that with Cu-yCCS (data not shown), corroborating the in-gel assay result. The lower activity of SOD1 after CuSO₄ treatment is consistent with the previous study (Lyons *et al*, 1998) where simple copper salts were not able to fully reconstitute apo-ySOD1. The disulfide status of SOD1 is readily evaluated by the selective reaction of the free thiol groups with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), which adds ca.500 Da per reactive thiol and thus shifts the mobility of the protein on a denaturing gel. Formation of the ySOD1 disulfide by either CuSO₄ or Cu-yCCS is shown by electrophoresis after AMS treatment (Bader *et al*, 1998) (lanes 1–3, Figure 1C). ySOD1 has two Cys residues, Cys57 and Cys146, and ySOD1^{SH} with two molecules of AMS runs ca. 1 kDa higher molecular weight than that of the respective AMS-unmodified or oxidized forms.

In contrast to WT, no activity of C146S ySOD1 was detected after incubation with either Cu-yCCS or CuSO₄. While the detection limit of the in-gel assay is ~2 ng (Rae *et al*, 1999), 200 ng of the protein was loaded. Two other disulfide mutant proteins, C57S and C57S/C146S, and the respective Ala mutants, C57A and C146A, were also completely inactive after incubation with either Cu-yCCS or CuSO₄ both in the in-gel and spectrometric assays (data not shown). Inductively coupled plasma atomic emission spectrometric (ICP-AES) analysis showed that C146S and C57S/C146S mutants have similar metal-binding properties with WT ySOD1; 40–70% of the Cu and Zn sites were occupied with the respective metal ions after 1 h incubation with CuSO₄ and ZnSO₄ at 37°C. Thus, abrogation of activity in the disulfide mutants is not due to the absence of metal cofactors. These results suggest that the disulfide bond itself is essential for manifestation of SOD1 activity. Its role is not known; however, the disulfide bond between Cys57 and Cys146 has been suggested to stabilize structurally a hydrogen bond between the backbone amide of Cys57 and Arg143 (Fisher *et al*, 1994), which may then orient the positively charged side chain for optimal interaction with superoxide anion. To place this disulfide within the context of physiological boundary conditions, we examined the thermodynamics and kinetics of disulfide formation by copper salts, O₂, and CCS.

Disulfide formation is slow in the absence of Cu-yCCS

Among the various oxidants present in aerobically grown cells, it is well known that dissolved oxygen can oxidize Cys residues, leading to disulfide bond formation; O₂ has significantly higher reduction potential ($E^{\circ} \sim +0.70$ V) than typical disulfide-containing proteins ($E^{\circ} = -0.12$ to -0.45 V) (Gilbert, 1995). After aerobic incubation for an hour, however, E,E-ySOD1^{SH} still remains in the reduced state (see Supplementary Figure S1), indicating a slow reaction. We also attempted to use another physiological oxidant, GSSG or GSH/GSSG buffer, but aerobic incubation with an equimolar amount of GSSG or with a physiological GSH/GSSG buffer did not complete disulfide formation within an hour (see Supplementary Figure S2). Compared to the slow oxidation of the thiol groups by O₂ or glutathione, analysis with ICP-AES showed that zinc was readily incorporated into E,E-ySOD1^{SH} under anaerobic conditions and reached 50–70%

Figure 1 The in-gel assay of the SOD1 activity: E,Zn-ySOD1^{SH} WT (lanes 1–3) and C146S (lanes 4–6). Reaction mixture contained 13 μ M ySOD1, 100 μ M EDTA, and 100 μ M BCS in 50 mM HEPES, pH 7.2, with or without 13 μ M Cu-yCCS and was prepared in a glove box (lanes 1, 2, 4, and 5). In lanes 3 and 6, the solution contains 13 μ M CuSO₄ without EDTA and BCS. After aerobic incubation at 37°C for an hour, an aliquot containing 200 ng of ySOD1 was applied to (A) 10% NATIVE-PAGE for the activity assay or (B) 12.5% SDS-PAGE under reducing conditions for Western blotting. (C) The thiol-disulfide status was also checked with 12.5% SDS-PAGE after modification with AMS.

occupancy after an hour or less. Disulfide formation is thus not a prerequisite for zinc acquisition. Similarly, E,Zn-ySOD1^{SH} was not oxidized after aerobic incubation for an hour; more than 6 h was needed for air oxidation to complete the disulfide formation (Figures 2A and B). The oxidation by glutathione was also not complete within an hour (data not shown). It has been reported that human and bovine Cu,Zn-SOD1 exhibits the thiol oxidase activity (Winterbourn *et al*, 2002); however, we cannot observe any disulfide formation in E,Zn-ySOD1^{SH} even after an hour incubation with the holo enzyme (Cu,Zn-ySOD1^{S-S}) (Figure 2B, inset). The sluggishness of the O₂ and GSSG oxidation of SOD1 led us to explore the possibility that these conserved cysteines had an unusual thermodynamic stability relative to the cytoplasmic redox potential.

The reduction potential of E,Zn-ySOD1 is estimated by monitoring the fraction of protein reacting with AMS in redox-buffered conditions. This varies sigmoidally with the ratio of GSH and GSSG in solution (Figure 3), and an E° value of -0.23 ± 0.02 V for the redox potential was obtained by fitting the concentration-dependent equilibrium position to eq. 1 (see Materials and methods). No mixed disulfide species between ySOD1 and glutathione were detected by the MALDI-TOF mass spectrometry. This redox potential is well below that of dissolved oxygen but in line with the potentials of cytosolic disulfide-containing enzymes such as thioredoxin. This result indicates that the Cys in E,Zn-ySOD1 has no unusual thermodynamic stability and that disulfide formation cannot be completed by the redox equilibration with the cytosolic glutathione buffer. Interestingly, the incubation time required for the redox equilibrium was significantly

Figure 2 (A) Effects of the dissolved oxygen on the disulfide formation in E,Zn-ySOD1^{SH}. Protein was incubated for the given time at 37°C, and following modification with AMS, 2 μg of ySOD1 was loaded on the 12.5% SDS-PAGE under nonreducing conditions. (B) The fraction of the reduced form plotted against the incubation time: the time course of the disulfide formation in 13 μM E,Zn-ySOD1^{SH} with equimolar (□) CuSO₄, (■) CuSO₄ in 100 μM EDTA, (○) Cu(CH₃CN)₄(PF₆), and (●) Cu(CH₃CN)₄(PF₆) in 100 μM BCS. The results of aerobic incubation of 13 μM E,Zn-ySOD1^{SH} (▲) are also shown. (Inset) The time course of the disulfide formation in 13 μM E,Zn-ySOD1^{SH} with 5 μM Cu,Zn-ySOD1^{S-S}.

Figure 3 Thiol-disulfide redox potential determination for E,Zn-ySOD1. (A) One of the SDS-PAGE gels for the measurement of the redox equilibrium: 1 μM E,Zn-ySOD1^{S-S} was mixed with 10 μM GSSG and 0.2–40 mM GSH (from left to right). After anaerobic incubation at 30°C for 65 h, the reaction mixtures were quenched by 10% TCA of total. After modification with AMS, 2 μg of protein was loaded on 12.5% SDS-PAGE under nonreducing conditions. (B) Fraction of the reduced form of ySOD1 was plotted against [GSH]²/[GSSG]. Each plot was fitted with eq. 1 to determine the redox potential, $E_{\text{SOD1}}^{\text{SH/S-S}}$. Dotted, broken, and solid curves colored red represent the fit for the plot after 14, 38, and 65 h incubation of E,Zn-ySOD1^{SH}, respectively, while the blue dotted and solid curves after 15 and 65 h incubation of E,Zn-ySOD1^{S-S}.

different for the reduced and oxidized forms of the protein. When E,Zn-ySOD1^{SH} is the starting material, it takes approximately 70 h to reach the redox equilibrium, but only ca. 15 h is enough in the case of E,Zn-ySOD1^{S-S} (Figure 3B). Given that the typical doubling time of a yeast cell is ~2 h (Sinclair *et al*, 1998), it is clear that a catalyst is required if the disulfide-bonded form of SOD1 is to persist in the cytoplasm where the ratio of GSH to GSSG is ca. 30- to 100-fold. The slow oxidation rate of E,Zn-ySOD1^{SH} led us to test the possibility that the disulfide remains reduced until reaction with O₂ and Cu-yCCS.

The disulfide bond could be formed during the copper incorporation into the protein because Cu²⁺ ion itself is known to be a good oxidant for disulfide formation (Kachur *et al*, 1999). As expected, disulfide formation in ySOD1 can be accomplished by aerobic addition of one equivalent of CuSO₄ or Cu¹⁺(CH₃CN)₄PF₆ per monomer within an hour (Figure 2B); however, when free copper ions were complexed by EDTA ($K_d \sim 10^{-19}$ M for Cu²⁺) or bathocuproine sulfonate (BCS, $K_d \sim 10^{-20}$ M for Cu¹⁺), disulfide formation was not observed (Figure 2B). Thus while free copper ions in aerobic buffers are potent oxidants for the rapid disulfide formation in the test tube, the chelated ions are not able to catalyze this oxidation.

Cu-yCCS accelerates the disulfide formation in ySOD1

When the reaction of ySOD1 with Cu-yCCS was performed under anaerobic conditions, the Cys residues of E,Zn-ySOD1^{SH} remain in the reduced state even after 2 h incubation at 37°C (Figure 4A). Upon exposure to air for as little as 3 min, in contrast, the Cys residues in E,Zn-ySOD1^{SH} were almost completely converted into the disulfide state

Figure 4 (A) Disulfide formation in E,Zn-ySOD1^{SH} with Cu-yCCS. The reaction mixture contains 13 μ M E,Zn-ySOD1^{SH}, 13 μ M Cu-yCCS in 50 mM HEPES, pH 7.2, with 100 μ M EDTA and 100 μ M BCS. After modification with AMS, 2 μ g of protein was loaded on 12.5% SDS-PAGE without β -ME. (B) The time course of the disulfide formation with Cu-yCCS or Cu¹⁺-GSH. (▲) Cu-yCCS, (■) Cu-yCCS with 8 mM GSH/0.16 mM GSSG, (□) Cu¹⁺-GSH, and (△) Cu¹⁺-GSH with 100 μ M BCS. The reaction of 13 μ M E,Zn-ySOD1^{SH} (H48F) with equimolar Cu-yCCS is also shown (●). (C) Time course of the disulfide formation by an equimolar amount of (□) totally reduced apo-yCCS, (■) oxidized apo-yCCS, and (○) reduced yCCS after reconstitution with Cu¹⁺. (D) Peptide-mapping analysis of oxidized apo-yCCS with and without E,Zn-ySOD1^{SH} after modification with the thiol-specific reagent, iodoacetamide (IA). After digestion by Asp-N, the sample was purified by ZipTip C18 (Millipore). The peptide fragment, 207–240, containing Cys229 and Cys231 (*m/z* 3851) is shifted to *m/z* 3968 after modification with IA. The peptide fragment, 116–152, is also shown in the figure (*m/z* 3999).

(Figure 4A). This is directly parallel to the enzyme activity time course reported by Brown *et al* and indicates a molecular role for dissolved O₂ in the activation by Cu-yCCS

(Brown *et al*, 2004). It should also be emphasized that the disulfide formation under these conditions is significantly accelerated by Cu-yCCS (Figure 4B) compared to that of the free copper ion (Figure 2B). Unlike the reactions with copper salts, the presence of the chelators (0.1 mM EDTA and 0.1 mM BCS) did not affect disulfide formation by Cu-yCCS and the kinetics overlaps with the curve for the reaction in the absence of the metal chelators shown in Figure 4B (data not shown). These controls demonstrate that disulfide formation in these assays is not the result of copper dissociating from Cu-yCCS, and are thus consistent with a direct role of copper ion bound within the SOD1/CCS complex. Cu-yCCS can also introduce the disulfide bond in the H48F ySOD1 mutant (Figure 4B), which cannot bind Cu ion (Lamb *et al*, 2000). These results show that Cu-yCCS itself can promote the O₂-dependent formation of the disulfide bond in ySOD1 even under stringent copper-limiting conditions.

We next examined whether the apo-yCCS protein could support disulfide exchange reaction with SOD1 in the absence of copper. yCCS contains seven Cys residues, three pairs of which are able to form intramolecular disulfides. Aerobic incubation of E,Zn-ySOD1^{SH} with totally reduced apo-yCCS does not lead to disulfide formation in ySOD1 (Figure 4C). After the reduced yCCS protein is oxidized by O₂ in an overnight incubation, in contrast, the resulting protein (oxidized apo-yCCS) can form the disulfide bond in ySOD1 under anaerobic conditions albeit inefficiently (Figure 4C). After the reduced form of apo-yCCS binds a Cu(I) ion, it significantly accelerates disulfide formation in E,Zn-ySOD1 (open circle in Figure 4C). Peptide-mapping analysis with the iodoacetamide modification (Figure 4D) shows that oxidized apo-yCCS contains the disulfide in domain III, and that this disulfide can be transferred to SOD1. This result demonstrates that the oxidized apo-yCCS protein is competent to undergo a specific disulfide isomerization reaction with SOD1. Given that the reaction does not go to completion in a physiological time frame, the result also underscores the importance of the Cu-bound form of CCS in reactions of CCS/SOD1 complex with O₂.

Before the discovery of CCS, Rotilio and co-workers (Ciriolo *et al*, 1990) proposed Cu¹⁺-GSH as a candidate for the copper donor to apo-SOD1 in the cell. Aerobic incubation of E,Zn-ySOD1^{SH} with Cu¹⁺-GSH leads to activation of ySOD1 in this assay (data not shown), and concomitantly about 70% of the ySOD1 forms a disulfide within an hour *in vitro* (Figure 4B). In comparison, the apparent rate of disulfide formation in the presence of Cu-yCCS was significantly (>30-fold) faster than in the presence of the Cu¹⁺-GSH complex. When experiments with the Cu¹⁺-GSH complex were repeated in the presence of the copper chelators, no active ySOD1 was obtained, and the disulfide formation was further decelerated. The small amount of the disulfide form may be due to the build-up of GSSG in the aerobic reaction mixture (Figure 4B). This is in sharp contrast to the Cu-yCCS reaction under aerobic conditions where rapid disulfide formation was unaffected by the presence of copper chelators. The bulk of SOD1 is localized in the cytosol where the excess of GSH over GSSG leads to a strongly reducing condition (Hwang *et al*, 1992). Such a reducing environment generally disfavors disulfide formation. Despite this, in the aerobic conditions, Cu-yCCS can form the ySOD1 disulfide in the presence of 8 mM GSH and metal chelators with little

perturbation on its formation kinetics (Figure 4B). Taken together, these data are consistent with a model in which CCS provides a privileged environment for copper within the docked CCS/SOD1 complex, particularly one that supports rapid air oxidation of the Cys residues even under copper-limiting and strongly reducing conditions such as those found in the eukaryotic cytosol. If these *in vitro* results are also relevant *in vivo*, the reduced form of ySOD1 should accumulate in yeast, which lacks yCCS.

yCCS facilitates ySOD1 disulfide formation in the cytosol

To test for an *in vivo* role of yCCS in the disulfide formation, we examined the thiol-disulfide status of ySOD1 using SY1699 (WT) and *ccs1Δ* (formerly referred to as *lys7Δ*) yeast strains, which exhibit no SOD1 activity (Culotta *et al*, 1997). Acid lysis of intact cells followed by the AMS modification prevents nonspecific disulfide scrambling during and after lysis (Frand and Kaiser, 1999). In the WT strain, ySOD1 can be detected in the Western blot and was not modified with AMS (lanes 3 and 4 of Figure 5A), indicating an intact disulfide bond in ySOD1. In contrast, the reaction of *ccs1Δ* lysate with AMS reveals bands corresponding to the AMS-modified ySOD1 proteins (lanes 5 and 6, Figure 5A), suggesting that >90% of the ySOD1 Cys residue remains in the reduced state in the absence of yCCS. The amounts of reduced ySOD1 in *ccs1Δ* cell lysate are significantly more than that expected from its redox potential (~50%). This is best explained by the results shown in Figure 3B, where disulfide formation through redox equilibration takes a long time, ~70 h, in the absence of CCS.

It is also noteworthy that the ySOD1 band in *ccs1Δ* lysate is a doublet in the absence of AMS (lane 5, Figure 5A). This

doublet band can be seen when the purified E,Zn-ySOD1^{SH} was loaded on SDS-PAGE gel without any reductants such as β-mercaptoethanol (β-ME) (lane 1, Figure 5B). During migration in the gel, a pair of Cys residues can be oxidized to the disulfide by radical initiators remaining after gel polymerization (Edwards and Maloy, 2001). The lower doublet band showed the same mobility as that of E,Zn-ySOD1^{S-S} (lanes 1 and 2, Figure 5B). On the other hand, the mobility of the upper band was the same as that of C146S ySOD1 or of E,Zn-ySOD1^{SH} with β-ME (lanes 1, 3, and 4, Figure 5B). Thus, the upper band in the ySOD1 doublet corresponds to ySOD1^{SH}. The assignment of the doublet band in *ccs1Δ* lysate corroborates the conclusion from the AMS experiment that formation of the ySOD1 disulfide *in vivo* is inefficient in the absence of yCCS. In addition, a significant portion of the SOD1 migrates as dimers or aggregates in *ccs1Δ* lysate, which is not treated with AMS (lane 5, Figure 5A). Since reaction of the lysate with AMS prevents the formation of these bands, they are best ascribed to disulfide-crosslinked species that are formed upon entry of reduced SOD1/extract mixture into oxidant-containing polyacrylamide gel matrix. These results underscore the susceptibility of the reduced Cys in SOD1 to mild oxidation.

It is also notable that a small amount of disulfide-bonded form of ySOD1 (~10% of total ySOD1) was observed even in the absence of yCCS (lane 6, Figure 5A), which raises the question of whether Cu-yCCS can activate ySOD1 once the disulfide forms in the copper-free protein (i.e. E,Zn-ySOD1^{S-S}).

Cu-yCCS can activate the reduced, but not oxidized, form of E,Zn-ySOD1

As shown in Figures 6A and B (lane 5), E,Zn-ySOD1^{S-S} is activated with CuSO₄, which is consistent with the previous *in vitro* experiments (Dunbar *et al*, 1984). In contrast, Cu-yCCS cannot act on E,Zn-ySOD1^{S-S} under conditions where E,Zn-ySOD1^{SH} is successfully activated (lanes 2 and 4, Figures 6A and B). These data suggest that Cu-yCCS distinguishes between ySOD1^{SH} and ySOD1^{S-S}. A standing controversy is how CCS recognizes SOD1; SOD1 is exclusively found in the dimeric state, which has been proposed to inhibit the heterodimeric interaction with CCS (Hall *et al*, 2000). Noting that the interface of the SOD1 dimer involves the loop structure connected with the disulfide bond, we tested the possibility that reduction of the disulfide could shift the monomer-dimer equilibrium in SOD1 using size-exclusion chromatography.

Figure 6C shows the elution profiles of E,Zn-ySOD1^{S-S} and E,Zn-ySOD1^{SH} in the gel filtration. E,Zn-ySOD1^{S-S} eluted as a major peak (~70%) at 14.9 ml, which corresponds to ca. 30 kDa, consistent with dimer. The monomer also exists but is a minor species (~20%) at 15.9 ml (19 kDa). The estimated molecular weight of the monomer and dimer is in good agreement with that reported (Hartz and Deutsch, 1972). In contrast, E,Zn-ySOD1^{SH} largely exists as monomer (~70%) with a minor fraction of dimer (~20%). The C146S ySOD1 mutant also showed a similar elution profile, in which about 70% of total protein stayed in the monomer (data not shown). These results indicate that the thiol-disulfide status in ySOD1 is a major determinant controlling monomer-dimer equilibrium. Heterodimer formation with Cu-yCCS is thus more probable for E,Zn-ySOD1^{SH} than for E,Zn-ySOD1^{S-S} given that the latter

Figure 5 (A) Western blotting of *in situ* modification of ySOD1 with AMS using the cell lysate. Lane 1: E,Zn-ySOD1^{S-S}; lane 2: E,Zn-ySOD1^{SH} modified with AMS. Lanes 3 and 5 are the cell lysate of SY1699 and *ccs1Δ*, respectively, while the lysate after modification with AMS is shown in lanes 4 (SY1699) and 6 (*ccs1Δ*). The samples were loaded on 12.5% SDS-PAGE under nonreducing conditions. (B) Comparison of the ySOD1 band mobility on SDS-PAGE. The samples are as follows: lane 1, E,Zn-ySOD1^{SH}; lane 2, E,Zn-ySOD1^{S-S}; lane 3, C146S-ySOD1; lane 4, E,Zn-ySOD1^{SH} loaded with β-ME.

Figure 6 After 13 μ M E,Zn-ySOD1^{SH} or E,Zn-ySOD1^{S-S} was incubated with equimolar Cu-yCCS or CuSO₄, about 200 ng of the samples was loaded on (A) 10% NATIVE-PAGE gel for in-gel assay and (B) 12.5% SDS-PAGE gel under reducing conditions for Western blotting analysis. (C) The gel filtration chromatograms of E,Zn-ySOD1^{S-S} and E,Zn-ySOD1^{SH}. (D) MALDI-TOF mass spectra of the heterodimer: C146S ySOD1 ($m/z^{calc} = 15\,707$, $m/z^{obs} = 15\,708$), Cu-yCCS ($m/z^{calc} = 27\,198$, $m/z^{obs} = 27\,192$), and C146S/yCCS heterodimer ($m/z^{calc} = 42\,905$, $m/z^{obs} = 42\,921$). Peaks denoted by * are from +2-charged states of the proteins. (Inset) The formation of the disulfide-linked heterodimer was further examined using 12.5% SDS-PAGE. Molecular marker (lane 1) and E,Zn-C146S ySOD1 aerobically incubated for an hour with Cu-yCCS at 37°C, loaded (lane 2) with or (lane 3) without β-ME, are shown.

undergoes self-association to form the dimer. This is also supported by the fact that aerobic incubation of the C146S ySOD1 mutant with Cu-yCCS produces a mass peak corresponding to the heterodimeric complex in the MALDI-TOF

mass spectrum (Figure 6D). As shown in the SDS-PAGE gel (inset of Figure 6D), this species disappears upon reduction with β-ME, corroborating formation of a disulfide-linked heterodimer between C146S ySOD1 and yCCS. Intriguingly, a corresponding disulfide-linked heterodimer is not observed in the reaction between C57S ySOD1 and Cu-yCCS (data not shown), indicating that intermolecular disulfide formation is not a random reaction, but is regio-specific. These results reveal that the disulfide status of ySOD1 plays an important role in recognition by Cu-yCCS, but it is not clear how this may be of value to the cell. To address this, we examined the relative susceptibility of the thiol and disulfide forms of SOD1 to aggregation.

Reduced form of SOD1 is prone to aggregation upon oxidative stress

Surface-accessible Cys residues are one of the most oxidizable groups in the cellular environment, and the aberrant thiol oxidation can lead to protein aggregation, which has been associated with some neurodegenerative diseases (Lee and Eisenberg, 2003). Since protein aggregates involving SOD1 are commonly observed in fALS, we further examine if oxidative stress can alter the aggregation state of SOD1. When H₂O₂ is used as a model for oxidative stress, a significant amount of the reduced E,E-ySOD1(WT) is converted to higher-order multimeric forms (Figure 7A). These bands disappear upon addition of the reductant, β-ME, before electrophoresis (Figure 7B), indicating that these multimers are crosslinked via an interprotein disulfide. In the Western blot, we observe some dimer species even in the absence of H₂O₂, suggesting that aerobic incubation of E,E-ySOD1^{SH} can lead to formation of a disulfide-linked dimer (Figure 7). No such bands are observed at a dimer position when E,Zn-ySOD1^{SH} was used. When either Cys57 or Cys146 is mutated to Ser, no formation of multimers higher than the dimer is

Figure 7 Western blotting of the ySOD1 multimer formed by addition of H₂O₂. E,E-ySOD1^{SH} (10 μ M) was aerobically incubated with or without 100 μ M H₂O₂ for an hour, as indicated. After modification with AMS, the protein solutions were mixed with the sample buffer (A) without or (B) with β-ME and loaded on 12.5% SDS-PAGE. Each band position of monomeric species is tentatively assigned on the basis of the apparent electrophoretic mobility.

observed upon addition of H_2O_2 . Furthermore, removal of all cysteines in SOD1, C57S/C146S double mutation, prevents formation of any disulfide linked aggregates even after addition of $100\ \mu\text{M}$ H_2O_2 . We concluded that reduced SOD1 is unusually susceptible to oxidative damage for a cytosolic protein, which underscores the fact that the thiol-disulfide status in SOD1 is important in determining the aggregation state of the protein.

Discussion

All SOD1 enzymes identified to date have one conserved disulfide bond per monomer, and this study shows that this disulfide is indispensable to the enzymatic dismutase activity. Given that eukaryotic SOD1 is typically ca. 1% of total protein and thus a major cytosolic protein, the question of how the disulfide is formed and maintained in the reducing cytosolic environment is enigmatic. Some of these questions are resolved by the new findings here for SOD1's cytosolic copper chaperone, CCS; Cu-CCS can facilitate O_2 -dependent redox chemistry in SOD1 disulfide formation (Figure 4). The results reported here led us to propose a new model in which disulfide formation is a critical step in the post-translational processing that ultimately leads to a holo-enzyme of extraordinary stability toward physical and oxidative stress.

Proposed catalytic cycle for the SOD1 activation by CCS

The first step in activation of the nascent SOD1 polypeptide is not disulfide formation; we propose that incorporation of Zn^{2+} into the reduced protein precedes both disulfide formation and Cu insertion. Gel filtration studies reveal that E,E-ySOD1^{SH} tends to aggregate much more readily than the more mature forms (Y Furukawa and T O'Halloran, unpublished data), but incorporation of Zn^{2+} ion can lead to formation of the distinct ySOD1^{SH} monomer (Figure 6C). Zn^{2+} ion would thus serve a structural role in organizing the monomeric SOD1 polypeptide to a state suitable for reaction with Cu-CCS. While E,E-ySOD1^{SH} is quite susceptible to oxidative aggregation, it is nonetheless of some physiological importance: Culotta and co-workers have shown that this form is most readily imported in the mitochondrial IMS (Field *et al*, 2003).

Apo-CCS at first captures Cu^{1+} ion from unidentified sources through the Cys residues in its domain III (Step I in Figure 8). Upon copper binding, CCS undergoes conformational changes that enhance its interaction with SOD1 (Rae *et al*, 2001). Next Cu-CCS and E,Zn-SOD1^{SH} are proposed to form a noncovalent heterodimeric complex (Step II). Importantly, E,Zn-SOD1^{S-S} is not a substrate for CCS. The subsequent reaction of the docked heterodimer with dissolved O_2 , which affords the oxidizing equivalents, leads to formation of a disulfide-linked heterodimer of CCS and SOD1 (Step III). This is supported by the results that the disulfide-linked heterodimer can be trapped by using the C146S ySOD1 mutant (Figure 6D). We further suggest that the intermolecular disulfide seen in the crystal structure of a heterodimeric complex between yCCS and the H48F ySOD1 mutant (Lamb *et al*, 2001) is not an artifact of crystallization: the data strongly indicate that a disulfide-bridged heterodimeric intermediate involving Cys57 of SOD1 is on the physiological pathway for the post-translational regulation of cellular SOD1 activity.

Cellular response to oxidative stress involving disulfide formation has been recently described for the molecular chaperone, Hsp33 (Graumann *et al*, 2001; Hoffmann *et al*, 2004). Upon exposure to oxidative stress, an intramolecular disulfide is formed among the Cys residues with concomitant release of Zn^{2+} ion, leading to activation of the Hsp33's chaperone function. In the case of CCS, the interaction of SOD1 with Cu-CCS would bring the redox active copper ion into close proximity of the Cys residues in SOD1. Given the redox properties of a Cu^{1+} ion, it is also plausible that O_2 attacks the Cu^{1+} ion bound to CCS-SOD1 complex, and the resulting intermediate facilitates oxidation of thiol groups, ultimately giving rise to a disulfide between Cys57 in SOD1 and one of the Cu-ligating Cys residues in CCS (Step III in Figure 8). Changes in the oxidation state of the copper ion in Cu-CCS may also facilitate metal translocation into the His-rich SOD1 active site while at the same time serving as an O_2 reaction site that initiates formation of the disulfide.

At this time, we cannot further define the chemical steps in the intimate oxidation mechanism; however, after the oxidation step, the intermolecular disulfide is envisioned to undergo exchange to give an intramolecular disulfide in SOD1 thus

Figure 8 Proposed mechanism of the O_2 -dependent post-translational modification of ySOD1 by Cu-yCCS (see text). The inset shows the heterodimeric structure between H48F ySOD1 and yCCS (1JK9) (Lamb *et al*, 2001). The green residues indicate the copper-binding site of ySOD1, and the intermolecular disulfide bond is represented as a yellow stick.

releasing active enzyme (Step IV). The intramolecular disulfide bond in the fully mature Cu,Zn-SOD1 seems to be thermodynamically more stable or at least kinetically more inert than that in E,Zn-SOD1. Cu,Zn-ySOD1 expressed in WT yeast maintains a persistent disulfide even in the strongly reducing condition of the cytosol, but E,Zn-ySOD1 in *ccs1Δ* cell does not (Figure 5A). Inspection of the structures of E,Zn- and Cu,Zn-SOD1 (human) also shows that the loop containing Cys57 (loop IV) is significantly ordered upon binding of Cu ion (Banci *et al*, 2002). Namely, the Cu binding could thereby stabilize the intramolecular disulfide and account for the resistance to reduction. Copper binding in the SOD1 site thus induces the conformational changes especially around the Cys residues, facilitating a protein disulfide isomerase-like reaction from the intermolecular to the intramolecular SOD1 disulfide. Finally, we note that the thermodynamic preference of the disulfide-bonded SOD1 protein for the dimeric state also drives product release, that is, dissociation of the docked complex to the individual proteins, Cu,Zn-SOD1^{S-S} and apo-CCS (Step IV). While multiple turnovers have yet to be documented *in vitro*, the results indicate that the copper-loaded form of CCS greatly accelerates maturation of SOD1 by O₂ and furthermore establish several elementary steps that are consistent with a catalytic role for CCS in the activation of SOD1.

Physiological role of the SOD1 disulfide

These results provide a mechanism that accounts for how major swings from normoxic to hypoxic or anoxic conditions can lead to a post-translational change in SOD1 activity. This has physiological implications *vis-a-vis* the proposal that reactive oxygen species observed in exercise-physiology studies lead to increases in the total activity of antioxidant protein including SOD1 (Rush *et al*, 2003). One path in which physiological oxidative stress could stimulate a post-translational increase in SOD1 activity involves the mechanisms proposed here, in which O₂ triggers Cu loading and disulfide formation in SOD1 by CCS.

These results reveal that SOD1 is part of a more complex biochemical pathway than previously envisioned and have several implications for the toxic gain of function observed for over 90 different SOD1 mutations in fALS patients (Cleveland and Rothstein, 2001). A variety of models have been proposed to explain how the fALS mutations in SOD1 give rise to motor neuron diseases, including alterations in metal affinity and reactivity (Estevez *et al*, 1999) or the formation of the toxic SOD1 aggregates (Bruijn *et al*, 1998); however, no consensus has yet emerged. While CCS-independent pathways for the activation of mammalian apo-SOD1 may be operative (Subramaniam *et al*, 2002; Carroll *et al*, 2004), our results raise the new possibility that fALS mutations have altered rates in one or several steps in the maturation cycle shown in Figure 8. This could allow build-up of an immature form of SOD1 that is more prone to aggregation. Alternatively, if the fALS mutants sequester CCS, then nascent immature SOD1 may be susceptible to oxidative aggregation in the cytosol or mitochondrial IMS (Field *et al*, 2003). Given that half-life of the SOD1 protein must exceed the transport time, ~500 days for motor neurons with a meter-long axon, SOD1 in the neuronal cells will be exposed to the prolonged oxidative stress that could damage reduced SOD1 and lead to formation of disulfide-

linked aggregates (Figure 8). Further tests of this model using fALS mutants are underway.

Materials and methods

Protein preparation

The expression vector, pET3d, containing the ySOD1 clone was transformed into *Escherichia coli* BL21, which was grown in LB culture in a shaken flask overnight at 37°C. Induction of the protein expression was performed by addition of 1 mM IPTG. After harvesting the cells by centrifugation, the cells were lysed by the freeze-thaw method and suspended in 2.5 mM K-Pi, pH 7.8. The precipitation resulting from the addition of 50–75% ammonium sulfate was collected, redissolved in 2.5 mM K-Pi, pH 7.8, and was desalted by using the HiPrep™ Desalting column (Amersham Pharmacia Biotech). The desalted protein fractions were loaded onto the anion exchange column, Uno-Q12 (Bio-Rad), equilibrated with 2.5 mM K-Pi, pH 7.8, and the ySOD1 protein was eluted by a linear gradient of 50 mM K-Pi and 100 mM NaCl, pH 7.8. All of the above chromatograms were performed by monitoring the absorbance at 215 nm.

The pET3d expression vector for WT ySOD1 was used to generate the mutants with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). yCCS and its Cu-bound form were obtained as described previously, and protein concentrations were determined with a calibrated Bradford (Bio-Rad) curve using IgG as the standard (Rae *et al*, 1999). The ySOD1 concentration was further checked with the absorption at 280 nm using 1490 M⁻¹ cm⁻¹ per monomer as the extinction coefficient (Lyons *et al*, 1998).

Preparation of the E,E-ySOD1^{SH/S-S} and E,Zn-ySOD1^{SH/S-S} proteins

A new method for the preparation of E,E-ySOD1^{SH} differs from previously published routes (McCord and Fridovich, 1969). The as-isolated ySOD1 protein was treated with 200 mM DTT at 37°C for an hour in a glove box to reduce the disulfide bond. The protein solution was acidified by 0.4% trifluoroacetic acid (TFA) to reduce the reactivity of thiol groups and the binding of metal ions with ySOD1. Then, the metal chelators (20 mM EDTA, 2 mM BCS, and 1 mM N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine) and the organic solvents (15% CH₃CN and 10% CH₃OH) were added to remove the metal ions and denature the protein, which was incubated at room temperature for 2 h. The protein solution was further purified by reverse-phase HPLC through a C4 Vydac 214TP54 column equilibrated with 0.1% TFA in water. The fractions containing ySOD1 were eluted with a linear gradient of 0.1% TFA in CH₃CN and dried under vacuum. The protein was redissolved in the chelex-treated buffer, 50 mM HEPES, pH 7.2, in a glove box. Metal content of E,E-ySOD1^{SH} was checked by ICP-AES using a Thermo Jarrell Ash Atomscan Model 25 Sequential ICP Spectrometer, and Zn and Cu ions were less than 10 and 100 nM, respectively, in the 2 μM protein sample.

E,Zn-ySOD1^{SH} was prepared by infusing an equimolar amount of ZnSO₄, which was incubated at 37°C for an hour in a glove box. E,Zn-ySOD1^{S-S} was made by addition of 10-fold molar excess of Fe(CN)₆³⁻ to the solution of E,Zn-ySOD1^{SH}, and excess amount of the oxidant was removed with Centrifugal Filtration Device (Millipore). The resultant E,Zn-ySOD1^{S-S} has 0 and 70–90% occupation of Cu- and Zn-binding sites, respectively.

AMS modification

To quench the thiol/disulfide exchange reaction, proteins were at first acidified and precipitated with 10% trichloroacetic acid (TCA), which is centrifuged at 12 000 g for 10 min. The pellet was washed with acetone and resuspended in 50 mM HEPES, pH 7.2, 25 mM AMS, 2.5% SDS, and 1 mM EDTA. The sample was anaerobically incubated at 37°C for an hour and run on SDS-PAGE.

Electrophoresis: SDS-PAGE

For analysis using SDS-PAGE, proteins were separated on a 12.5% polyacrylamide Tris-HCl resolving gel with a 5.4% polyacrylamide stacking gel. Samples were dissolved in Laemmli buffer with or without β-ME and boiled at 95°C for 2 min before loading. The gels were run at a constant voltage of 150 V, and the separated protein bands were stained by Coomassie blue.

Electrophoresis: SOD1 activity assay

SOD1 activity was monitored with nondenaturing gel electrophoresis. In order to prevent the loading of free copper ion to apo-SOD1, Cu-yCCS was at first incubated with 100 μ M each of EDTA and BCS at 37°C for 30 min in a glove box. Then, 100–200 ng of the SOD1 protein, which is an equimolar amount to Cu-yCCS, was added and further incubated at 37°C for an hour in aerobic conditions. Proteins were separated in the 10% NATIVE-PAGE gel and stained by riboflavin, NBT, and TEMED (Beauchamp and Fridovich, 1971). A 1 mM portion of EDTA was included in the sample and running buffers.

Thiol-disulfide redox potential measurements

A 1 μ M portion of E,Zn-ySOD1^{SH} was incubated with 10 mM GSH and different concentrations of GSSG (20 μ M–1.4 mM) in 50 mM HEPES, pH 7.2, at 30°C, and the redox potential of the bulk solution was varied by modulating the concentration of GSH and GSSG. Incubation time in the glove box ranged from 3 to 72 h to check the equilibrium. Achievement of the equilibrium was further confirmed by using E,Zn-ySOD1^{S-S}, that is, 1 μ M E,Zn-ySOD1^{S-S} was incubated with 10 μ M GSSG and different concentrations of GSH (0.2–40 mM). After the AMS modification, the fraction of E,Zn-ySOD1^{S-S} and E,Zn-ySOD1^{SH} was examined by the SDS-PAGE analysis. After scanning gels, the images were analyzed by Adobe Photoshop and ImageJ software.

The redox potential of the thiol/disulfide in ySOD1, $E_{\text{SOD1}}^{\text{SH/S-S}}$, can be quantified as follows:

$$E_{\text{SOD1}}^{\text{SH/S-S}} = E_{\text{GSH}}^{\circ} - \frac{RT}{2F} \ln \left(\frac{f_{\text{R}} [\text{GSH}]^2}{1 - f_{\text{R}} [\text{GSSG}]} \right) \quad (1)$$

where f_{R} is the fraction of E,Zn-ySOD1^{SH} at the equilibrium, and [GSH] and [GSSG] are the concentrations of GSH and GSSG, respectively. E_{GSH}° is the standard reduction potential of the GSH/GSSG couple, −0.24 V (Gilbert, 1995). R , T , and F are the gas constant, temperature, and the Faraday constant, respectively.

AMS modification of SOD1 in cell lysate

In this study, *S. cerevisiae* cells, SY1699 (α , *leu2*, *ura3*, *ade1*, *his3*) and *ccs1Δ* (formerly referred to as *lys7Δ*, α , *leu2*, *ura3*, *ade2*, *his3*, *lys7::LEU2*), were used. To reduce reactivity of thiol groups during the lysis process, about 5 μ l of the cell pellet was suspended in 100 μ l of 10% TCA and lysed by using glass beads. After

centrifugation, protein precipitates were washed with acetone and dried up under vacuum. Then, 100 μ l of 25 mM AMS containing 0.5% SDS was added to suspend the precipitates, and pH of the solution was adjusted to about 7.5 by 1 M NaOH. The solution contained 1 mM EDTA and 1 mM BCS to avoid the thiol oxidation by free copper ion in the cell lysate. Following removal of insoluble materials by centrifugation, the solution was incubated at 37°C for an hour. Proteins were again extracted by the TCA procedure and redissolved in 10 μ l of 50 mM HEPES, pH 7.2. The protein samples were separated by SDS-PAGE, transferred from the gel to PVDF membrane (Bio-Rad), blocked for an hour and analyzed by Western blot using the polyclonal antibody against ySOD1 as the primary antibody (1:5000 dilution) and anti-rabbit IgG horseradish peroxidase-linked whole antibody (from donkey) (Amersham Pharmacia Biotech) as the secondary antibody (1:10 000 dilution). Blots were developed with the ECL+ plus Western Blotting Detection System (Amersham Biosciences).

Gel filtration experiments

A 200 μ l portion of 16 μ M E,Zn-ySOD1^{SH/S-S} protein was loaded on Superose 12 HR 10/30 (Amersham Pharmacia Biotech). The column was pre-equilibrated with 50 mM K-Pi, pH 7.8, and the flow rate was 0.5 ml/min. To prevent the possible air oxidation of the thiol groups, 1 mM DTT was further included in the experiments using E,Zn-ySOD1^{SH}. The chromatogram was obtained by monitoring the absorbance at 215 nm. The calibration of the column for the estimation of molecular weight was performed using 0.25 g/l bovine serum albumin, horse skeletal myoglobin, horse cytochrome c, and *E. coli* thioredoxin as protein standards.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

Yeast SOD1 gene was a generous gift of J Valentine. We also thank V Culotta for providing yeast strains, N Brown and K Inabo for helpful discussions, and A Herrreiter and A Nadimpali for preparation of several protein samples. This work was supported by grants from NIH (GM 54111) and from the ALS Association (to TVO). YF was supported by a Japan Society for the Promotion of Science Postdoctoral Fellowship for Research Abroad.

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