

## Copper-Zinc Superoxide Dismutase is Activated through a Sulfenic Acid Intermediate at a Copper-Ion Entry Site

Morgan M. Fetherolf<sup>1</sup>, Stefanie D. Boyd<sup>2</sup>, Alexander B. Taylor<sup>3,4</sup>, Hee Jong Kim<sup>6</sup>, James A. Wohlschlegel<sup>6</sup>, Ninian J. Blackburn<sup>7</sup>, P. John Hart<sup>3,4,5</sup>, Dennis R. Winge<sup>1</sup> and Duane D. Winkler<sup>2,\*</sup>

<sup>1</sup>Department of Medicine, University of Utah Health Sciences Center School of Medicine, 30 North 1900 East, Salt Lake City, UT 84132-2408, USA; Department of Biochemistry, University of Utah, 15 North Medical Drive East, Salt Lake City, UT 84112-5650, USA.

<sup>2</sup>Department of Biological Sciences, University of Texas at Dallas, 800 W. Campbell Road, Richardson, TX 75080, USA.

<sup>3</sup>Department of Biochemistry and <sup>4</sup>X-ray Crystallography Core Laboratory, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA; <sup>5</sup>Geriatric Research, Education, and Clinical Center, Department of Veterans Affairs, South Texas Veterans Health Care System, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA.

<sup>6</sup>Department of Biological Chemistry, David Geffen School of Medicine at the University of California at Los Angeles, 615 Charles E. Young Drive South, Los Angeles, CA 90095, USA

<sup>7</sup>Institute of Environmental Health, Oregon Health and Science University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97239, USA.

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\*To whom correspondence should be addressed: Duane D. Winkler, Department of Biological Sciences, University of Texas at Dallas, 800 W. Campbell Road, Richardson, TX 75080, USA.

Email: [duane.winkler@utdallas.edu](mailto:duane.winkler@utdallas.edu) Tel: 972-883-3533, Fax: 972-883-2409

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### ABSTRACT

Metallo-chaperones are a diverse family of trafficking molecules that provide metal ions to protein targets for use as cofactors. The copper chaperone for Sod1 (Ccs1) activates immature copper-zinc superoxide dismutase (Sod1) by delivering copper and facilitating the oxidation of the Sod1 intramolecular disulfide bond. Here, we present structural, spectroscopic, and cell-based data supporting a novel copper-induced mechanism for Sod1 activation. Ccs1 binding exposes an electropositive cavity and proposed “entry site” for copper ion delivery on immature Sod1. Copper-mediated sulfenylation leads to a sulfenic acid intermediate that eventually resolves to form the Sod1 disulfide bond with concomitant release of copper into the Sod1 active site. Sod1 is the predominant disulfide bond requiring enzyme in the cytoplasm and this copper-induced mechanism of disulfide bond formation obviates

the need for a thiol/disulfide oxidoreductase in that compartment.

The copper chaperone for superoxide dismutase (Ccs1) activates newly translated copper-zinc superoxide dismutase (Sod1) proteins (1). Sod1 is a critical antioxidant enzyme that detoxifies superoxide, a byproduct of cellular respiration, through redox cycling at its catalytic copper ion [ $2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ ] (2). Nascent Sod1 is inactive and monomeric (3-7), but is converted to its active homodimeric form through its interaction with Ccs1 (1, 6, 8). Yeast lacking Ccs1 are deficient in Sod1 enzymatic activity unless exogenous copper is added to the culture medium (9-11). Like *ccs1Δ* yeast, *Drosophila melanogaster* deficient in Ccs1 lack Sod1 activity and are hypersensitive to oxidative stress (12). Expression of human Sod1 in yeast *ccs1Δ* cells results in limited Sod1 activity (~25%) through the ability of a subset of hSod1 proteins to acquire copper independent of Ccs1 (12, 13).

Ccs1 proteins are comprised of three domains (D1, D2, and D3), each of which plays a specialized role in Sod1 activation (6, 8, 9). The N-terminal domain (D1) contains a MxCxxC copper-binding motif that is required under copper-limiting *in vivo* yeast growth conditions and *in vitro* activation of human Sod1 (6, 9). Ccs1 D1 appears to acquire Cu(I) from the membrane transporter Ctr1 (9, 14, 15). The second domain (D2) possesses significant sequence and structural similarity with Sod1 that permits it to mediate interaction with the immature molecule via residues analogous to those found at the Sod1 homodimeric interface (8, 16-19). Like D2, the C-terminal domain (D3) is essential for Sod1 activation and contains an invariant CxC motif (1, 9). Cells containing Ccs1 proteins lacking the D3 CxC motif possess Sod1 proteins devoid of copper and intra-subunit disulfide bonds (8, 9, 20). However, *in vitro* studies on Sod1 activation revealed that a Ccs1-variant containing only D1 and D2 was competent to transfer Cu(I) to Sod1 but failed to mediate the disulfide bond formation in Sod1 (8). Disulfide bond formation in Sod1 is dependent on Ccs1 D3 (8).

Multiple studies have sought to delineate the mechanism by which Ccs1 activates Sod1. Experiments in yeast demonstrated that Ccs1 acts on newly synthesized Sod1 to facilitate Cu(I) ion insertion and oxidation of the Sod1 intra-subunit disulfide bond (6, 8, 21, 22). The presence of a disulfide bond in Sod1 is unusual for an enzyme found predominantly in the highly reducing cytosolic environment. The two major cellular compartments in eukaryotes in which disulfide oxidation is commonly observed are the lumen of the endoplasmic reticulum and the mitochondrial intermembrane space, both of which contain thiol/disulfide oxidoreductases [reviewed in (23)]. Although the mechanism of disulfide oxidation in nascent cytosolic Sod1 had been elusive until now, it is known that the disulfide bond is essential for full catalytic activity because it anchors the side chain of R<sub>143</sub> adjacent to the catalytic copper ion where it helps to guide superoxide into the active site (24, 25).

Copper ion delivery and disulfide bond oxidation appear to be linked processes (6, 26), but may occur sequentially (8). In Ccs1-containing yeast, Sod1 mutants unable to bind copper in the active site remain largely disulfide

reduced (27). The addition of Cu-Ccs1 to Zn-Sod1 fails to induce oxidation of the disulfide bond anaerobically, but this process is facile in the presence of oxygen or superoxide (21). A previous yeast Sod1•Ccs1 crystal structure revealed an intermolecular disulfide between C<sub>57</sub> of Sod1 and C<sub>229</sub> of Ccs1, suggesting the intermolecular disulfide may be an intermediate that eventually resolves to the C<sub>57</sub>-C<sub>146</sub> disulfide via a disulfide exchange reaction (19).

Unlike mammalian Sod1 proteins, yeast Sod1 proteins cannot acquire copper via the Ccs1-independent pathway in normal growth medium (10, 28). The electrostatic loop of yeast Sod1 contains two proline residues that preclude the low levels of Ccs1-independent activation observed in the human and mouse enzymes (13, 29). Replacement of the Pro residues (P<sub>142</sub> and P<sub>144</sub>) in the yeast enzyme with their cognate residues from mammalian Sod1 proteins permits yeast enzyme activation via a Ccs1-independent pathway (13). Likewise, ~25% of the Sod1 pool is active in *ccs1* *-/-* null mice (22). The activation of Sod1 proteins without Pro at these positions in *ccs1Δ* yeast cells (12, 28) or in *ccs1* *-/-* null mice suggests the copper ion may be provided by labile Cu(I) complexes in the yeast cytosol (e.g. Cu-glutathione) (30, 31). These results also highlight the link between copper acquisition and disulfide bond formation.

Here, we present structural, biochemical, and spectroscopic data that suggest a mechanism of Sod1 activation not previously described. Crystallographic studies on a new Sod1•Ccs1 complex reveal a cavity with positive electrostatic potential exposed by a previously unobserved β-hairpin conformation of Ccs1 D3 that embraces the Sod1 "disulfide loop," displacing it from the β-barrel. Spectroscopic studies establish that Cu(I) is held at a (Cys)<sub>2</sub>(His) "entry site" adjacent to the electropositive cavity and Sod1•Ccs1 interface. *In vivo* studies of yeast Sod1 and its variants strongly suggest that the "entry site" is functionally relevant and that disulfide formation and Cu delivery into the Sod1 active site are linked processes dependent on sulfenylation of C<sub>146</sub>. This new model is consistent with the vast majority of the biochemical data on Ccs1-mediated activation in the literature.

## RESULTS

*Role(s) of Ccs1 D3 in Sod1 activation.* Previous studies have highlighted the importance of the CxC motif in Ccs1 D3 for Sod1 activation (8, 9, 32); however, debate continues on its exact role. Early studies suggested that the D3 CxC motif mediated Cu(I) transfer from D1 to Sod1 (9), but subsequent studies point to a more prominent role in Sod1 disulfide bond formation (6, 8, 21). We attempted to glean additional insights into the role of D3 by structurally characterizing the Sod1•Ccs1 heterocomplex and evaluating residue spacing within the CxC motif.

Previously, Lamb and colleagues determined the structure of a yeast H48F Sod1•Ccs1 heterotetramer containing two Sod1•Ccs1 heterodimers in the asymmetric unit related by two-fold noncrystallographic symmetry (NCS) (19). Heterotetramer assembly was mediated by reciprocal "swapping" interactions in which a highly conserved Ccs1 D3 <sup>237</sup>WEER<sub>240</sub>  $\alpha$ -helical motif bound to grooves on D2 of NCS-related heterodimers (**Fig. S1**) (19). To probe the importance of the <sup>237</sup>WEER<sub>240</sub> motif, we created a series of mutants targeting these residues. One variant, E<sub>238</sub>A/E<sub>239</sub>A/R<sub>240</sub>A (<sup>237</sup>WAAA<sub>240</sub>) demonstrated ~5-fold greater yield and enhanced resistance to the trypsinolysis observed at K66 in the linker region between D2 and D3 in wild type Ccs1 (18). H<sub>46</sub>R/H<sub>48</sub>Q Sod1 possesses an ablated copper-binding site (27) and forms a highly stable, H<sub>46</sub>R/H<sub>48</sub>Q Sod1•E<sub>238</sub>A/E<sub>239</sub>A/R<sub>240</sub>A Ccs1 heterocomplex useful here for structural analysis.

*New structure of the Sod1•Ccs1 complex.* Structure of the H<sub>46</sub>R/H<sub>48</sub>Q Sod1•E<sub>238</sub>A/E<sub>239</sub>A/R<sub>240</sub>A Ccs1 complex was determined by molecular replacement and refined in space group P2<sub>1</sub>2<sub>1</sub>2 to a resolution of 2.35 Å. Table 1 provides the X-ray diffraction and structure refinement statistics. Though the asymmetric unit contains two Sod1•Ccs1 heterodimers related by noncrystallographic symmetry, the D3 conformation and heterodimeric orientations are dramatically different from the previous yeast H48F Sod1•Ccs1 heterotetramer structure (**Fig. S2**) (19). The extended  $\alpha$ -helical conformation of Ccs1 D3 is unlikely to exist within the confines of a stable Sod1•Ccs1 heterodimer. Previous data

consistently indicates that the Sod1•Ccs1 complex exists as a heterodimer in solution (17, 27) and in contrast to the  $\alpha$ -helical and/or disordered conformations of D3 observed in earlier work (18, 19), D3 in the new structure adopts a compact  $\beta$ -hairpin conformation that strengthens the Sod1•Ccs1 interaction (**Fig. 1A, B**).

Multiple interactions with residues coming from Ccs1 D1 and D2 stabilize the  $\beta$ -hairpin conformation of Ccs1 D3 (**Fig. 1C**). The indole ring of W<sub>222</sub> stacks with the side chain of R<sub>105</sub> from D2, immobilizing the residues immediately N-terminal to the  $\beta$ -hairpin (**Figs. 1A, C**). The buried location of W<sub>222</sub> is dramatically different from the solvent exposed environment observed in a previous Sod1•Ccs1 complex structure (**Fig. 1B**). The invariant <sup>237</sup>WEER<sub>240</sub> tryptophan fits nicely into a hydrophobic pocket on D2 formed by residues I<sub>83</sub>, V<sub>100</sub>, and L<sub>103</sub> and seemingly acts as a "latch" stabilizing the D3  $\beta$ -hairpin (**Figs. 1A, C**). Ccs1 D3 highlights the major structural differences between the Sod1•Ccs1 complex presented here and the previous structure from the Rosenzweig group. The two differing forms of Sod1 used in the compared structures are determined to be nearly identical in their conformation with an overall RMSD of 0.55 over 781 atoms (**Fig. S3C**). The other side of the major dimeric interface (Ccs1 D2) aligns with an even lower RMSD of 0.48 over 839 atoms (**Fig. S3C**).

Previous studies in solution reported that Ccs1 D3 is required not only for Sod1 activation, but also for robust Sod1•Ccs1 interaction (8, 33). The Sod1•Ccs1 complex structure presented here provides a clear molecular basis for the latter observation. **Figure 1C** highlights the interface between the Sod1 "disulfide loop" region and the Ccs1 D3  $\beta$ -hairpin as a stereo-image of the representative  $\alpha$ A electron density with coefficients  $2mFo-DFc$  contoured at 1.2  $\sigma$  superimposed on the refined model. The large number of interactions between Sod1 and Ccs1 (**Table S1**) can occur only when the Sod1 disulfide bond is reduced, consistent with previous observations of stable Sod1•Ccs1 binding only under reducing conditions (27). These interactions, together with the unanticipated intercalation of the D3 CxC motif cysteine (C<sub>231</sub>) between the disulfide loop and  $\beta$ -barrel of Sod1,

create a cysteine-rich “notch-into-groove” complementary surface (**Fig. S3**).

*Spacing of D3 CxC residues is critical for Sod1 activation.* By altering the spacing of the Ccs1 D3 CxC cysteines, we can begin to differentiate the roles of copper delivery and disulfide bond formation catalyzed by Ccs1. Yeast mutants lacking mature (e.g. metal bound, disulfide oxidized) Sod1 are lysine auxotrophs and exhibit impaired growth in -Lys medium. Growth of the CxC variant yeast strains were largely compromised (**Fig. 2A, B and S4**) and lysates from these cells exhibited nominal Sod1 activity (**Fig. 2C**). Since only 2% of endogenous Sod1 activity is required for cell growth suppressing the lysine auxotrophy (34), the impaired growth of most Ccs1 CxC variants suggest that Sod1 activation is compromised to greater than 98%. However, cells expressing the C<sub>230</sub>C<sub>231</sub> Ccs1 variant exhibited significant growth in -Lys medium at both 30° and 37°C and lysates exhibited upwards of 7% Sod1 activity (**Fig. 2B, C**). With a dithiol configuration, only a C<sub>230</sub>C<sub>231</sub> Ccs1 variant shows any significant Sod1 activation. These results suggest that two thiolates are necessary in Ccs1 D3 for Sod1 activation and that a strong positional effect is observed for both. The C<sub>230</sub>C<sub>231</sub> Ccs1 variant, which cannot form an intramolecular D3 disulfide, ostensibly rules-out prior models implicating Ccs1 D3-mediated shuffling of a preformed disulfide bond to Sod1 during activation (8, 21, 35).

To assess the redox status of the Sod1 disulfide bond in these studies, thiol-specific alkylation experiments were performed. Modification of cysteines with polyethylene glycol modified N-ethyl maleimide (m-PEG) selectively adds 2 kDa per free thiol. Sod1 present in WT cells is resistant to m-PEG modification, whereas Sod1 in *ccs1Δ* cells is readily modified (**Fig. 2D**). Likewise, Sod1 modification is similar for cells harboring Ccs1 CxC spacer mutants (**Fig. 2D**), suggesting the bulk of cellular Sod1 in these strains exists in the disulfide reduced inactive state. However, there is likely a small fraction of oxidized Sod1 in the cells expressing Ccs1 C<sub>230</sub>C<sub>231</sub> that cannot be visualized by alkylation which gives rise to the cell viability seen on plate assays.

*Stalled Sod1•Ccs1 complexes.* Previous studies have noted and our current Sod1•Ccs1 structure deciphers that immature forms of Sod1 are the primary targets of Ccs1 (**Fig. 1C**) (17, 27). **Figure 3** shows the results from Strep-Sod1•Ccs1 pull-downs from yeast where Ccs1 does not significantly co-purify with WT Strep-Sod1 (**Fig. 3A**). Co-purification of stalled Sod1•Ccs1 complexes was enhanced in cells expressing the Sod1 variant C<sub>146</sub>S and to a lesser degree with C<sub>57</sub>S or the catalytically inactive R<sub>143</sub>E mutant (**Fig. 3A**). The Ccs1 D3 CxC variant C<sub>231</sub>S, but not C<sub>229</sub>S, enhanced co-purification relative to WT Ccs1 (**Fig. 3B**). Enhancement persists when the Sod1 variant C<sub>146</sub>S, is mixed with Ccs1 D3 CxC variants (**Fig. 3C**). However, D3 CxC spacing mutants with C<sub>231</sub> still in place did not affect complex stalling (**Fig. 3D, E**). The positional requirement of C<sub>231</sub>, but not C<sub>229</sub>, posits deviating roles during the Sod1 activation process that may be explained by their relative arrangements in our Sod1•Ccs1 complex (**Fig. 1A, C**). The structure shows C<sub>231</sub> is near the proposed entry site as C<sub>229</sub> points away and does not share a proximate relationship with the other proposed entry site residues.

*Sod1 disulfide formation secures copper delivery.* Next, we further examined the link between disulfide bond formation and metal delivery to Sod1. Various affinity-purified heterocomplexes were assessed on non-reducing SDS-PAGE for the presence of an *inter*-molecular disulfide bond. A ~45 kDa molecular mass band containing both Sod1 and Ccs1 was not only observed in samples when C<sub>146</sub>S Sod1 were utilized, but also when C<sub>229</sub>S or C<sub>231</sub>S Ccs1 were present (**Fig. 4A**). Without both Ccs1 D3 CxC cysteines present, the process guiding formation of the Sod1 C<sub>57</sub>-C<sub>146</sub> intra-subunit disulfide bond likely becomes trapped as an *inter*-molecular disulfide intermediate. Interestingly, though a high mass Sod1•Ccs1 complex was not visualized with Sod1 C<sub>57</sub>S, significant levels of Ccs1 were co-purified with this Sod1 mutant (compare **Fig. 4A** to **Fig. 3A**). However, when we purify Sod1 after the addition of 20 mM N-ethyl maleimide (NEM) to the lysate, a clear crosslinked Sod1-Ccs1 adduct is seen with the C<sub>57</sub>S Sod1 sample (**Fig. S5**). The intermolecular crosslink between C<sub>57</sub>S Sod1 and Ccs1 in the absence of NEM is susceptible to



reduction or resolution via disulfide exchange reactions. In contrast, the intermolecular disulfide linking C<sub>146</sub>S Sod1 and Ccs1 is more resistant to reduction. The addition of NEM resulted in all of the single mutants forming the high molecular weight complex, nonetheless at least one of the D3 CxC cysteines is necessary (**Fig. S5**). Interestingly, a Ccs1 D3 variant lacking both Cys residues was attenuated in its interaction with Sod1 consistent with an intermolecular disulfide in the stalled Sod1-Ccs1 interaction. In order to rule out that the stalled heterocomplexes are bridged by Cu(I) coordination, the Cu(I) chelator BCS was added to the reactions and stalled complexes persisted even in the presence of excess BCS (**Fig. 4B**).

To gauge whether Sod1 in the Ccs1 mutant cells contained bound metal ions, N-terminally Strep-tagged Sod1 purified from cells with WT and mutant Ccs1 variants were probed for metals by ICP-OES (**Fig. 4C**). WT Sod1 contained ~1 mol equiv. of both copper and zinc, whereas Sod1 purified from *ccs1Δ* cells contained essentially no copper but ~2 mol equiv. of zinc. Sod1 purified from cells containing Ccs1 D3 variants or Sod1 with a C<sub>57</sub>S substitution were largely devoid of copper. In contrast, the C<sub>146</sub>S Sod1 was isolated with appreciable levels (~0.3 mol equiv.) of copper relative to *ccs1Δ* cells. The difference in the copper content of C<sub>57</sub>S vs. C<sub>146</sub>S Sod1 may suggest that these two cysteines play differing roles in copper loading and/or retention. These *in vivo* studies support the previous *in vitro* studies showing the importance of the Sod1 cysteines in copper acquisition (36). Taken together, the variant forms of Sod1 or Ccs1 that hinder efficient disulfide bond formation also hinder the ability of Sod1 to receive its full complement of copper.

*Cysteine residues from Sod1 and/or Ccs1 form a Cu(I) “entry site.”* Next, we wanted to address the position and coordination of copper during delivery to Sod1. WT and variant forms of Strep-Sod1 were purified from yeast cells containing either WT or mutant Ccs1. Again, the mutant Sod1 samples, but not the WT, co-purify with Ccs1. X-ray absorption spectroscopy was then utilized to probe copper coordination. Absorption edge features can distinguish Cu(I) from Cu(II) and digonal from trigonal complexes by

Cu(I) and Cu(II) model complexes (37). **Figure 5A** shows the XAS Cu K-edge for WT and mutant Sod1. The absorption edge exhibited a feature at ~8983 eV consistent with a 1s→4p transition of the Cu ion. For all three samples, the energy and intensity of this near-edge feature relative to the absorption continuum are characteristic of trigonal Cu(I) complexes (37).

**Figure 5B** shows the copper K-edge EXAFS spectra and Fourier transform of the EXAFS for WT Sod1. The Fourier transform consists of a major peak at 1.96 Å and two outer shell scatter peaks at ~3 and ~4 Å. Simulations with different ligand types reveal a best fit for the 1.96 Å peak with three N/O ligands, which agrees well with the known three histidyl ligand coordination of Cu(I) ion in the Sod1 active site (38). The outer shell scatter peaks correspond to backscattering effects of the imidazole ring carbons. Analyses of the H<sub>46</sub>R/H<sub>48</sub>Q Sod1 mutant, which cannot bind copper at the active site, was carried out after purification from yeast containing WT Ccs1 or the mutant Ccs1 containing only the D3 CxC cysteines (**Fig. 5C** and **5D**, respectively). The copper K-edge feature in both samples was indistinguishable from WT Sod1, suggestive of trigonal Cu(I) coordination (**Fig. 5A**). In contrast to WT Sod1, the EXAFS analyses of the two variant samples reveal broader first shell peaks that best fit to mixed sulfur and N/O coordination of the Cu(I) ion. Cu(I) binding to Ccs1 consists of only thiolate ligands, so the candidate nitrogen ligand is likely from Sod1. The EXAFS Fourier transform of the mutant Sod1 purified with WT Ccs1 is best fit with a model containing two sulfur ligands at 2.21 Å (2.22 Å for Cys variant Ccs1) and a single N/O at 1.92 Å (1.93 Å for Cys variant Ccs1) (**Fig. 5C, D**). The Cu(I) site thiolate ligands are not fully distinguished, but are consistent with a model in which Cu(I) is coordinated at an “entry site” generated by the interaction between immature Sod1 and Ccs1.

*Ccs1 exposes an electropositive cavity on immature Sod1.* The reduced disulfide bond in Sod1 permits the intercalation of the Ccs1 D3 β-hairpin between the “disulfide loop” and β-barrel, which exposes an electropositive cavity on the Sod1 surface inaccessible in the disulfide oxidized/mature enzyme (**Fig. 6A, B**). The positive surface potential arises primarily through

the amide nitrogen atoms of A<sub>60</sub>, T<sub>116</sub>, and L<sub>117</sub>. The cavity is immediately adjacent to the S $\gamma$  atom of C<sub>231</sub> coming from the Ccs1 D3 CxC motif and Sod1 residues C<sub>57</sub> and C<sub>146</sub> that become oxidized to form the disulfide bond. Replacement of the glutamine residue at position 48 in this structure with the normal active site histidine suggests a structural equivalence between H<sub>48</sub> and H<sub>120</sub> such that either could provide a ligand in a putative (Cys)<sub>2</sub>His Cu(I) “entry site” (**Fig. 6C** and **S6**). Sod1 disulfide bond formation likely drives concomitant copper delivery to the active site, as mutants where the disulfide bond is not formed are defective in copper acquisition/retention (**Fig. 4c**). These structural details provide relevance for our hypothesis that an electropositive cavity and proximal copper ion “entry site” exposed on Ccs1-bound Sod1 link copper ion delivery and disulfide oxidation.

*Copper-dependent sulfenylation at the Sod1 “entry site.”* The dismutation of superoxide anions by Sod1 yields both molecular oxygen and hydrogen peroxide. Thiols are particularly prone to modification by reactive oxygen species in the cell and sulfenic acid is one product produced upon oxidation by H<sub>2</sub>O<sub>2</sub> (39, 40). The propensity of cysteine residues to sulfenylation is influenced by its nucleophilicity, accessibility to H<sub>2</sub>O<sub>2</sub> and the protein microenvironment (41, 42). The formation of sulfenic acid on glutathione peroxidase 3 is a critical precursor for the subsequent disulfide transfer to the transcriptional activator Yap1 under oxidative stress (43, 44). Could the Cu(I) entry site Sod1 intermediate with its electropositive hole mediate cysteine sulfenylation?

Purified Sod1 treated with dimedone, which specifically traps sulfenic acid modifications, was analyzed by western blot using a dimedone adduct specific antibody. A gel band visualized by this technique comigrated with a band imaged with a Sod1 antibody (**Fig. 7A**). Notably, sulfenylation was observed on mutant Sod1 proteins purified from yeast also containing untagged WT Sod1, so the modification did not arise from a buildup of cellular reactive oxygen species. The sulfenylated Sod1 intermediate is only observed in samples purified without the addition of a reductant. In the presence of reductant, no sulfenylated Sod1 is seen. The sulfenylated adduct is detectable in all

Sod1 mutants except for C<sub>146</sub>S, advocating a preferred site for adduct formation (**Fig. 7B**). In mature Sod1, R<sub>143</sub> is important for electrostatic guidance of superoxide towards the active site. During the maturation process, the role of R<sub>143</sub> is not well understood. Interestingly, sulfenylation is more prevalent in the R<sub>143</sub>E variant. Architectural effects of this mutation on the “entry site” may enhance the lifetime of the adduct, though addition of a negative charge at this site may simply help direct superoxide towards the vicinal electropositive cavity and ‘entry site.’

Sod1 sulfenylation is attenuated in yeast cells that are rendered copper-deficient by inclusion of the copper chelator BCS in the growth medium or selective knockout of the influx copper transporter Ctr1 (**Fig. 7C**). This strongly suggests that sulfenylation at Sod1 C<sub>146</sub> is a copper-mediated reaction. Additionally, Sod1 isolated from *ccs1* $\Delta$  cells is sulfenylated implying that the primary role of Ccs1 may not be in copper delivery. Sulfenylation at this site likely promotes C<sub>57</sub>-C<sub>146</sub> disulfide oxidation in Sod1. We cannot be certain whether the sulfenylation of C<sub>146</sub> or the subsequent disulfide bond formation triggers release of copper from its “entry site” ligands into the nearby Sod1 active site (**see Fig. 5C**).

*Sod1 activation under copper-replete conditions.* For yeast Ccs1 (but not necessarily mammalian), D1 is required only under copper limiting conditions *in vivo* (9, 10, 32). The conditional necessity of D1 in yeast Ccs1 suggests that the cellular environment may alter the mechanism of copper delivery. During normal cellular conditions, copper ions are present in the cytosol as small molecule complexes with reduced glutathione (GSH) or metallothionein scavengers (45). However, only mammalian cells are known to utilize Cu(I)-GSH as an alternate route for Sod1 activation (13). **Figure 7D** shows the results for an *in vitro* yeast Sod1 activation assay incorporating Cu(I)-GSH. The addition of Cu(I)-GSH to E,Zn-ySod1 results in only minimal Sod1 activation (lane 2). When E,Zn-ySod1 is mixed with apo-Ccs1 and Cu(I)-GSH, abundant Sod1 activity is detected (lane 4); a level of activity similar to apo-Sod1 mixed with Cu(I)-Ccs1 (lane 5). In addition, an apo-Ccs1 D1 MxCxxC variant (C<sub>17,20</sub>A) can still promote copper delivery by Cu-GSH to E,Zn-ySod1 (**Fig. 7D**, lower panel, lane

7), yet the D3 CxC variant (C<sub>229,231</sub>A) fails to do so (lane 8). These results strongly support a facilitatory role for Ccs1 in the copper delivery process of reduced glutathione without copper coordination by Ccs1 D1. Relatedly, the addition of dimedone to in vitro Sod1 activation assays preclude complete Sod1 activation, likely through inhibition of disulfide exchange at the “entry site” and the associated copper delivery (**Fig. S7**). These data suggest that when copper is readily available Ccs1 is not required to directly coordinate copper by D1 and may simply expose the electropositive cavity and Cu(I) “entry site” at the Sod1·Ccs1 interface to allow entry of Cu(I) from either Ccs1 or a labile source such as glutathione and then facilitating disulfide formation, as described above.

*Entry-site specific cysteine sulfonylation examined by MS.* Sulfonylation reactions were analyzed by mass spectrometry to identify peptides with dimedone adducts. Briefly, reactions were digested by trypsin, fractionated online using reversed-phase chromatography, and analyzed by tandem mass spectrometry. Dimedone-modified peptides were identified by a characteristic mass shift of +138.068080 on the modified cysteine residue. Entry site cysteine residues on Sod1 (C<sub>57</sub> and C<sub>146</sub>) were both modified in the yeast Sod1 sample. For human Sod1, the C<sub>57</sub> site was modified. No cysteines outside of the “entry site” (eg C<sub>6</sub> and C<sub>111</sub>) on either form of Sod1 were modified, showing specificity for the additions. Annotated MS2 spectra for each modified peptide (**Fig. S8**) as well as its relative abundance across the set of reactions is shown in **Fig. 8A, B**.

## DISCUSSION

Based upon the data presented here, Ccs1-mediated Sod1 maturation appears to proceed via a Cu-mediated sulfonylation intermediate that resolves to form the stable Sod1 disulfide bond. Our new Sod1·Ccs1 complex structure demonstrates that upon binding to immature Sod1, interactions between Ccs1 and the disulfide loop of Sod1 orient the D3  $\beta$ -hairpin towards Sod1. The indole side-chain of W<sub>237</sub> fits into a hydrophobic pocket on Ccs1 D2 securing the D3  $\beta$ -hairpin. The intercalation of C<sub>231</sub>, together with hydrogen bonding interactions between the D<sub>52</sub>

side chain of Sod1 with the indole nitrogen of W<sub>222</sub>, pulls the disulfide loop away from the Sod1  $\beta$ -barrel and exposes a copper ion “entry site” and adjacent electropositive cavity. A Cu(I) ion coming from Ccs1 D1 or a labile Cu(I) pool can now be coordinated at the “entry site” and either superoxide or H<sub>2</sub>O<sub>2</sub> is attracted towards the electropositive cavity harboring Sod1 C<sub>146</sub>. If superoxide anion is attracted, copper ion oxidation (e.g. Cu(I)  $\rightarrow$  Cu(II)) reduces the superoxide to hydrogen peroxide. This newly generated H<sub>2</sub>O<sub>2</sub> molecule promotes sulfonylation of a cysteine, likely Sod1 C<sub>146</sub>, although sulfonylation could initially occur on a Ccs1 D3 cysteine. Alternatively, attraction of H<sub>2</sub>O<sub>2</sub> to the Cu(I) entry site could directly sulfonylate the cysteine with no change in the copper oxidation state. Sulfonylation subsequently mediates disulfide bond formation through disulfide exchange reactions involving the Ccs1 D3 cysteines. This reaction likely drives the entry site copper ion to the active site. Sulfonylation of Sod1 is not dependent on Ccs1 (**Fig. 7B**), but the resolution to a disulfide bond is dependent on Ccs1. The driving force for copper migration to the tetra-histidine environment of the mature catalytic site may be either sulfonylation of C<sub>146</sub> or disulfide bond formation. Sod1 disulfide oxidation and copper ion release into the active site expels the Ccs1 D3  $\beta$ -hairpin as the formation of the disulfide bond closes the electropositive cavity and terminates interaction between Sod1 and Ccs1.

The Sod1·Ccs1 interaction during the maturation process is likely short-lived and is terminated by successful intra-subunit C<sub>57</sub>-C<sub>146</sub> disulfide bond formation in Sod1. However, this transient binding event can be trapped as an inter-molecular disulfide linked intermediate in cells containing either C<sub>146</sub>S Sod1 or C<sub>231</sub>S Ccs1, yet substitution of both Ccs1 D3 CxC cysteines expectedly attenuates trapping. Enhanced trapping of the Sod1·Ccs1 interaction observed with Sod1 catalytic site His mutants blocks copper migration to the active site. This may lead to resolution of the C<sub>146</sub> sulfenic acid by a Ccs1-derived thiol, which may in turn generate intermolecular cross links of the type observed by Lamb et al. (46) and our present results showing that at least one Ccs1-derived thiol is required for formation of the cross-link. Our data support a model where Ccs1-

mediated disulfide exchange reactions between cysteine residues at the Cu(I) “entry site” drive Sod1 maturation to completion.

Clear evidence for a Cu(I) “entry site” on immature Sod1 bound by Ccs1 is provided by X-ray absorption and related techniques. Cells harboring a Sod1 H<sub>46</sub>R/H<sub>48</sub>Q variant unable to bind copper at the active site, coordinate Cu(I) using a (Cys)<sub>2</sub>His pseudo-trigonal planar geometry. Consistent with this geometry and ligand set, the new heterocomplex structure reveals that the S $\gamma$  atoms of C<sub>231</sub>, C<sub>57</sub>, and the position of Cu in the activated enzyme are coplanar, with liganding nitrogen atoms from H<sub>48</sub> and H<sub>120</sub> equidistant and normal to this plane (**Fig. 6C**). Thus, the entry site may be fluxional, sampling some or all of the four possible thiolate ligands. A Cu(I) resting state observed in the WT EXAFS analysis is consistent with *in vivo* NMR analysis performed on the maturation steps of Sod1 (6, 7). In separate *in vitro* analysis, transfer of copper from Cu(I)-Ccs1 to Sod1 in the presence of oxygen showed a stable Cu(II)-Sod1 (26). The difference between these two experiments may be that H<sub>2</sub>O<sub>2</sub> driven sulfenylation may predominate *in vivo* yielding a Cu(I)-Sod1 complex. This is not unduly surprising since it is likely that concentration of free H<sub>2</sub>O<sub>2</sub> vastly exceeds that of free superoxide in the cytosol. However, we emphasize that both pathways are chemically plausible and it may be that both are operative, perhaps under different cellular conditions.

A readily accessible Cu(I) “entry site” requires direct delivery and “hand-off” by a copper ion carrier. Cu-GSH complexes have shown the ability to activate human Sod1 in the absence of Ccs1; a process not replicable for yeast Sod1 due to proline residues just upstream of C<sub>146</sub> (13). Here, we show evidence that Cu-GSH can deliver copper to yeast Sod1, but requires interaction with metal-free Ccs1. Under normal cellular conditions, the major role of Ccs1 may be to pull the disulfide loop away from the Sod1  $\beta$ -barrel, thereby exposing the electropositive cavity and “entry site” on immature Sod1.

The copper dependency of the sulfenylation reaction may arise from two effects. If superoxide is the substrate, peroxide is generated through a copper redox reaction. The copper dependency of a H<sub>2</sub>O<sub>2</sub>-mediated

sulfenylation reaction may occur orienting the thiols at the reaction site or enhancing the cysteine nucleophilicity. Finally we note that coordination may increase the fidelity of the sulfenylation reaction at the critical thiol (C<sub>146</sub>) via a specific interaction with the Cu(I) which may electronically activate this thiol for reactivity.

In conclusion, our biochemical studies and Sod1•Ccs1 complex structure suggest a novel mechanism for Sod1 maturation. Copper delivery to an exposed “entry site” on immature Sod1 leads to a copper-mediated sulfenylation intermediate. Copper donation may occur either through Ccs1 D1 or via a small labile Cu(I) complex. In the later case, Ccs1 may still facilitate entry site copper binding through its stabilization of a nascent conformation of Sod1. Ccs1 directs Sod1 disulfide formation through sulfenylation of C<sub>146</sub>, which keys disulfide exchange reactions concluding in a stable Sod1 C<sub>57</sub>-C<sub>146</sub> disulfide, copper ion placement in the active site, and termination of the interaction with the now mature Sod1. The maturation path is a concerted process requiring a specific conformation of thiolates in the entry complex, accompanied by coupled sulfenylation, copper ion migration, and resolution to disulfide, with the driving force dependent on all three molecular events. In this context, the Cu(I) “entry site” and electropositive cavity are integral to normal Sod1 function. Our data and ensuing mechanism also require that Sod1 maturation is initiated by oxidative chemistry as previously reported by the O’Halloran group (47). This latter conclusion is significant since it implies that Sod1 metallation and activation is regulated by the presence of its substrate such that its precious copper cargo is only loaded when the cell or organelle experiences oxidative stress.

## MATERIALS and METHODS

**Materials.** Monobasic and dibasic potassium phosphate, acetonitrile (Optima grade), formic acid, sodium hydroxide, yeast extract, peptone, dextrose (glucose), EDTA, sodium chloride, and sodium acetate were obtained from Fischer Scientific. Ammonium sulfate was purchased from US Biological. Polyethylene glycol 1000 came from Fluka. Tris was purchased from Research Products International. Primers came



from Invitrogen. Agarose, glycerol, DEAE Sephadex, DTT, and PMSF were obtained from Sigma. *Pfu* DNA polymerase and deoxyribonucleotides were purchased from Stratagene. Glass beads were obtained from Biospec. Crystallization screening kits and crystal growth trays were purchased from Hampton Research. Phenyl Sepharose and Sephadex G-75 came from Pharmacia. All solutions were prepared using de-ionized water passed through a Millipore ultra-purification system.

*Sod1 Cloning, Expression and Purification.* DNA fragments encoding the human H46R/H48Q Sod1 double mutant were amplified by PCR and ligated into the YEP351-hSOD plasmid, where expression of the Sod1 protein is directed under the control of its own promoter. The protein was expressed, purified, and characterized as previously described (27) with the addition of a DEAE-Sephadex chromatography step between the hydrophobic interaction chromatography and gel filtration column steps. The metal content of purified Sod1 and Ccs1 proteins was determined using inductively coupled plasma mass spectrometry (ICP-MS) at the Chemical Analysis Laboratory at the University of Georgia.

*Ccs1 Cloning, Expression and Purification.* DNA fragments encoding yCcs1 were generated by polymerase chain reaction (PCR) from plasmids generously supplied by J.S. Valentine (UCLA). Ccs1 constructs were cloned into a pKA6H vector, which contains an inducible *lacZ* promoter, a 6x-N-terminal His-tag, and a tobacco etch virus (TEV) cleavage site. Residues <sup>237</sup>WEER<sup>240</sup> in D3 were substituted to <sup>237</sup>WAAA<sup>240</sup> via quick-change mutagenesis and these substitutions dramatically enhanced the overall yield of recombinant Ccs1 (~5-fold) while simultaneously obviating the instability and proteolysis observed in wild type Ccs1 otherwise identically prepared. Yeast Ccs1 was expressed in *Escherichia coli* BL21 (DE3). Transformed cells were grown in LB media at 37° C to an OD<sub>600</sub> of 0.6 to 0.8. After induction with 0.1 mM IPTG, the cells were transferred to 30° C for an additional 4 hours before being harvested. Ccs1 was purified using a HisTrap HP Ni<sup>2+</sup> affinity column purchased from GE. After purification, the hexa-His-tag was removed by digestion overnight with TEV protease produced

in-house and engineered to contain its own non-cleavable hexa-His tag. After digestion, the cleaved His tag and TEV protease was removed from the Ccs1 sample by a final pass through the nickel column. This procedure leaves a two residue (Gly-His) extension on the Ccs1 N-terminus.

*Crystallization, Structure Determination, and Refinement.* The H46R/H48Q Sod1•E238A/E239A/R239 Ccs1 complex was prepared in solution as previously described (27). All crystals were grown at room temperature using the hanging drop vapor diffusion method. Purified Sod1•Ccs1 complex at 20 mg/mL in 50 mM MES, pH 6.5, 150 mM sodium chloride, 10 mM TCEP and 50  $\mu$ M ZnSO<sub>4</sub> was mixed with an equal volume of reservoir solution containing 100 mM Bis-Tris, pH 6.5, 200 mM ammonium sulfate, and 25% w/v PEG 3350. Rectangular crystals grew in space group P2<sub>1</sub>2<sub>1</sub>2 within two weeks at room temperature. Suitable specimens were soaked in a cryoprotectant consisting of reservoir solution made 10% (v/v) in ethylene glycol before flash-cooling by plunging into liquid nitrogen. X-ray diffraction data were measured to a resolution of 2.5 Å resolution at the Argonne National Laboratory NE-CAT beamline 24-ID-E, which is equipped with microfocus MD-E microdiffractometer. All diffraction data were processed using the HKL2000 program suite (48). The structure was determined by molecular replacement with the program MOLREP (49) using the structures of human S134N Sod1 [PDB: 1OZU (50)] and yCcs1 D2 [PDB: 1JK9 (19)] as the search models. The Sod1 component was positioned in the unit cell first, then yeast Ccs1 DII followed by rigid body refinement using the program PHENIX (51). The model was refined iteratively in PHENIX without noncrystallographic symmetry restraints, invoking simulated annealing in the early rounds of refinement and alternating with manual model adjustment into  $\sigma_A$ -weighted electron density map using the program COOT (52). No stereochemical restraints were applied to the metal-ligand distances or bond angles. All structural figures were created using the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

**Yeast Growth, Strains and Plasmids.** *S. cerevisiae* strains were grown in SC-glucose (dextrose) (SCD) media at 30°C unless otherwise noted. BY4741 *ccs1Δ::KanMX* knockout was generously provided by Dr. Val Culotta. The *sod1Δ* mutant in BY4741 as well as in the *ccs1Δ::KanMX* background was generated with a *URA3* disruption. Dr. Culotta generously provided both pRS313-*CCS1* and YEP351-*ySOD1* plasmid for *in vivo* and EXAFS experiments. N-terminal Strep tag (WSHPQFEK) with a 2 Gly linker and all subsequent mutations in *ySOD1* were generated by Phusion mutagenesis PCR.

**Analysis of Sod1 Disulfide Redox State and Activity Measurements.** *ccs1Δ* were transformed with *CCS1*-containing plasmids harboring specific spacing mutations. 50 mL cultures were harvested at OD<sub>600nm</sub>~1. Pellets were resuspended in 8M Urea, 3mM EDTA, 0.5% Triton X-100, 50 mM Tris, pH 8.0 and split into 2 aliquots containing or lacking mPEG-Mal (PEG Maleimide, MW 2000) purchased from NANOCs. The cells were lysed by bead beating and centrifuged at 12,000 x g for 10 min. The supernatant was placed at 37°C for 1 h and ~ 30 µg of lysate was analyzed by western blot. Sod1 activity was measured using the SOD assay kit purchased from Sigma.

**Sod1 Purification.** For Strep-tag affinity purification, *ccs1Δsod1Δ* cells were co-transformed with vectors encoding Sod1-Strep and Ccs1 variants and grown to OD<sub>600nm</sub>~1 in SCD media. Cells were resuspended in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0 with Roche cOmplete protease inhibitor cocktail tablets. Cells were lysed by bead beating and centrifuged at 12,000 x g for 10 min. One mg of supernatant was diluted to 500 µl of buffer and 50 µl bead volume of Strep-Tactin Superflow plus, purchased from Qiagen, was added and incubated for either 4 h or overnight. For non-reducing alkylation experiments, 20 mM N-ethyl maleimide (NEM) was added to overnight incubation of lysates. To test whether copper was involved in heterodimer complex, 500 µM BCS was added to lysates in overnight incubation. Eluates were then analyzed by western blot analysis. For purification of Sod1 H<sub>46</sub>R H<sub>48</sub>Q, cells were lysed by French pressure cell press and centrifuged at 10,000 x g to clarify lysates. Ammonium sulfate was added to lysates

at 4°C while stirring to a final concentration of 60%. Upon reaching 60% saturation, an equal volume of methanol was added to the solution and stirred vigorously for 10 min. The supernatant was centrifuged at 10,000 x g for 30 min and the supernatant was dialyzed overnight into 50 mM phosphate buffer, 150 mM NaCl, pH 7.0. Following dialysis, the eluate was loaded onto a Superdex 75 FPLC column and the major peak containing Sod1 was dialyzed into 20 mM Tris, pH 8.0.

**Sod1 Metal Analysis.** Sod1-Strep tagged constructs were grown in SCD media to OD<sub>600nm</sub> 1.5 and harvested. Cells were lysed by French pressure cell press and centrifuged at 50,000 x g for 1 h at 4°C. The protein was purified using Strep-Tactin Superflow plus resin. Eluted proteins were dialyzed overnight in 20 mM Tris pH 8.0 and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) for metal content.

**Sod1 Sulfenylation.** Sod1 was purified as described above. 5,5-dimethyl-1,3-cyclohexanedione (dimedone), purchased from Sigma Aldrich, was added to a final concentration of 20 mM to the eluate or to lysates. The protein was incubated at 37°C for 1 h, and analyzed by western blot analysis using an anti-cysteine sulfenic antibody purchased from EMD Millipore which reacts with cysteine sulfenic acids derivatized with dimedone. For experiments to test the role of copper in sulfenic acid formation, cells were grown overnight in low Cu/Fe media to an OD~1. Cells were back diluted to OD ~1 in low Cu/Fe medium, plus or minus 150 µM BCS and harvested at OD~1.

**X-ray Absorption Data Collection and Analysis.** Copper K-edge (8.9 KeV) extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) data were measured at the Stanford Synchrotron Radiation Lightsource operating at 3 GeV with currents between 300 and 500 mA operated in continuous top-up mode. Samples were measured on beamline 7-3 using a Si[220] monochromator and an upstream Rh-coated mirror with 13 keV energy cutoff in order to reject harmonics. Data were collected in fluorescence mode using a

Canberra 30-element Ge array detector with maximum count rates per array element set to less than 120 kHz. A Z-1 nickel (NiO) filter and Soller slit assembly inserted in front of the detector were used to reduce elastic scattering relative to the Cu K $\alpha$  fluorescence. Four scans of a buffer blank were averaged and subtracted from the averaged data for each protein sample to remove the Ni K $\beta$  fluorescence and produce a flat pre-edge baseline. Samples were measured as aqueous glasses in 20% ethylene glycol at 10K. Data reduction and background subtractions were performed using the program modules of EXAFSPAK (53). Spectral simulation was carried out using EXCURVE version 9.2 (54, 55) as described previously (56-58). The best fit of the simulations of the EXAFS data supported a mixed-shell coordination model consisting of imidazole from histidine and sulfur from cysteine coordination where appropriate. The threshold energy, E<sub>0</sub>, was chosen at 8985 eV, and refinement of structural parameters included distances (R), coordination numbers (N), and Debye-Waller factors ( $2\sigma^2$ ), and included multiple scattering contributions from outer-shell atoms of imidazole rings.

*Mass Spectrometry Data Collection and Analysis.* Sample digestion and data acquisition were performed as described in Zempel et al. (59) Modified peptides were identified by the MSGF+ search algorithm using a differential modification search that considered a mass shift of +138.068080 on cysteine residues and performed against a yeast or human database depending on the proteins of interest (60). The search results were combined and analyzed using Percolator and filtered at a spectrum-level q-value of 0.01.(61) Dimedone site localization and site probability was determined using PTMRS algorithm and filtered at 95% confidence level or higher.(62) The spectrum for each peptide was manually annotated using SeeMS spectrum viewer.(63) The Skyline software was used to evaluate the presence of each dimedone-modified peptide across reactions and plot the intensity-based bar plot.(64)

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**Author Contributions:** MMF, SBS, ABT and DDW conducted the research presented; MMF, NJB, DDW, PJH and DRW contributed to data analysis and interpretation, MMF, NJB, DDW, PJH and DRW drafted and edited the manuscript; and DDW, NJB and DRW contributed ideas for experimentation. All authors approved the final version of the manuscript.

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## FIGURE LEGENDS

**Figure 1:** Structures of Sod1•Ccs1 complexes. In all panels, the Sod1  $\beta$ -barrel is shown in green with the electrostatic loop orange and disulfide loop purple, Ccs1 D1 is marine, D2 is grey, and D3 is red. The Sod1 zinc ions are shown as orange spheres. **A)** The heterodimeric complex between human H<sub>46</sub>R/H<sub>48</sub>Q Sod1 and yeast E<sub>238</sub>A/E<sub>239</sub>A/R<sub>240</sub>A Ccs1 determined in the present work [pdb 5U9M]. The reduced disulfide bond of H<sub>46</sub>R/H<sub>48</sub>Q Sod1 between C<sub>57</sub> and C<sub>146</sub> permits the displacement of the disulfide loop from the Sod1  $\beta$ -barrel where it interacts extensively with residues coming from Ccs1 D2 and D3. The  $\beta$ -hairpin portion of D3 begins just downstream of N<sub>224</sub> with C<sub>231</sub> presiding on the loop between the two short  $\beta$ -strands. **B)** The heterodimeric complex between yeast H<sub>48</sub>F Sod1 and yeast Ccs1 [pdb 1JK9] (19). **C)** Intercalation of the D3  $\beta$ -hairpin between the Sod1  $\beta$ -barrel and disulfide loop. Sod1 is shown as an electrostatic surface contoured at  $\pm 4$  kT. The reduced C<sub>57</sub>-C<sub>146</sub> Sod1 disulfide bond exposes an electropositive “hole” near C<sub>146</sub>. Domains 1 (marine) and 2 (grey) of Ccs1 are shown as surfaces. The stabilizing positioning of the conserved Ccs1 tryptophan residues W<sub>222</sub> and W<sub>237</sub> from D3 are highlighted.

**Figure 2:** The spacing of the D3 CxC Cys residues is critical for Sod1 activation. **A)** Mutations were made in the CxC motif of Ccs1 to alter the spacing to either a CC or a CxxC motif without disrupting downstream residues. **B)** Viability tests were performed by plating cells on synthetic complete media with or without lysine at 30°C or 37°C. **C)** Sod1 activity for various Ccs1 mutants was quantified. **D)** The status of the disulfide bond was visualized by lysing cells in the presence of a PEG-maleimide alkylating agent that selectively reacts with free thiols.

**Figure 3:** Stalled Sod1•Ccs1 complexes. **A)** Lysates from *sod1* $\Delta$  cells transformed with Sod1-Strep mutants were subject to affinity purification using Strep-Tactin resin, where 2% (20  $\mu$ g) of total input, and 20 % of total elution were loaded and analyzed by western blot. **B)** Strep affinity purification from *ccs1* $\Delta$  cells harboring Ccs1 CxC motif mutants, **C)** Strep affinity purification from *ccs1* $\Delta$  *sod1* $\Delta$  cells harboring Sod1 C<sub>146</sub>S-Strep and co-transformed with vectors encoding various Ccs1 CxC mutants, **D)** and **E)** Strep affinity purification from lysates containing Ccs1 CxC spacing mutants coexpressing Sod1-Strep.

**Figure 4:** Sod1 can form an intermolecular disulfide with Ccs1. **A)** Cell lysates from *ccs1* $\Delta$  *sod1* $\Delta$  cells co-transformed with WT or Ccs1 CxC mutants and WT Sod1-Strep or Sod1-Strep mutants were subject to affinity purification, where 2% (20  $\mu$ g) of total input, and 20% of total elution were loaded and analyzed by western blot. Reducing SDS PAGE gels were run (top) as well as non-reducing SDS-PAGE gel (bottom) and blotted for Sod1 or Ccs1. **B)** WT Sod1-Strep or mutants were purified in the presence or absence of 500  $\mu$ M bathocuproine disulfonic acid (BCS) and analyzed for Ccs1 interaction. **C)** Sod1-Strep (WT) or containing various Sod1 mutations were affinity purified and metal ratios were determined by ICP-OES.

**Figure 5:** Cysteine residues from Sod1 and/or Ccs1 form a novel Cu(I) “entry site.” Sod1 was purified from yeast lysates containing the indicated Sod1 and Ccs1 mutations. X-ray absorption spectroscopy was employed to determine the Cu coordination environment of Sod1. **A)** The Cu K edge of WT samples along with WT Ccs1 + Sod1 H<sub>46</sub>R H<sub>48</sub>Q and Ccs1 C<sub>17/20/27/64/159</sub>S + Sod1 H<sub>46</sub>R H<sub>48</sub>Q, **B)** EXAFS region for WT samples, **C)** EXAFS region for sample containing WT Ccs1 + Sod1 H<sub>46</sub>R H<sub>48</sub>Q, **D)** EXAFS region for sample containing C<sub>17/20/27/64/159</sub>S + Sod1 H<sub>46</sub>R H<sub>48</sub>Q.

**Figure 6:** Electropositive cavity and copper ion “entry site” on immature Sod1. **A)** WT Sod1 (colored as in Figs. 1 and 2) shown in its disulfide oxidized mature conformation (left). The electrostatic surface is contoured at  $\pm 4$  kT (right). **B)** The disulfide reduced, copper-free Sod1 molecule from the current Sod1•Ccs1 complex with residues 46 and 48 exchanged back to histidine for demonstrative purposes. The box in the right panel highlights an electropositive cavity on Sod1 made accessible through the



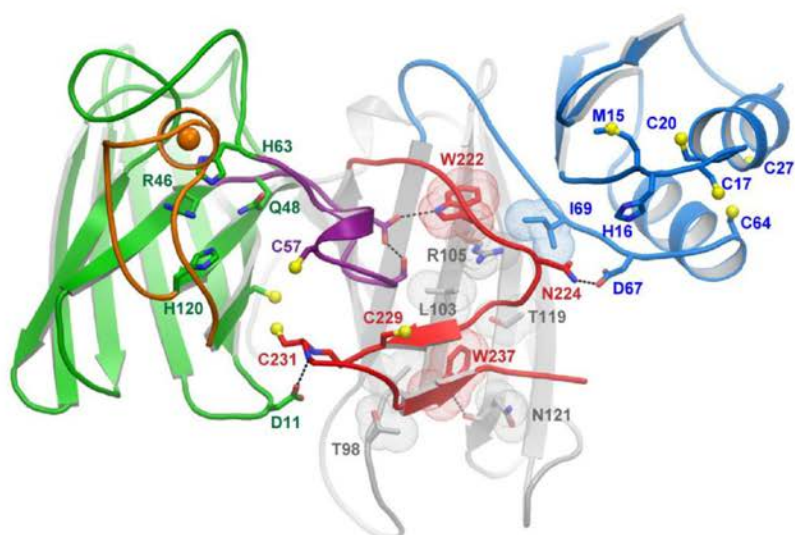
intercalation of the Ccs1 D3  $\beta$ -hairpin. **C)** The Cu(I) “entry site” on immature Sod1 (solid black lines), as shown here, contains two cysteine sulfhydryl groups (C<sub>57</sub>, C<sub>146</sub> from Sod1) and one histidyl side chain from H<sub>48</sub> or H<sub>120</sub>. The left panel shows a semi-transparent electrostatic surface covering the “entry site” from the view of the Ccs1 D3  $\beta$ -hairpin. The right panel is rotated back and counterclockwise (both  $\sim 90^\circ$ ) from the left panel and now the electropositive cavity surface is shown to emphasize its depth and positioning with regard to the “entry site” ligands.

**Figure 7:** Copper-dependent sulfenylation at the Sod1 “entry site” and a role for reduced glutathione (GSH) **A)** WT Sod1-Strep purified from yeast cells and treated with DMSO vehicle or dimedone to a final concentration of 20 mM. Coomassie stain of the purified Sod1 samples are on left panel and western blot using a cysteine sulfenic antibody or Sod1 antibody on the panels on the right. **B)** Purified Sod1 with various mutations were reacted with dimedone and visualized by western blot analysis. **C)** WT cells or *ctr1 $\Delta$*  cells were transformed with a Sod1-Strep-encoding plasmid. The cells were grown in the absence or presence of 150  $\mu$ M BCS and subject to Strep-Tactin affinity purification, where 2% (20  $\mu$ g) of total input, and 40% of total elution were loaded and analyzed by western blot. **D)** *In vitro* activation of yeast Sod1. Zn-Sod1 (copper free, disulfide-reduced) was mixed with either CuGSH, Cu-Ccs1 or apo-Ccs1 aerobically in 50 mM Tris pH 7.6, 100 mM NaCl and loaded on the gel for an in gel Sod1 enzymatic activity. Top panel: lane 1- Zn-Sod1; lane 2- Zn-Sod1 + CuGSH; lane 3- Zn-Sod1 + apo-Ccs1; lane 4- Zn-Sod1 + CuGSH + apo-Ccs1; lane 5- Zn-Sod1 + CuCcs1. Bottom panel: the same Sod1 activation assay as the top panel, but includes the Ccs1 D1 MxCxxC (lanes 4 and 7) and D3 CxC (lanes 5 and 8) mutants. **E)** Copper-mediated sulfenylation of yeast Sod1. Apo-Sod1 was preloaded with 1 mol equiv Zn(II) and mixed with yeast Ccs1 either pre-loaded with 1 mol equiv Cu(I), Co(II) or Zn(II). After mixing the samples were incubated with 20 mM dimedone prior to loading on a gel. The top panel is the visualization of the dimedone sulfenylated Sod1 adduct using the anti-sulfenic acid antibody, the bottom panel shows the level of Sod1 protein as visualized with an anti-Sod1 antibody. **F)** Copper-mediated sulfenylation of human Sod1. Apo-human Sod1 was preloaded with 1 mol equiv Zn(II) and incubated with yeast Ccs1 either pre-loaded with 1 mol equiv Cu(I), Co(II) or Zn(II). The conditions and 2 panels are as described in panel **E**.

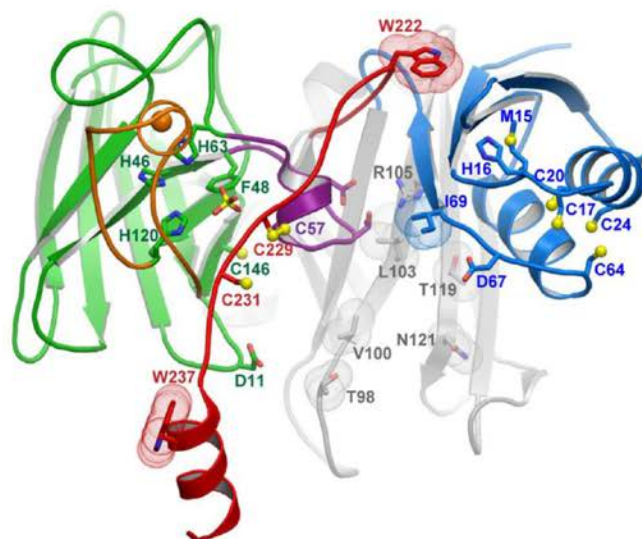
**Figure 8:** Relative abundance of dimedone-modified Sod1 peptides. Calculated from the extracted ion chromatogram for each peptide across the five reactions. Rxn 1: 5uM Apo-Sod, BCS activity buffer (50mM Tris pH 7.6, 100mM NaCl, 0.5mM TCEP, and 200uM BCS). Rxn 2: 5uM Apo-Sod, 5uM H<sub>2</sub>O<sub>2</sub>, 5mM Dimedone, 20uM Zn<sub>2</sub>SO<sub>4</sub>, 25uM Cu1-yCcs, BCS buffer. Rxn 3: 5uM Apo-Sod, 25uM H<sub>2</sub>O<sub>2</sub>, 5mM Dimedone, 20uM Zn<sub>2</sub>SO<sub>4</sub>, 25uM Cu1-yCcs, BCS buffer. Rxn 4: 5uM Apo-Sod, 5mM Dimedone, 20uM Zn<sub>2</sub>SO<sub>4</sub>, 25uM Cu1-yCcs, BCS buffer. Rxn 5: 5uM Apo-Sod, 20uM Zn<sub>2</sub>SO<sub>4</sub>, 25uM Cu1-yCcs, BCS buffer. In **A**, 1-5 indicates yeast SOD1 reactions while, in **B**, 1-5 indicates human SOD1 reactions.

**Figure 1**

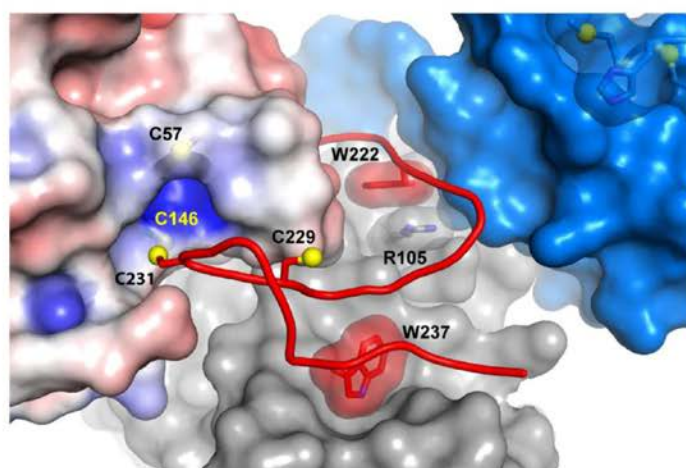
**A**



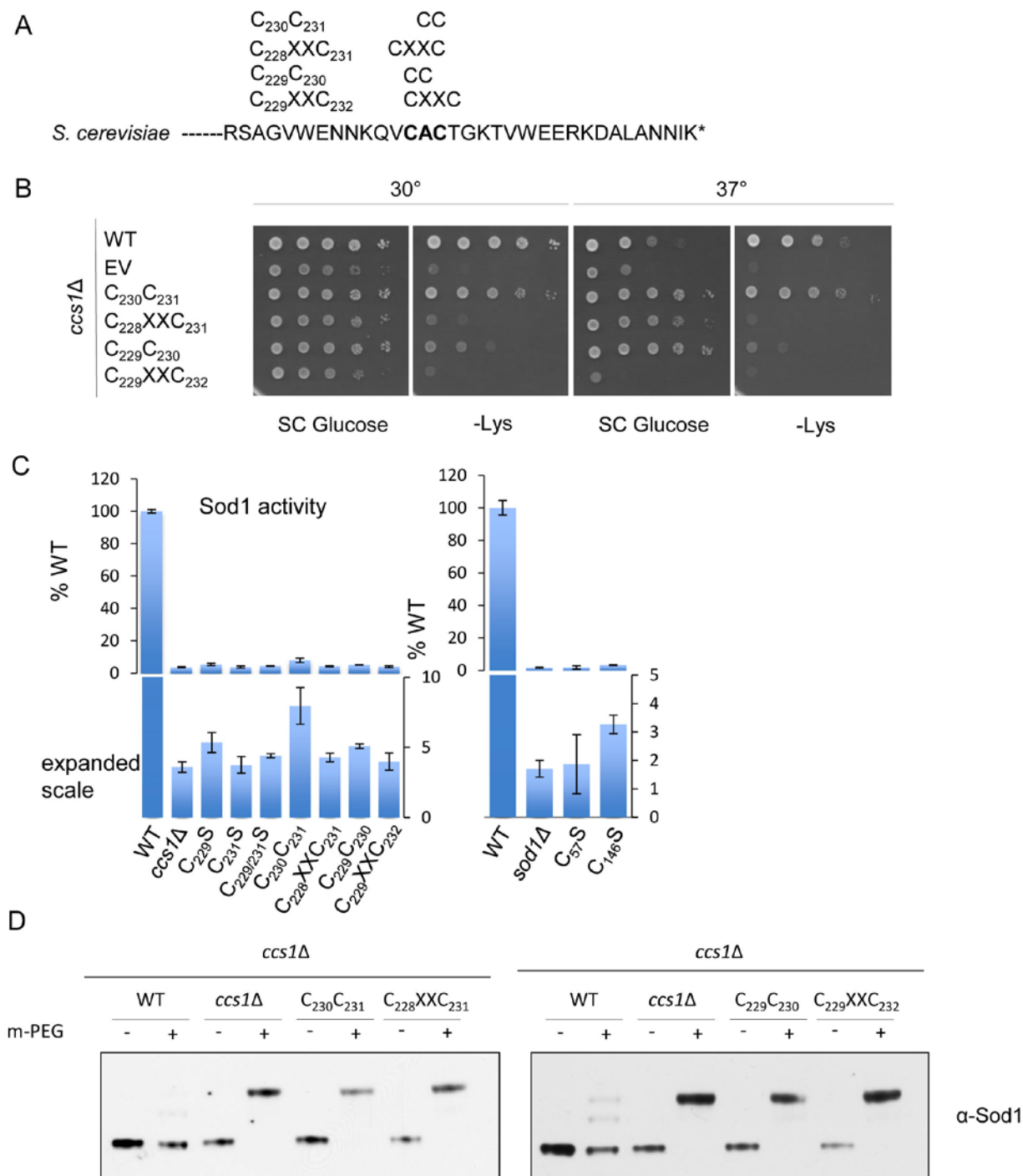
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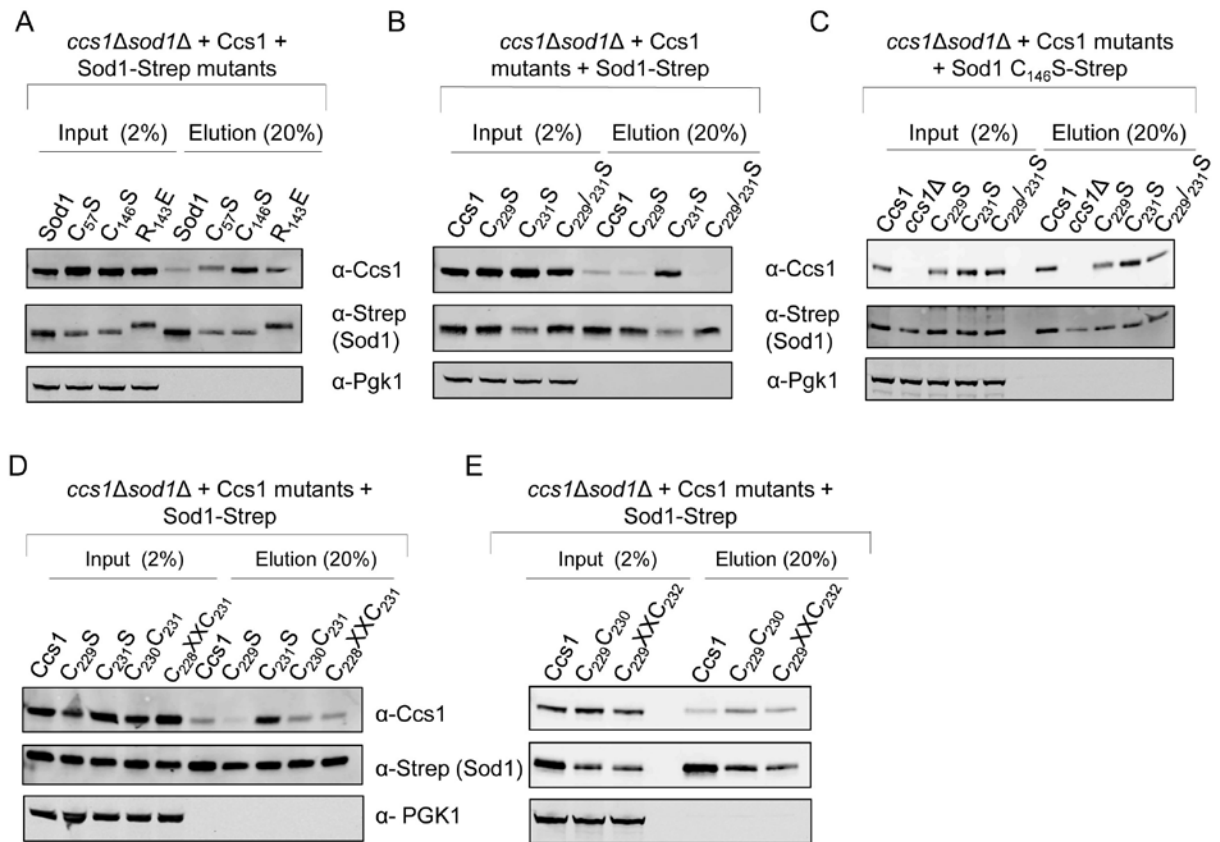
**C**



**Figure 2**

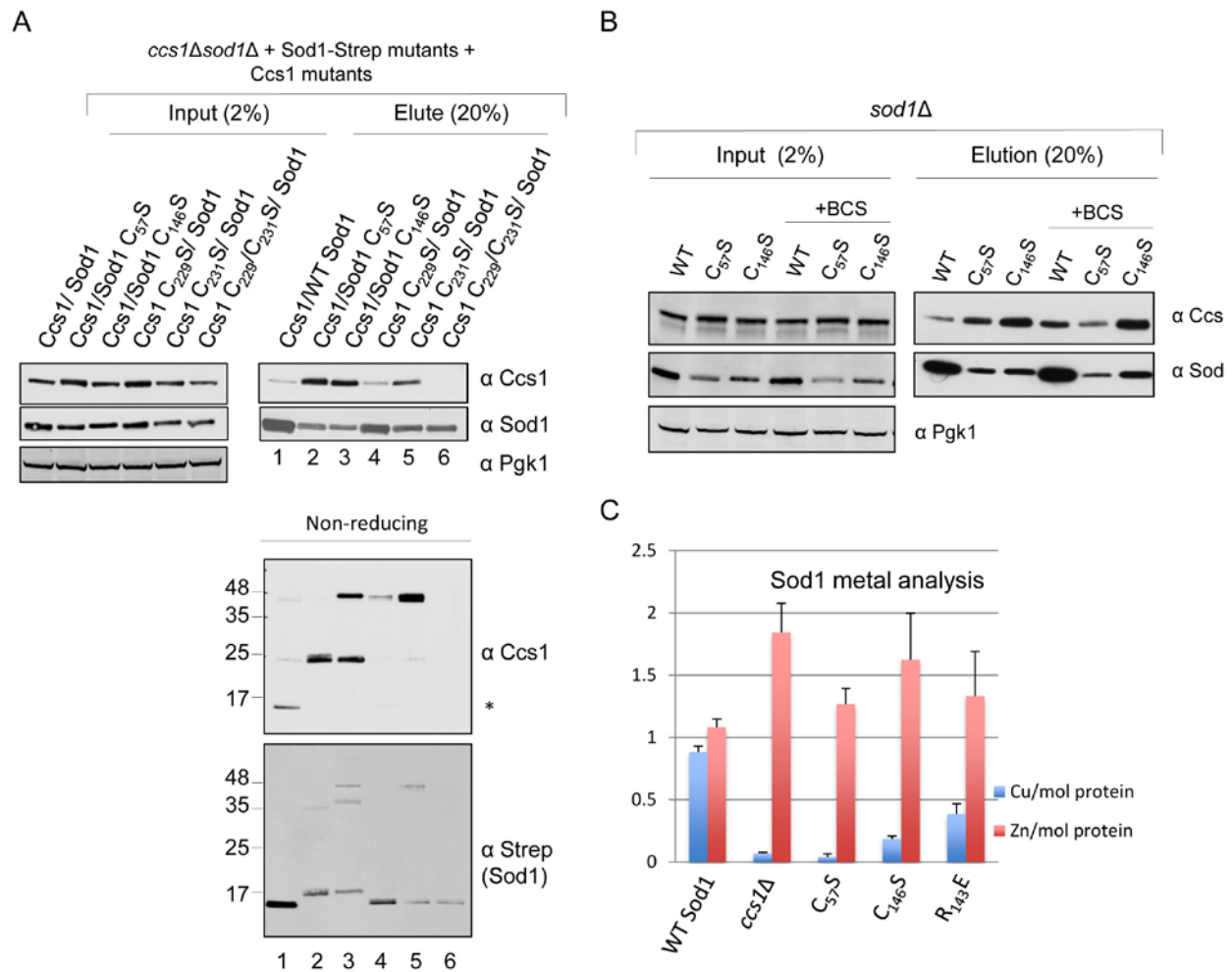


**Figure 3**

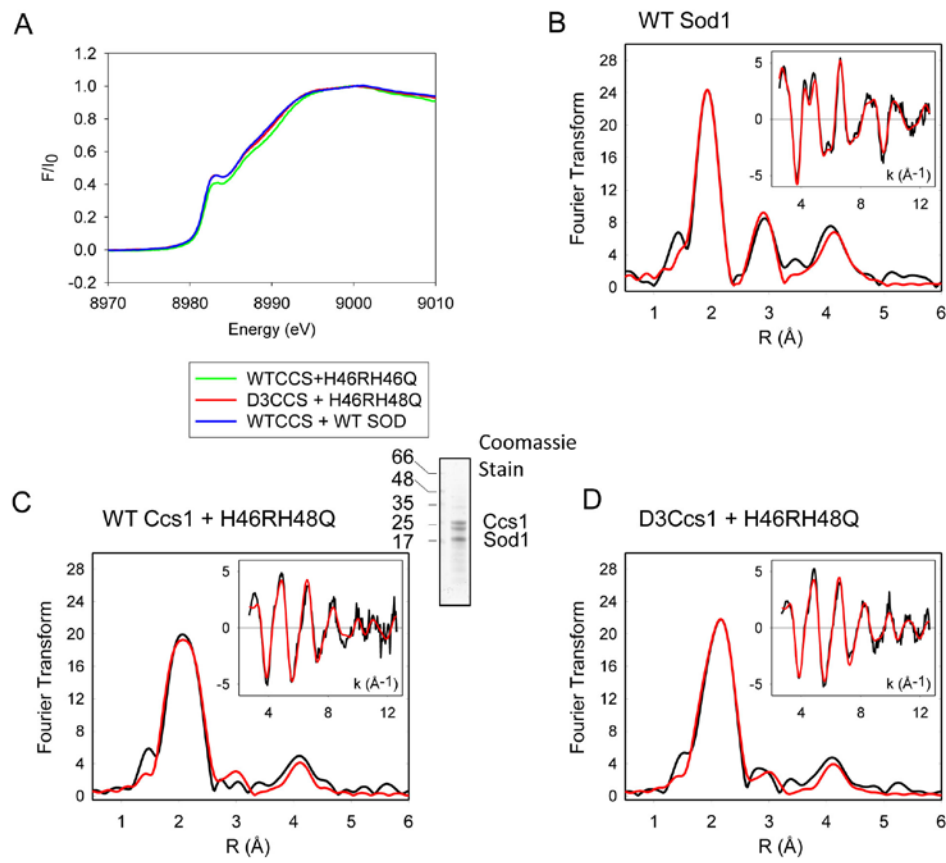




**Figure 4**



**Figure 5**



**Figure 6**

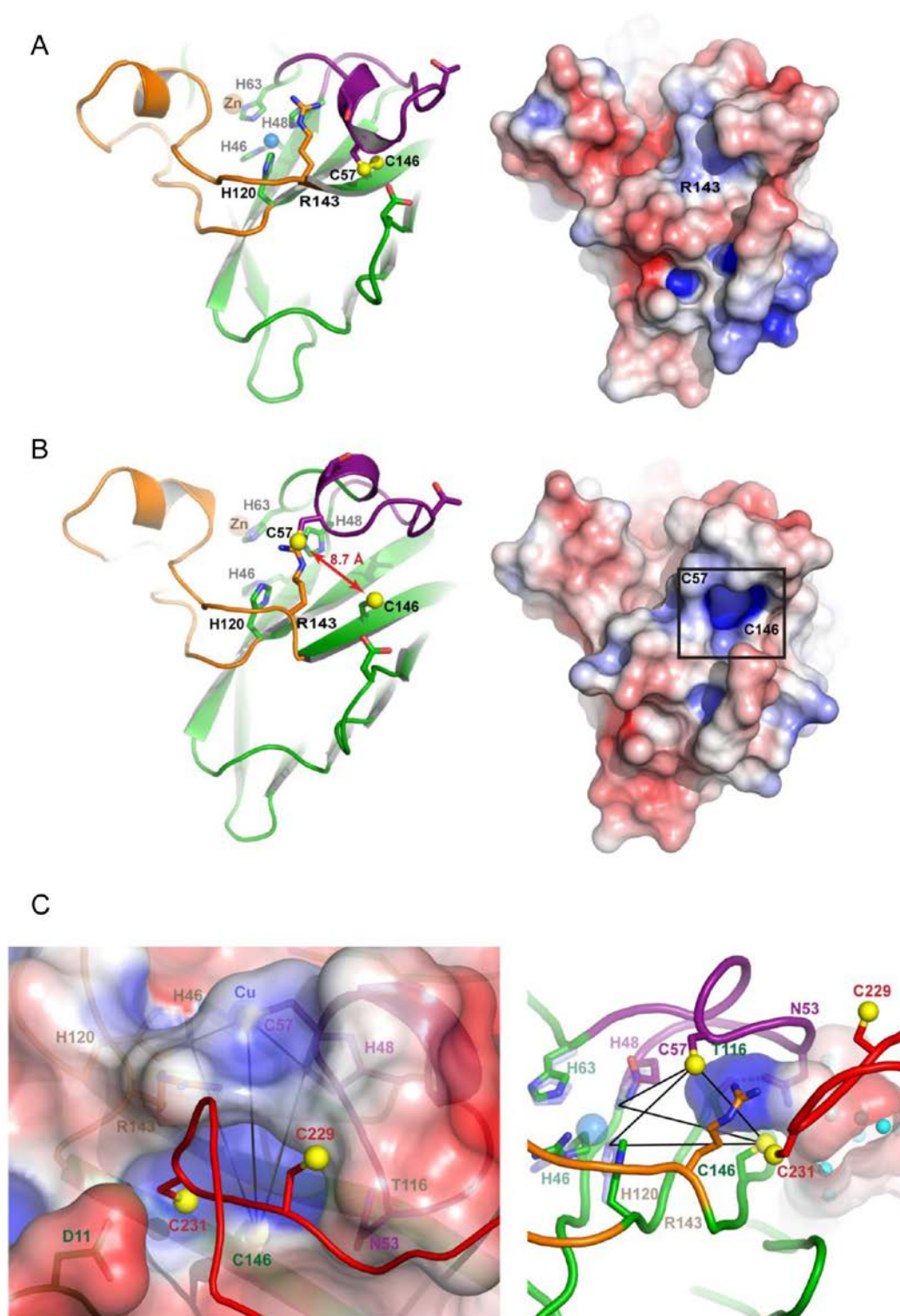
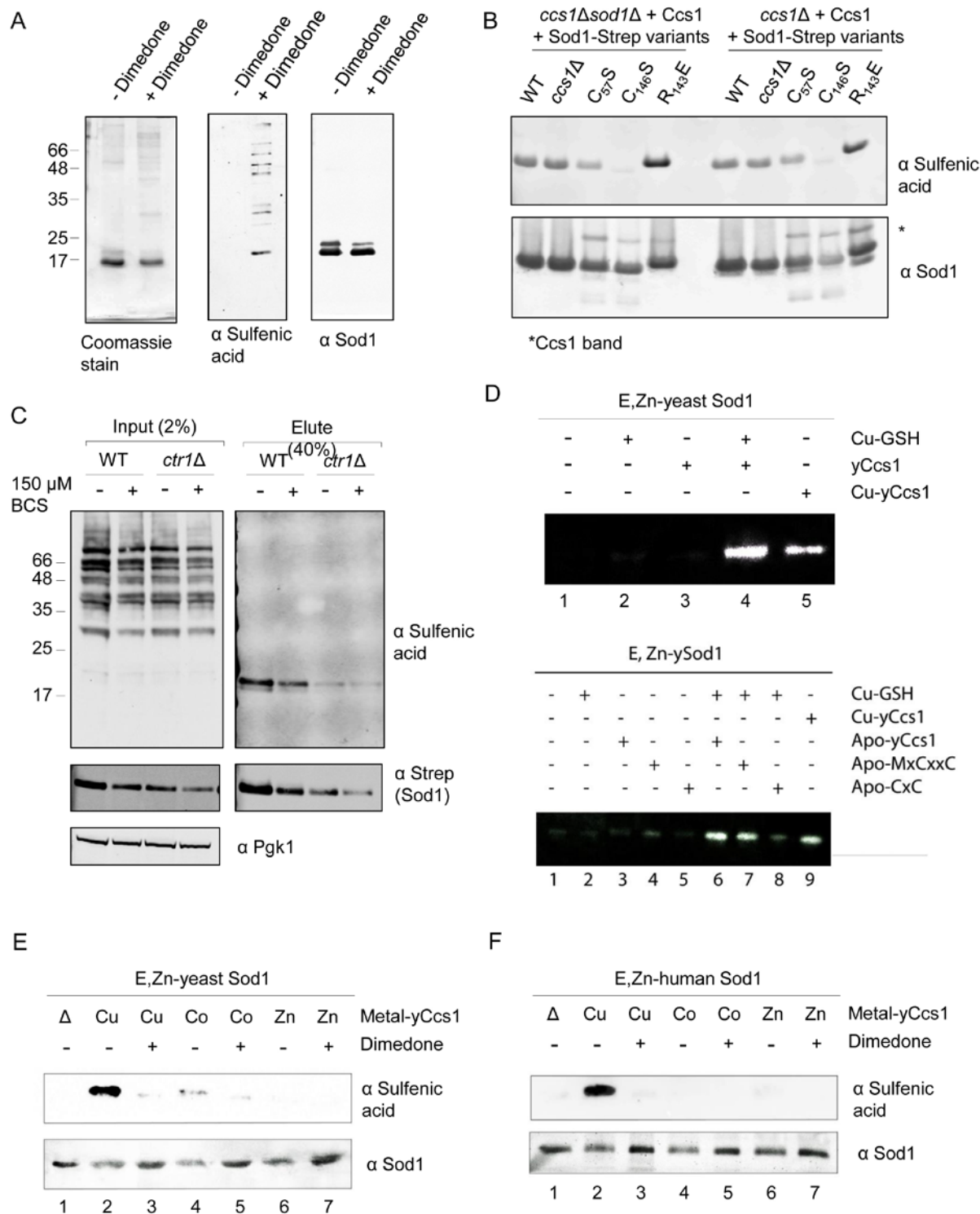
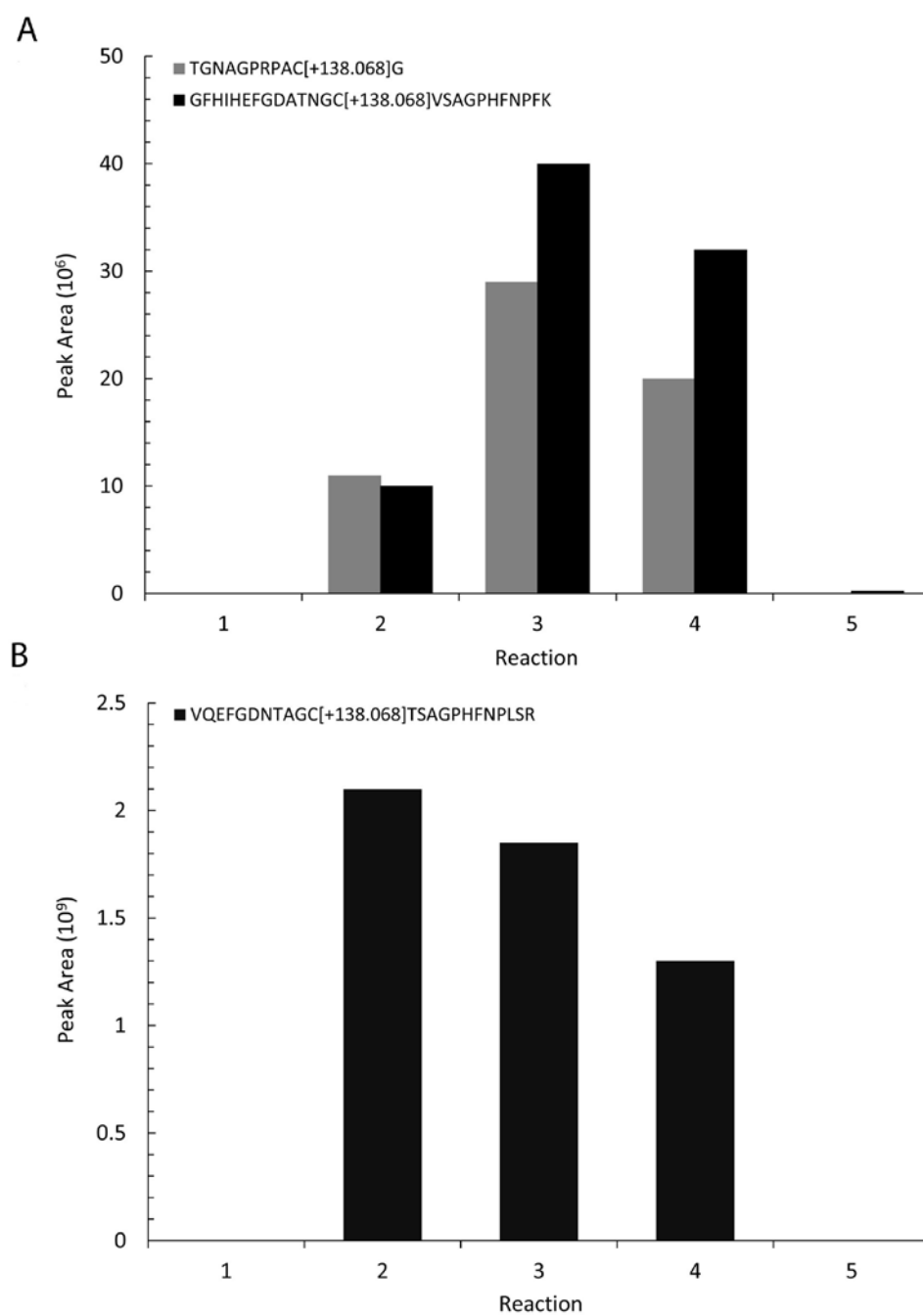


Figure 7





**Figure 8**



**Table 1**

Data collection, phasing and refinement statistics [PDB ID: 5U9M]

<b>Data collection</b>	
Space group	$P2_12_12$
Cell dimensions	
$a, b, c$ (Å)	99.1, 184.4, 48.5
$\alpha, \beta, \gamma$ (°)	90, 90, 90
Wavelength (Å)	0.97918
Resolution (Å)	48.51-2.35 (2.48-2.35)*
$R_{\text{sym}}$	0.112 (1.125)
$R_{\text{pim}}$	0.051 (0.480)
Mean ( $I/\sigma I$ )	13.8 (2.0)
Completeness (%)	99.9 (100)
Redundancy	7.0 (7.3)
<b>Refinement</b>	
Resolution (Å)	46.92-2.35
No. reflections	37,948
$R_{\text{work}} / R_{\text{free}}$	0.186/0.235
No. atoms	
Protein	5,734
Ligand	3 $\text{Zn}^{2+}$
Solvent	120
$B$ -factors	
Protein	57.4
Ligand	73.0
Solvent	42.9
R.m.s deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.142
Ramachandran statistics	
Favored, allowed, outliers (%)	98.7, 1.3, 0.0

\*Values in parentheses are for the highest-resolution shell.

**Copper-zinc superoxide dismutase is activated through a sulfenic acid intermediate at a copper-ion entry site**

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