A Gain of Superoxide Dismutase (SOD) Activity Obtained with CCS, the Copper Metallochaperone for SOD1

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Paul J. Schmidt‡, Minerva Ramos-Gomez‡, and Valeria Cizewski Culotta†

From the Department of Environmental Health Sciences, Johns Hopkins University School of Public Health, Baltimore, Maryland 21205

The incorporation of copper ions into the cytosolic superoxide dismutase (SOD1) is accomplished in vivo by the action of the copper metallochaperone CCS (copper chaperone for SOD1). Mammalian CCS is comprised of three distinct protein domains, with a central region exhibiting remarkable homology (approximately 50% identity) to SOD1 itself. Conserved in CCS are all the SOD1 zinc binding ligands and three of four histidine copper binding ligands. In CCS the fourth histidine is replaced by an aspartate (Asp200). Despite this conservation of sequence between SOD1 and CCS, CCS exhibited no detectable SOD activity. Surprisingly, however, a single D200H mutation, targeting the fourth potential copper ligand in CCS, granted significant superoxide scavenging activity to this metallochaperone that was readily detected with CCS expressed in yeast. This mutation did not inhibit the metallochaperone capacity of CCS, and in fact, D200H CCS appears to represent a bifunctional SOD, capable of self-activation with copper. The aspartate at CCS position 200 is well conserved among mammalian CCS molecules, and we propose that this residue has evolved to preclude deleterious reactions involving copper bound to CCS.

In eukaryotic cells, copper is delivered to specific protein targets via the action of a family of copper carrier proteins termed “metallochaperones” (1). These molecules are well conserved between yeast and humans and serve to guide the metal to discrete cellular locations and facilitate incorporation of the cofactor into target metalloenzymes (reviewed in Refs. 2–4). One such copper chaperone, COX17, acts in the delivery of copper to mitochondrial cytochrome oxidase (5–8). A second soluble metallochaperone, ATX1, escorts copper strictly to transport ATPases in the secretory pathway (1, 9–12). Thirdly, copper delivery and incorporation into cytosolic superoxide dismutase 1 (SOD1) is mediated by the soluble copper carrier, CCS (copper chaperone for SOD), also known in Saccharomyces cerevisiae as LYS7 (13, 14). Studies with the yeast metallochaperone have shown that CCS directly incorporates copper into SOD1 despite exquisitely low levels of available free copper (15).

The target of the CCS metallochaperone, SOD1, is a homodimeric copper- and zinc-requiring enzyme that acts to disproportionate superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and oxygen in a reaction catalyzed by the redox cycling of bound copper (16). However, SOD1 is also capable of catalyzing deleterious reactions involving the redox active copper cofactor. The Cu(I) form of SOD1 can react with H$_2$O$_2$ to generate the highly toxic hydroxyl radical (OH·) (17–19). In fact, it has been suggested that this inherent peroxidase activity of SOD1 may be involved in cases of familial amyotrophic lateral sclerosis in which disease results from dominant mutations in SOD1 (20–24).

It is noteworthy that the human CCS metallochaperone harbors a polypeptide region bearing striking resemblance to SOD1. This region, found in the central 16-kDa portion of CCS, is postulated to serve in target recognition of SOD1 (25), whereas smaller segments at the N and C terminus of CCS are thought to facilitate the binding and release of copper into SOD1 (26). Homology between the central portion of CCS and SOD1 approaches 50% identity and 60% similarity (25). Based on this curious conservation of sequence, CCS was originally postulated to be “SOD,” the fourth mammalian SOD (GenBankTM accession number 1608528).

The concordance of sequence between SOD1 and CCS was the focus of current studies. CCS molecules from diverse mammals contain all of the zinc binding ligands found in SOD1 and three of four histidine copper binding ligands; the fourth histidine is always substituted by an aspartate. We demonstrate here that the presence of this single aspartate prohibits CCS from functioning as a SOD. Substituting this aspartate with a histidine results in a CCS molecule with significant superoxide scavenging activity. Furthermore, this variant of CCS appears to be a bifunctional SOD, capable of self-activation with copper.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The isogenic wild type SY1699 and lys7 null SY2950 strains were previously described (27), as was the sod1Δ sod2Δ strain KS100 (28). VC107 and VC279 are sod1Δ::TRP1 derivatives of SY1699 and SY2950, respectively, and PS120 is a lys7Δ::URA3 derivative of KS100. Stocks of yeast strains were maintained by growth on enriched YPD medium (29) in anaerobic culture jars (BBL, GasPak), whereas growth tests for lysine auxotrophy utilized a synthetic minimal medium (29).

The vector for expression of human CCS in yeast cells is pCCS-HIS, containing the human CCS coding sequence under the control of the S. cerevisiae PGK1 (phosphoglycerol kinase) promoter. pCCS-HIS was constructed by mobilizing the PGK1-CDS fusion from pSMCCS (13) by digestion with BamHI and SalI and by insertion at these same sites into pRS423 (His3 2µ; Ref. 30). Human SOD1 was expressed in yeast by the pLC1 vector (URA3 2µ) where the SOD1 cDNA was placed under the control of the PGK1 promoter as described (31). pSM703 is the PGK1 promoter-containing vector (URA3 2µ) that served as the parent plasmid for construction of all human SOD1 and CCS expression plasmids. Plasmids pMR002 and pPS030 for the expression of D200H CCS...
and H120D SOD1 were derived from pCCS-HIS and pLC1, respectively, through use of the Quick Change Site-Directed Mutagenesis Kit (Stratagene) per manufacturer’s instructions.

**Biochemical Analysis—**Cell lysates were prepared essentially as described (33). Strains transformed with the appropriate plasmids were grown approximately 18 h at an absorbance of 1.5 in 50 ml of selecting SD medium (29); cells were harvested and were lysed by glass bead homogenization in 0.2–0.5 ml of a lysis buffer containing 10 mM NaPO4 (pH 7.8), 0.1 mM EDTA, 0.1% Triton, 50 mM NaCl, 20 μg/ml leupeptin, 10 μg/ml pepstatin, and 1.0 mM phenylmethylsulfonyl fluoride. Glycerol was added to the final lysates at a concentration of 5%. As needed, lysates were concentrated by Microcon-10 Microconcentrator (Amicon) columns per manufacturer’s instructions. For analysis of SOD activity, extracts were applied directly without boiling to a nondenaturing 12% precast polyacrylamide gel (Novex). Following electrophoresis, the gel was subject to nitro blue tetrazolium (NBT) staining for superoxide (32) in a 75-ml solution containing one tablet of NBT (10 mg/tablet), 50 mM KPO4 (pH 7.8), 0.1 mg/ml riboflavin, and 1 μM TEMED. Western blot analyses of lysates were carried out as described (33, 34) using a denaturing 14% polyacrylamide gel. A polyclonal rabbit anti-human SOD1 serum (kind gift of D. Borchelt, Johns Hopkins University) or preimmune serum (1:100 dilution) in a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.1% Nonidet P-40, as needed. Following incubation for 2 h at 4 °C, 20 μl of a 50% suspension of A-Sepharose beads (Sigma) was added, and incubation continued for an additional hour. The mixture was subjected to microcentrifugation at 1 min at 4 °C, and the resulting supernatants were concentrated on Microcon-10 Microconcentrator (Amicon) columns.

**RESULTS**

The central region of human CCS encompassing amino acids 78 to 232 shares nearly 50% identity with human SOD1. All four of the zinc binding ligands of SOD1 and three of four histidine copper binding ligands are present in CCS; the fourth histidine is replaced by an aspartate residue, which is also a possible ligand for copper (35) (Fig. 1A). Interestingly, this precise pattern of homology to SOD1 is observed with murine CCS (36). Based on sequence analysis alone it would seem plausible that mammalian CCS should possess SOD activity.

To address this question in vivo, human CCS was expressed in a yeast strain lacking SOD1 and was assayed for the ability to overcome the oxidative damage of these cells. Yeast sod1Δ mutants cannot synthesize lysine when grown in air due to oxidative damage of lysine biosynthetic component(s) (37–39). This defect is readily corrected by expression of the heterologous human SOD1 (Fig. 1C). However, expression of human CCS failed to complement the lysine auxotrophy of the sod1Δ mutant (Fig. 1C) even though the protein accumulated to substantial levels (Fig. 1B). By a NBT gel assay for SOD activity (32), CCS exhibited no detectable scavenging of superoxide (Fig. 2A, lane 5). Therefore, despite the exquisite homology between SOD1 and human CCS, the copper chaperone appears nonfunctional for SOD activity.

The most noteworthy difference between SOD1 and the SOD1 counterpart in human CCS is the substitution of a copper binding histidine with an aspartate (Fig. 1A). To test whether this single amino acid variation precludes SOD activity, the aspartate at CCS position 200 was mutated to a histidine. By Western blot analysis, D200H CCS accumulated to wild type levels when expressed in yeast (Fig. 1B). Surprisingly, expression of this mutant CCS molecule effectively complemented the yeast sod1Δ mutation (Fig. 1C), suggesting that this metallochaperone had acquired SOD activity. D200H CCS was directly examined for superoxide scavenging activity by the NBT gel assay. These studies were conducted in the back-
superoxide scavenging activity was detected in lysates from activity (Fig. 2).

On nondenaturing gels, the superoxide scavenging activity associated with D200H migrates to a position that lies between dimeric SOD1 and tetrameric SOD2. The strong inhibitory effect of the H120D mutation on SOD1 activity supports the notion that the aspartate residue at CCS position 200 is sufficient to prevent this molecule from functioning as a SOD.

Because the D200H CCS molecule retains its function as a copper chaperone and also exhibits superoxide scavenging activity, it is possible that this SOD-like molecule can act as its own metallochaperone? To address this, the mutant CCS molecule was expressed in a strain lacking both SOD1 and the yeast copper chaperone LYS7. This strain cannot grow on medium lacking lysine; however, the D200H CCS mutant rescued oxidative damage and supported lysine independent growth (Fig. 5A). By the NBT gel assay, it is evident that D200H CCS is capable of superoxide scavenging even in the absence of the yeast copper chaperone LYS7 (Fig. 5B, lanes 2–4). Therefore, the D200H mutant of CCS appears to self-activate itself for superoxide scavenging activity.

DISCUSSION

The mammalian CCS metallochaperone has evolved with a domain exhibiting remarkable homology to its target of copper delivery, SOD1. Based on this high degree of sequence identity, SOD activity would not have been an unreasonable assump-
tion. In fact, a number of copper-containing complexes have been shown to scavenge superoxide (43–45). However, CCS exhibits no detectable superoxide scavenging activity, and prohibition of the deleterious peroxidase activity typical of SOD1 (18, 20) yet if this were the case, the metal appears incapable of the redox cycling needed for superoxide scavenging. As an added benefit, self-oxidation of the copper chaperone or oxidation of its histidine is highly susceptible to oxidation, whereas the loss of this metal binding site prohibits the deleterious peroxidase activity typical of SOD1 (42), the presence of a SOD1-like copper site in CCS may contribute to the overall domain structure needed for target activity. In fact, a number of copper-containing complexes have shown that at the analogous position in human SOD1 (His120) the histidine is highly susceptible to oxidation, whereas the remaining three histidine copper ligands are not. Hence, the presence of Asp200 in CCS appears perfectly designed to prohibit self-oxidation of the copper chaperone or oxidation of its intimate partner, SOD1. Although mammalian CCS exhibits high homology to SOD1, far less homology is evident when one compares the central domain of yeast CCS to that of yeast SOD1 (approximately 25% identity at the amino acid level). Furthermore, as revealed through x-ray crystallographic studies by Rosenzweig and co-workers (48), the central domain of yeast CCS is devoid of a metal binding cavity, even though the overall structure is remarkably similar to SOD1. The loss of this metal binding site in yeast CCS may reflect a unique requirement for activation of yeast SOD1. Valentine and co-workers (42) have demonstrated that unlike mammalian SOD1, which forms a symmetrical homodimer, the yeast enzyme adopts an asymmetrical conformation in which there is unequal metallation of the two subunits (42). As fungal SOD1 diverged from the human enzyme, it is likely that their respective metallochaperones evolved concomitantly to conform to specific metallation requirements.

Why do separate molecules exist for SOD1 and its metallochaperone? Our studies here with human D200H CCS indicate that it is possible to manufacture a self-sufficient SOD molecule that can charge itself with copper. Although more energy-consuming, the synthesis of separate molecules for superoxide scavenging and metal incorporation may allow for control of SOD1 activity at the post-translational level. In general, the SOD1 polypeptide is ubiquitously expressed, but the fraction of apo- to holoSOD1 can vary greatly among cell types and tissues (49–52). As such, CCS represents a potentially effective means for rapidly controlling SOD1 activity in response to cellular needs.

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