Copper Stabilizes a Heterodimer of the yCCS Metallochaperone and Its Target Superoxide Dismutase*

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The copper chaperone for superoxide dismutase (CCS) activates the antioxidant enzyme Cu,Zn-SOD (SOD1) by directly inserting the copper cofactor into the apo form of SOD1. Neither the mechanism of protein–protein recognition nor of metal transfer is clear. The metal transfer step has been proposed to occur within a transient copper donor/acceptor complex that is either a heterodimer or heterotetramer (i.e. a dimer of dimers). To determine the nature of this intermediate, we generated a mutant form of SOD1 by replacing a copper binding residue His-48 with phenylalanine. This protein cannot accept copper from CCS but does form a stable complex with apo- and Cu-CCS, as observed by immunoprecipitation and native gel electrophoresis. Fluorescence anisotropy measurements corroborate the formation of this species and further indicate that copper enhances the stability of the dimer by an order of magnitude. The copper form of the heterodimer was isolated by gel filtration chromatography and contains one copper and one zinc atom per heterodimer. These results support a mechanism for copper transfer in which CCS and SOD1 dock via their highly conserved dimer interfaces in a manner that precisely orients the Cys-rich copper donor sites of CCS and the His-rich acceptor sites of SOD1 to form a copper-bridged intermediate.

Copper, the third most abundant transition element after iron and zinc in most eukaryotes, is critical to the activity of many enzymes involved in reactions with dioxygen and other reactive oxygen species (1, 2). Copper is imported into cells or compartments by a series of metal transporters (3) and accumulates in yeast to total cellular concentrations in the 0.01–0.1 mM range (4). While the total amount of copper in a cell is high, the concentration of the free copper ion in the cytoplasm of Saccharomyces cerevisiae corresponds to less than 10^(-18) M, far less than one atom per cell. Thus, in the absence of physical and chemical stress there are no chemically labile copper species available for direct binding to the nascent form of copper proteins (4).

Several families of proteins are required for utilization of copper (5–7). The copper chaperones represent one of these families and protect and guide copper to specific protein targets in the cytoplasm (8–10). The LYS7 gene, which encodes yeast CCS,\(^1\) is required for the appearance of the copper-dependent SOD1 activity \(\textit{in vivo}\) (9, 11). Both the yeast (4) and human (12) forms of copper-loaded CCS activate SOD1 by directly inserting the copper cofactor \(\textit{in vitro}\).

Superoxide dismutase plays a key role in protecting cells against oxidative damage (1, 13). When this enzyme is compromised by mutation, there are significant metabolic consequences (14, 15). Over 70 mutant forms of SOD1 are known to play a significant but unclear role in Lou Gehrig's disease (15–18). Insights into biochemical mechanisms that control the activity of SOD1 inside the cell provide a basis for understanding the molecular basis of this neurological disorder.

Mechanisms for activation of SOD1 must account for protein–protein interactions with CCS chaperone. Biochemical assays reveal that the metallochaperone protects copper from the most extreme copper scavengers \(\textit{in vitro}\) but allows facile and direct transfer of the metal to SOD1. This observation leads to the proposal of an intermediate in which copper was simultaneously coordinated by side chains in both CCS and SOD1 (4). Biochemical and genetic studies indicate three functional domains in yCCS (19), and structural studies reveal key insights into the roles of two of the domains and possible mechanisms of protein recognition (20–23). Spectroscopic and biochemical studies are consistent with metal coordination to Cys residues in the first and third domains (19, 24, 25).

Recent two-hybrid experiments in \(S.\ cerevisiae\) provide the strongest evidence that SOD1 directly interacts with CCS inside the cell (26). While the two most C-terminal domains of yCCS are required for contact with SOD1 \(\textit{in vivo}\), it is not known how these proteins dock or how the metal is transferred. Alternative models for a heterodimeric or heterotetrameric intermediate between yCCS and apo-SOD1 have been proposed (12, 20–23). A central assumption in the heterotetramer model is that E,Zn-ySOD1 (where E indicates an empty copper-binding site) or E,E-ySOD1 remains a dimer upon encountering yCCS (22). Several characteristics of SOD1 suggest that this need not be the case. First metal occupancy influences the stability of the dimeric state of bovine SOD1 (bSOD1) (7, 27, 28). Chemical modification experiments indicate that the presence of Zn(II) in the active site (E,Zn-SOD1) restores the overall native structure of bSOD1 (29, 30) and that the E,Zn-SOD1 dimer is significantly favored relative to E,E-SOD1 (31). Both

\(\textit{\#}^1\) The abbreviations used are: CCS, copper chaperone for superoxide dismutase; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid; HRP, horseradish peroxidase; CPM, maleimide derivative of coumarin; TCEP, tris-(2-carboxyethyl)phosphine; wt, wild type; SOD, superoxide dismutase; ICP-AES, inductively coupled plasma atomic emission spectroscopy.

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E.E-SOD1 and E.Zn-SOD1 are less stable than holo-SOD1(31); however, E.Zn-ySOD is the dominant form in cells that lack yCCS (32).

In this study we employ a mutant form of yeast E.Zn-SOD1 in which one of the four copper binding histidine residues is replaced with a residue that cannot bind metal ions. Using this protein as bait we have delineated steps required for capture of a complex that contains the docked copper chaperone. The new experiments corroborate our preliminary report supporting a heterodimer (33) and further show that copper facilitates formation of the 1:1 complex between yCCS and E.Zn-SOD1. The significant thermodynamic stabilization of this otherwise transient complex by copper provides new energetic insights into the capture and release mechanism of metal transfer among these physiological partners.

**Experimental Procedures**

Preparation and Characterization of [H48F]-ySOD—The His-48 to Phe mutant was created in an expression vector for wild-type ySOD1 (WT ySOD1-pET3d) (34) by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The mutagenic primers 5'-CGTGGGTTCACATTTTGAGATGCC-3' and its reverse complement were used to create the mutation. Mutagenesis was confirmed by sequencing of the plasmid (ABI Prism). The plasmid was transformed into Escherichia coli strain BL21(DE3) cells, which were grown in Luria-Bertani medium with 100 μg/ml carbenicillin to an OD600 of 0.8 and then induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside for 2.5 h. Protein was extracted from the cell pellet by three cycles of freeze-thaw lysis into 2.5 mM K2HPO4, pH 7.8. The extract was treated with a combination of gel filtration chromatography on a pre-scale Superdex 75 column (Amersham Pharmacia Biotech) and anion exchange chromatography on a DEAE column (Pharmacia) with a gradient of 2.5 mM K2HPO4, pH 7.8 to 50 mM K2HPO4, 500 mM NaCl, pH 7.8 yielding ~50 mg of [H48F]-ySOD1 per liter of Luria-Bertani medium. Purification of [H48F]-ySOD1 (>95% by SDS-PAGE gel) and protein identity were confirmed by MALDI-TOF mass spectrometry with an observed mass of 15,733.2 Da (calculated mass: 15,732.5 Da) indicating processing of the N-terminal methionine.

The concentration of a stock solution of E.Zn-[H48F]-ySOD1 monomer (10 μM) was determined using a calculated extinction coefficient of ε280 = 1400 (mM•cm)⁻¹ from the Gill and von Hippel equation (35). The low absorbance at 280 nm of the purchased forms of ySOD1 in a Vydac C18 column using a gradient of water, 0.1% trifluoroacetic acid and acetonitrile, 0.1% trifluoroacetic acid. Peak fractions were combined, dried under vacuum, and resuspended in deionized water to be used in the inoculum. The identity and purity of the proteins were verified by MALDI-TOF mass spectrometry. The HPLC-purified yCCS and ySOD1 were used as antigens for polyclonal antibodies raised in rabbit (Bioscience International).

Immunoprecipitation Reactions—Immunoprecipitation reactions were performed by combining 50 μl of 100 μM apo- or Cu-yCCS with 50 μl of 100 μM H48F or apo-[WT]ySOD1 for 1 h on ice in buffer containing 50 mM Tris/ MES, pH 8.0. 1 μl of primary antibody for yCCS was added to the reaction and the sample was incubated for 30 min on ice. Pre-washed protein A-Sepharose beads were then added, and the mixture was incubated for additional 30 min on ice. The mixture was centrifuged for 5 min at 10,000 × g, the beads were washed with 100 μl 1× phosphate-buffered saline, then centrifuged for 5 min at 10,000 × g three times. The beads were boiled for 10 min in SDS-PAGE sample buffer containing SDS and β-mercaptoethanol. The mixture was then centrifuged for 5 min and run on a 12% Tris-denaturing SDS-PAGE gel. Proteins were transferred from gel to polyvinylidene difluoride membrane (Bio-Rad), blocked overnight with 5% nonfat dry milk, and analyzed by Western blot using ySOD1 polyclonal antibody as the primary antibody (1:8000 dilution) and goat anti-rabbit IgG HRP conjugate (Bio-Rad) as the secondary antibody (1:8000 dilution). The blot was developed using the Hybond ECL kit (Amersham Pharmacia Biotech).

Detection of Heterodimeric E.Zn-SOD1 with Apo-Copper Chaperone Complexes—Detection of the E.Zn-ySOD1, full-length wild-type ySOD1 and yCCS were incubated with 150 μl of 100 μM H48FySOD1 on ice for 0, 30, and 60 min. Reactions were analyzed by nondenaturing gel electrophoresis on 12% Tris-HCl precast native gels (Bio-Rad). 5 μl of each reaction mixture was combined with native PAGE sample buffer containing (tris, EGTA, Coomassie dye, and glycerol), and the electrophoresis was run for 1 h at 200 V.

The above reaction mixtures (250 μl) were also analyzed on a Superose 12 analytical gel filtration column (Amersham Pharmacia Biotech). Running buffer was Chelex-treated and consisted of 50 mM Tris/MES, 100 mM NaCl, pH 8.0. Gel filtration standards included 100 μM bovine serum albumin (mass, 66.3 kDa; elution volume, 12.3 ml), ovalbumin (mass, 42.9 kDa; elution volume, 13.4 ml), holo-hSOD1 (mass, 31.6 kDa; elution volume, 14.5 ml) and carbonic anhydrase (mass, 29.0 kDa; elution volume, 14.6 ml). Gel filtration of Cu-yCCS, apo-yCCS, and [H48F]-ySOD1 was performed using 250 μl of 100 μM protein. Absorbance in all cases was monitored at 254 nm due to the low extinction coefficient at 280 nm of [H48F]-ySOD1.

Detection of heterodimeric complexes—Complexes isolated by gel filtration, as described above, were analyzed relative to yCCS and H48F alone by native PAGE gel stained by Coomassie dye. Western blot of the native PAGE gel after transfer to polyvinylidene difluoride membrane and overnight blocking in 5% nonfat dry milk was performed using yCCS polyclonal antibody as the primary antibody (1.8000 dilution) and goat anti-rabbit IgG HRP conjugate as the secondary antibody (1.8000 dilution). The membrane was then stripped at 50 °C using 2% SDS, 100 mM N-mercaptoethanol, and 62.5 mM Tris-HCl as the stripping buffer, and re-blotted using ySOD1 polyclonal antibody as the primary antibody (1.8000 dilution) and goat anti-rabbit IgG HRP conjugate as the secondary antibody (1.8000 dilution).

Complexes isolated by gel filtration were analyzed by MALDI-TOF mass spectrometry. For MALDI-TOF, a sample of ~10 μM protein complex was diluted 1:4 into a solution of 30% CH3CN and 0.3% trifluoroacetic acid in water with sinapinic acid matrix (10 mg/ml). A myoglobin internal mass calibration standard was also added to this mixture prior to application on the MALDI-TOF sample plate.

Labeling of E.Zn-[H48F]-ySOD—The maleimide derivative of coumaryl (CFM) and the reducing agent tri(2-carboxyethyl)phosphine (TCEP) were purchased from Molecular Probes. Purified [H48F]-ySOD1 was reduced overnight with TCEP in Tris, pH 7.5 and 1.0 mM NaCl. CPM was added from a freshly prepared stock solution in Me2SO. The reaction proceeded at room temperature for 45 min. The mixture was dialyzed against buffer MES pH 6.0, 150 mM NaCl, 25 μM TCEP at 4 °C. Labeled protein concentration was determined using the NanoOrange kit from Molecular Probes. The labeling ratio of dye to protein (LR), under these conditions, is no more than 0.5, indicating that on the average only one of the two cysteines of the monomer is labeled. Equilibrium sedimentation studies on a Beckman XL A analytical ultracentrifuge equipped with UV absorbance optics conducted on 18,000 and 22,000 rpm; scans were taken at 280 nm.

Fluorescence Anisotropy—Fluorescence measurements were made on an ISS PC1 spectrofluorometer equipped with a 300 watt UV Xenon arc lamp. The absorption and emission wavelengths were 384 and 469, and the bandwidths were 8 and 16 nm, respectively. The experiments were conducted in 1.5 ml of MES pH 6, 150 mM NaCl, 1 mM dithiothreitol, 25 μM TCEP, 25 °C. CPM-labeled [H48F]-ySOD (10–12 nm) was titrated with apo-yCCS or Cu-yCCS and the anisotropy, using the T format, and total fluorescence intensities were measured at each protein concentration. CuSO4 or ZnSO4 was added to the SOD1 aliquot to a final concentration of 12 nM, which readily loads CCS under these conditions. Data were fit with Origin 4.1 software package from MICROCAL™ Software, INC. using the following binding expression in Equation 1,

\[ A = A_0 + (A_{\infty} - A_0) \frac{[K]}{1 + [K][I]} \]  

(1)
with the immunoprecipitation results shown in Fig. 1, we find results, the new complex appears in reaction mixtures containing [H48F]ySOD1 (Fig. 2). As with the immunoprecipitation reaction between CCS and SOD. Reactions were probed by Cu-yCCS. Immunoprecipitation assays using yCCS primary antibody shows no cross-reactivity of yCCS with ySOD1 primary antibody.

FIG. 1. [H48FlySOD1 forms a stable complex with apo- and Cu-yCCS. Immunoprecipitation results shown in Fig. 1, we find the new complex appears in reaction mixtures containing [H48F]ySOD1 (Fig. 2). As with the immunoprecipitation reaction between CCS and SOD. Reactions were probed by Cu-yCCS. Immunoprecipitation assays using yCCS primary antibody shows no cross-reactivity of yCCS with ySOD1 primary antibody.

RESULTS

Mutation of the copper binding histidine 48 to phenylalanine in ySOD1 disrupts the native copper binding site but, as anticipated, does not disrupt zinc binding. Under conditions where the wild-type protein binds both copper and zinc, the [H48FlySOD1 binds 1.02 mol of Zn(II) per mol of monomer and essentially no copper (<0.0015 mol Cu per mol monomer). This mutant thus cannot fully accept a copper ion in the native catalytic binding site and was used to trap a chaperone/enzyme complex.

Immunoprecipitation of a Heterocomplex—Immunoprecipitation experiments reveal that yCCS readily forms a complex with [H48F]SOD1 (Fig. 1). Under these conditions apo-[wt]-ySOD1 is not captured (data not shown). The sequence of binding and release reactions that lead to activation of the wild-type ySOD1 is potentially too rapid to be detected in this assay. Western blots using ySOD1 primary antibody show strong interaction of yCCS with [H48F]ySOD1 despite repeated washing with high salt buffer (1× phosphate-buffered saline). Apo-yCCS forms an apparently weaker complex indicated by a less intense band for [H48F]ySOD1 (Fig. 1). To characterize this complex, methods to isolate it from the individual proteins were established.

Detection of the Heterocomplex in the Native Gel and the Effect of Copper upon its Formation—A time course of the reaction between yCCS and [H48F]SOD1 reveals that a new band in native gels appears in less than 30 min of exposure to air at 4°C. No complex is observed if the reaction is performed and analyzed in the anaerobic chamber for as long as 24 h. This exposure to oxygen may stabilize either CCS or SOD1 via disulfide bond formation.2 The new band in the native gel exhibits an intermediate mobility between yCCS and [H48F]ySOD1 (Fig. 2A). As with the immunoprecipitation results, the new complex appears in reaction mixtures containing the copper-loaded or apo-form of the chaperone. Also in accord with the immunoprecipitation results shown in Fig. 1, we find the new band is more intense in the Cu-yCCS reaction than in the apo-yCCS case, again indicating that copper favors heterocomplex formation (Fig. 2A).

Analytical Gel Filtration Evidence for the Heterodimer—Removing metal from the wild-type SOD1 active site is known to weaken SOD1 homodimer stability (31). The purified reactants were thus examined by analytical gel filtration monitored at 254 nm to test whether the [H48F]SOD1 is a dimer or monomer under these conditions. In the previous study, SOD1 was not detected when fractions were monitored at 280 nm (33); however, it is readily observable at 254 nm. The elution volume of [H48F]ySOD1 was 14.5 ml and corresponds to an apparent mass of 32 kDa on the calibrated Superose 12 column (Fig. 2B). This indicates that the protein is predominately a dimer under these conditions. Similar analysis reveals the apo-yCCS is a monomer while the Cu(I)-loaded form exhibits both monomeric and dimeric states, consistent with previous observations (19).

Analytical gel filtration analysis of equimolar reaction mixtures (1:1 CCS/SOD1) reveals a new species with an apparent mass of 41 ± 2 kDa (Fig. 2B). This is within experimental error of the theoretical mass for a heterodimer containing yCCS and [H48F]ySOD1 (42.9 kDa); however, changes in metal occupancy or conformation of one of the proteins in the sample could lead to hydrodynamic changes that appear as a larger mass. The new peak fractions were therefore examined for protein and metal content by additional methods.

Characterization of the Heterocomplex—To further delineate protein content, all fractions from the gel filtration profiles were also analyzed by Western blot for SOD1 and by Bradford assay for total protein (Fig. 3). When [H48F]ySOD1/CyCCS mixtures were injected on the column, only fractions 26–33 contained protein; and, when [H48F]ySOD1 was injected alone, only fractions 30–32 contained protein. Western analysis of all fractions using ySOD1 primary antibody provides a sensitive test of whether larger oligomers such as heterotetramers are present in the early fractions. SOD1 is detected in earlier fractions (26–30 corresponding to mass range 50–30 kDa), but none was detected in fractions that would contain species larger than 66 kDa, ruling out the possibility that the observed complex corresponds to a heterotetramer (theoretical mass, 85.8 kDa). The mixture of apo-[H48F]ySOD1 and Cu-yCCS also elutes in fractions corresponding to 50–30 kDa. These shifts in elution volume are evidence of a [H48F]ySOD1 heterodimer with yCCS.

The fraction exhibiting the maximum absorbance for both mixtures and corresponding to a mass of 41 kDa (fraction 28) was further analyzed by nondenaturing native gel electrophoresis and Western blot using both yCCS and ySOD1 primary antibodies (Fig. 4). The intermediate band observed for fraction 28 (Fig. 4, first panel) has the same relative mobility as the band detected in the time course (Fig. 2A). Western blots demonstrate that after separation by gel filtration and native gel electrophoresis, the complex still contains both yCCS and ySOD1 (Fig. 4).

In the equimolar reaction mixtures, Cu-yCCS begins with approximately one copper atom per monomer and [H48F]SOD1 begins with approximately one zinc atom per monomer. Therefore, an intact heterodimer or heterotetramer should exhibit a 1:1 ratio of these elements. Other heterocomplexes (such as CuSOD1 to CCS, etc.) would exhibit different Cu/Zn ratios. To test this, the metal content of a dilute sample of the isolated heterocomplex (fraction 28) was directly determined by ICP-AES revealing a copper/zinc ratio of 0.93:1 (or concentrations of 0.28 and 0.30 μM, respectively). This result, coupled with the absence of higher mass aggregates by immunoblot analysis of fraction 28, supports the heterodimer assignment. Since yCCS does not bind

Zn(II), this metal remains bound in the [H48F]ySOD1 component of the heterodimer, but copper may be coordinated by side chains from either CCS or CCS and SOD1.

Copper Effect on the Energetics of CCS/SOD1 Interactions—The CPM-labeled SOD1 was titrated with unlabeled metallochaperone, and the fluorescence anisotropy change at each point was measured (Fig. 5). Equilibrium sedimentation experiments established that the CPM label did not alter the dimerization of SOD1 itself at concentrations $\geq 20 \mu M$ and that higher order oligomers were not observed unless $[SOD1]$ exceeded 40 $\mu M$. Thus under conditions of the anisotropy experiment (0.01 $M$), the labeled protein did not aggregate beyond a dimer state.

The anisotropy of labeled [H48F]ySOD1 increases as a function of added CCS, indicating the formation of a larger, more slowly rotating species in solution (Fig. 5). In the presence of copper the binding constant for CCS increases by approximately one order of magnitude relative to the value obtained in the presence of zinc or no metal at all (Table I). This is consistent with the results from immunoprecipitation and native gel experiments and as discussed below, provides a thermodynamic measure of the effect of copper on complex formation.

**DISCUSSION**

Two models for docking of the yeast CCS with its partner ySOD1 have been proposed. In one scenario, metal transfer occurs within a docked heterotetramer consisting of a chaperone homodimer and a SOD1 homodimer (20–22). In the other model, metal transfer is proposed to occur within a heterodimer made up of chaperone and SOD1 monomers (12, 20, 21). The data presented here provide new evidence for a heterodimer intermediate and show that copper stabilizes this interaction. This suggests a mechanism for the transfer of copper from yeast CCS to its target within a heterodimeric intermediate.

By changing the copper binding residue His-48 to another aromatic residue that could occupy the vacant metal site, we have captured a heterocomplex at a stage before release of the active SOD1. It may be that the off-rate of the chaperone from [H48F]ySOD1 is slower than from wild-type ySOD1, allowing...
for accumulation of the stable heterodimer in the former but not the latter case. An important issue here is that while the mutation clearly diminishes the ability of SOD1 to bind copper it does not impair the capacity of the protein for binding zinc.

Zinc has been shown to be a key stabilizing factor in the SOD1 structure. The E,E form of bSOD1 was shown to fold in a native-like conformation upon binding of zinc (29, 30). Immunoprecipitation, native gel PAGE, and analytical gel filtration reveal the presence of a heterodimer and suggest that when the copper ion is present there is a greater yield of this species. Under these conditions the heterodimer has a Cu/Zn mole ratio of 0.93:1. Fluorescence anisotropy (Table I) indicates that the two proteins interact in solution; however, in the presence of copper the binding strength is enhanced by one order of magnitude. Under the conditions employed in this study, the apo-γCCS protein does not readily form dimers (Fig. 2B), but monomeric and dimeric forms of the copper-loaded protein are readily observed, consistent with observations from previous studies (19). These results indicate that the driving force for interaction of the metallochaperone with its target is dependent upon the presence of the cargo metal.

Hall et al. (22) have proposed a key role for a heterotetramer in the metal transfer step and delineate several concerns with a model in which the heterodimeric intermediate employs the highly conserved dimer interfaces of γCCS and γSOD1. Two key arguments against the heterodimer model have been cited: 1) the γSOD1 dimer is too stable to allow a heterodimer formation; and 2) that in a heterodimer, the essential CXXC motif would be too far from the active site residues of SOD1 to allow direct metal transfer without extensive conformational changes. With respect to the first argument the results shown here indicate that E,Zn-γSOD1 need not remain in a dimeric state upon encountering the chaperone. A number of dimeric systems that are quite stable can nonetheless undergo rapid monomer exchange (12, 39).

The distance argument against the heterodimer is ameliorated if Domain III is flexible and can undergo a hinge-like motion (12). In fact, recent proteolysis and MALDI-TOF studies of γCCS (19) and hCCS (12) indicate that Domain III is quite accessible in the apo state and copper induces significant conformational changes. In both human and yeast CCS, Cu(I) binding leads to protection of Domain III against proteolysis. In hCCS, the protected region includes the putative metal binding residues in the CXC motif (12). Using the crystal structure of γyCCS determined by Rosenzweig and co-workers (20) as a

FIG. 3. Addition of Cu- or apo-γCCS to [H48F]γSOD1 shifts the elution volume of mutant SOD1. Gel filtration profiles and Western blots using ySOD1 primary antibody of all protein-containing gel filtration fractions from a 60-min incubation of apo- and Cu-γCCS with [H48F]ySOD1 (fractions 25–32) and from [H48F]ySOD1 alone. Heterodimer-containing fractions were primarily found in fractions 27–29. A trace amount of oxidized heterodimer remained intact in the apo-γCCS/[H48F]γSOD1 reaction (fractions 27–29) and is apparent as a higher molecular mass species in the Western blot. Fraction 28 represents the bulk of the heterodimer and was used in subsequent characterization. No larger oligomers containing [H48F]γSOD1 are detected beyond fraction 26, and no protein is detected in earlier fractions by Bradford assay.

FIG. 4. Isolation and characterization of the heterodimer of γyCCS and [H48F]γySOD1. Coomassie-stained nondenaturing 12% Tris-HCl gel of isolated heterodimer, Cu-γCCS and [H48F]γySOD1 (left) and Western blots using γCCS (middle) and γSOD1 (right) primary antibodies. Isolated heterocomplex from gel filtration fraction 28 shows immunoreactivity with both γCCS and ySOD1.

FIG. 5. Fluorescence anisotropy titration of CPM-labeled [H48F]γSOD with apo- and Cu-γCCS. Apo-CCS (open diamonds), Cu-CCS (filled diamonds) generated in situ by adding CuSO₄ to the strongly reducing buffer (1 mM dithiothreitol/25 μM TCEP) as described. The solid lines are the best fit theoretical curves with the following refined values: Aᵣ = 0.074 ± 0.0065, A₀ = 0.094 ± 0.0063, and Kₑₒₜₘₜₑₜₖₐₜ = 2.0 (± 0.4) × 10⁶ for apo-CCS; and with Aᵣ = 0.074 ± 0.0066, A₀ = 0.095 ± 0.0005, and Kₑₒₜₘₜₑₜₖₐₜ = 1.8 (± 0.3) × 10⁷ for Cu-CCS.
starting point for building heterodimer model, we find that Domain III is long enough to span the predicted distances. The distance of the last visible residue in Domain III (Trp-222) to the closest His in SOD1 is ca. 25 Å. Other heterodimer models give a similar result (23). Since an extended peptide from Trp-222 to the sulfur atoms of Cys-229 or Cys-231 is between 27 and 34 Å in length, it is clearly possible for the metal binding motif of Domain III peptide to reach the SOD1 active site. Also, the distance from Trp-222 to the CXXC motif in Domain I is 20 Å indicating that the Domain III CXC site can also reach the putative metal binding site in Domain I.

While these arguments suggest a specific Cu-bridged heterodimer intermediate, the data presented here indicate that Cu(I) is not required for chaperone/SOD1 interaction. These findings corroborate the in vivo two-hybrid studies of Culotta and co-workers (26) where CCS/SOD1 interactions are found under both copper replete and copper starvation conditions. Interestingly these studies further indicate that Domain III is required for CCS/SOD1 interaction in vivo. A precedent for this Domain III role can be seen in the crystal structure of another metal-handling protein, mercuric ion reductase (12, 40, 41), where a metal-binding C-terminal peptide extends across a dimerization interface into an adjacent active site. These considerations provide further support for a heterodimer intermediate in the metal transfer step. It is possible that initially a heterotetramer encounters species forms and subsequently rearranges from a dimer of homodimers to a dimer of heterodimers that are docked via the conserved dimerization interface, as suggested for hCCS activation of hSOD1(12).

A model in which the Domain III cysteines project into the active site of SOD1 can also account for the observed copper stabilization of the heterodimer. A copper ion could potentially be bound simultaneously to Cys thiols in CCS and one or more of the remaining His nitrogens in SOD1. In this case the coordinate-covalent Cu–S and –N bonds would provide some of the stabilization of the protein-protein complex. Alternatively, the copper-induced conformational change in CCS may have an allosteric effect on its interaction with SOD1. The oxidation state of the copper and of the remaining Cys side chains remain an open issue and are under evaluation. The fact that the apo wild-type protein and thereby will play a protective role in the activation of hSOD1 (12). The fact that yCCS binds tightly to a mutant ySOD1 plasmid, and R. Pufahl for yCCS antibody preparation. We also thank A. Rosenzweig and A. Lamb for helpful discussions and critical reading of the manuscript. MALDI-TOF instrumentation was purchased with National Institutes of Health support (S10RR13810) and the use of instrumentation in the Keck Biophysical Facility was supported by an NCI grant to the Robert H. Lurie Comprehensive Cancer Center.

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REFERENCES

Table I

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<td>87 nm CuSO_4</td>
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The concentration of labeled [H48F]SOD1 is 10–12 nm.
Copper Chaperone Complex with Superoxide Dismutase

Copper Stabilizes a Heterodimer of the yCCS Metallochaperone and Its Target Superoxide Dismutase
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