

Copper Stabilizes a Heterodimer of the γ CCS 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 *in vivo* (9, 11). Both the yeast (4) and human (12) forms of copper-loaded CCS activate SOD1 by directly inserting the copper cofactor *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 *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 γ CCS (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 γ CCS are required for contact with SOD1 *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 γ CCS 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 γ CCS (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

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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.

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 between these physiological partners.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of [H48F]ySOD1—The His-48 to Phe mutant was created in an expression vector for wild-type ySOD1 (WT ySOD1-pET 3d) (34) by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutagenic primers 5'-CGTGGGTTCACATTTT-TGATATGCC-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 μ M carbenicillin to $A_{600} = 0.8$ and then induced with 500 μ M 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 K_2HPO_4 , pH 7.8. The extract was treated with a combination of gel filtration chromatography on a prep scale Superdex 75 column (Amersham Pharmacia Biotech) and anion exchange chromatography on a DEAE column with a gradient of 2.5 mM K_2HPO_4 , pH 7.8 to 50 mM K_2HPO_4 , 500 mM NaCl, pH 7.8 yielding ~50 mg of [H48F]ySOD1 per liter of Luria-Bertani medium. Both the purity of [H48F]ySOD1 (>95% by SDS-PAGE gel) and protein identity were confirmed by MALDI-TOF mass spectroscopy 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 (10.0 μ M) was determined using a calculated extinction coefficient of $\epsilon_{280} = 1400 \text{ M}(\text{monomer})^{-1} \text{ cm}^{-1}$ from the Gill and von Hippel equation (35). The low absorbance at 280 nm arises from a single tyrosine residue in [H48F]ySOD1 with a minor contribution from two cysteines that are assumed to be bridged by a disulfide bond. The concentration of the same stock solution was determined to be 10.5 μ M by Bradford assay using IgG standard. Consequently, routine SOD1 concentrations were determined using values from the IgG/Bio-Rad assay multiplied by a factor of 0.95 (10.0 μ M/10.5 μ M). Purified [H48F]ySOD1 protein (10 μ M) was analyzed by ICP-AES for Cu and Zn and was also screened for SOD activity by standard native PAGE NBT assay (36). Analysis of 9.5 μ M purified [H48F]ySOD1 by ICP-AES revealed 9.7 μ M zinc and essentially no copper (less than 15 nM).

Preparation of Primary Antibodies—To prepare antibodies specific for yCCS and ySOD1, full-length wild-type ySOD1 and yCCS were purified as previously reported and then further purified by HPLC on a Vydac C_4 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 μ l of 100 μ M apo- or Cu-yCCS with 50 μ l of 100 μ M H48F or apo-[WT]ySOD1 for 1 h on ice in buffer containing 50 mM Tris/MES, pH 8.0. 1 μ l 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 an additional 30 min on ice. The mixture was centrifuged for 5 min at $10,000 \times g$. The beads were washed with 100 μ l $1 \times$ phosphate-buffered saline, then centrifuged for 5 min at $10,000 \times g$ 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 mem-

brane (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 (BioRad) as the secondary antibody (1:8000 dilution). The blot was developed using the Hybond ECL kit (Amersham Pharmacia Biotech).

Detection of Heterocomplex by Nondenaturing Gel Electrophoresis and Analytical Gel Filtration—150 μ l of 100 μ M Cu or apo-yCCS were incubated with 150 μ l of 100 μ M [H48F]ySOD1 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 μ l of each reaction mixture was combined with native PAGE sample buffer (containing Tris, EDTA, Coomassie dye, and glycerol), and the electrophoresis was run for 1 h at 200 V.

The above reaction mixtures (250 μ l) 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 μ M bovine serum albumin (mass, 66.3 kDa; elution volume, 12.3 ml), ovalbumin (mass, 42.8 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 μ l of 100 μ M protein. Absorbance in all cases was monitored at 254 nm due to the low extinction coefficient at 280 nm of [H48F]ySOD1.

Characterization of the Heterocomplex—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 $^{\circ}$ C using 2% SDS, 100 mM β -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 spectroscopy and ICP-AES. For MALDI-TOF, a sample of ~10 μ M protein complex was diluted 1:4 into a solution of 30% CH_3CN 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 coumarin (CPM) and the reducing agent tris-(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 M NaCl. CPM was added from a freshly prepared stock solution in Me_2SO . The reaction proceeded at room temperature for 45 min. The mixture was dialyzed against buffer MES pH 6.0, 150 mM NaCl, 25 μ M TCEP at 4 $^{\circ}$ 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 18–20 μ M samples of CPM-labeled and unlabeled [H48F]ySOD revealed average masses of 27.4 ± 1.5 and 29.2 ± 5.7 kDa respectively, indicating that neither the mutation nor the labeling causes aggregation beyond the dimer state (data not shown). A four-hole AN-60 rotor was used at 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 μ M TCEP, 25 $^{\circ}$ 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. $CuSO_4$ or $ZnSO_4$ was added to the SOD1 aliquot to a final concentration of 87 nM in the strongly reducing buffer. This condition leads to quantitative formation of Cu(I), which readily loads CCS under these conditions (4). In all the experiments the system came to equilibrium within min, and the anisotropy was subsequently measured for 30 and 60 s several times at each point and averaged for the final analyses. The data were fit with Origin 4.1 software package from MICROCALTM Software, INC. using the following binding expression in Equation 1,

$$A = A_f + (A_b - A_f) [K(x)/(1 + K(x))] \quad (\text{Eq. 1})$$

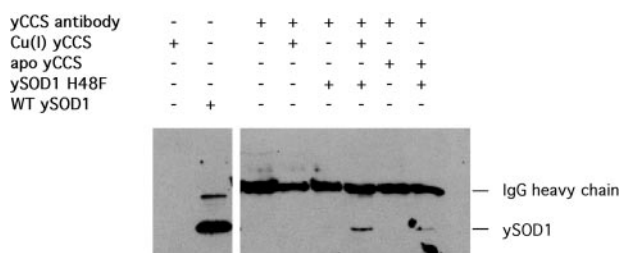


FIG. 1. [H48F]ySOD1 forms a stable complex with apo- and Cu-yCCS. Immunoprecipitation assays using yCCS primary antibody reveal interaction between CCS and SOD. Reactions were probed by Western blots with ySOD antibodies. Cu-yCCS and to a lesser extent apo-yCCS pull down [H48F]ySOD1 (lanes 6 and 8) consistent with heterocomplex formation. Controls of yCCS (lane 1) and ySOD1 (lane 2) show no cross-reactivity of yCCS with ySOD1 primary antibody.

where A is the measured anisotropy, A_f and A_b are the anisotropies of the free and bound fluorescent molecule CPM-[H48F]SOD1, X is the concentration of added CCS, and K is the observed equilibrium binding constant. The K , A_f , and A_b were the fitted parameters in the analyses. Values for A_b and A_f were found to be ~ 0.09 and 0.07 for all runs. Although small (~ 0.02 units), this increase in anisotropy is sufficient for analysis of binding reactions (37). No copper-dependent quenching of the CPM moiety was observed in the fluorescence intensity, which decreased by $\sim 20\%$ over the course of the titration of both the apo and copper cases.

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 [H48F]ySOD1 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 are 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 ($1\times$ 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 predominantly 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).

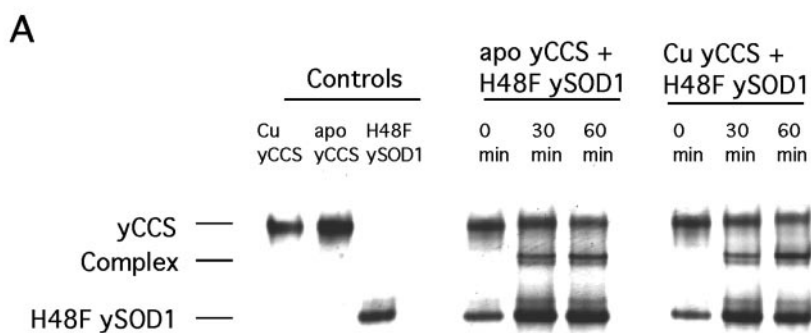
Analytical gel filtration analysis of equimolar reaction mixtures (1:1 CCS/SOD1) reveals a new species with an apparent mass of 41 ± 2 kDa (Fig. 2B). This is within experimental error of the theoretical mass for a heterodimer containing yCCS and [H48F]ySOD1 (42.9 kDa); however, changes in metal occupancy 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/yCCS 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).

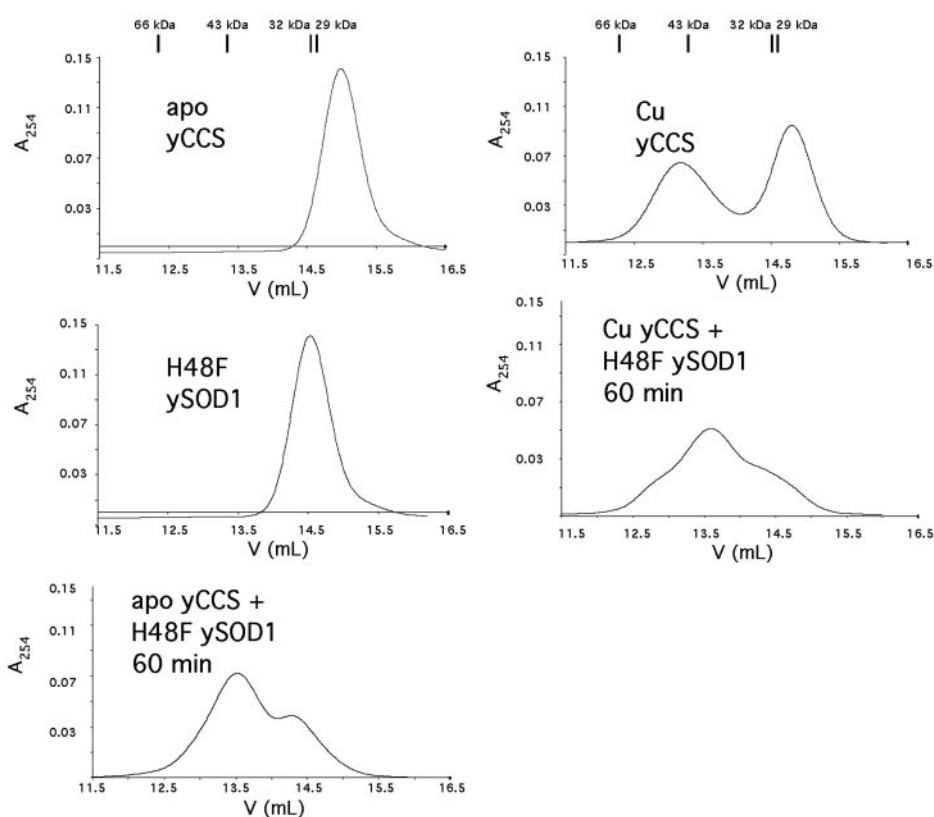
In the equimolar reaction mixtures, Cu-yCCS begins with approximately one copper atom per monomer and [H48F]ySOD1 begins with approximately one zinc atom per monomer. Therefore, an intact heterodimer or heterotetramer should exhibit a 1:1 ratio of these elements. Other heterocomplexes (such as 2:1 SOD1 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

² A. S. Torres, N. Brown, L. E. Lemont, and T. V. O'Halloran (2001), manuscript in preparation.



B

FIG. 2. Time course of complex formation and detection of a heterodimer of Cu-yCCS and [H48F]ySOD1. A, controls of 5 μ l of 100 μ M Cu-yCCS, apo-yCCS, and [H48F]ySOD1 were run in a 12% Tris-HCl nondenaturing gel (left) and compared with a time course of complex formation with either apo-yCCS (middle) or Cu-yCCS (right) and [H48F]ySOD1. Time points were taken at 0, 30, and 60 min. B, gel filtration profiles of 100 μ M of apo-yCCS, Cu-yCCS, and [H48F]ySOD1 alone and the resulting chromatograms after a 60-min incubation of either apo-yCCS (left) or Cu-yCCS (right) and [H48F]ySOD1. The new peak detected elutes at \sim 13.6 ml. This corresponds to a calculated mass of 41 kDa.



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 metal-chaperone, 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 μ M and that higher order oligomers were not observed unless [SOD1] exceeded 40 μ M. Thus under conditions of the anisotropy experiment (0.01 μ 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

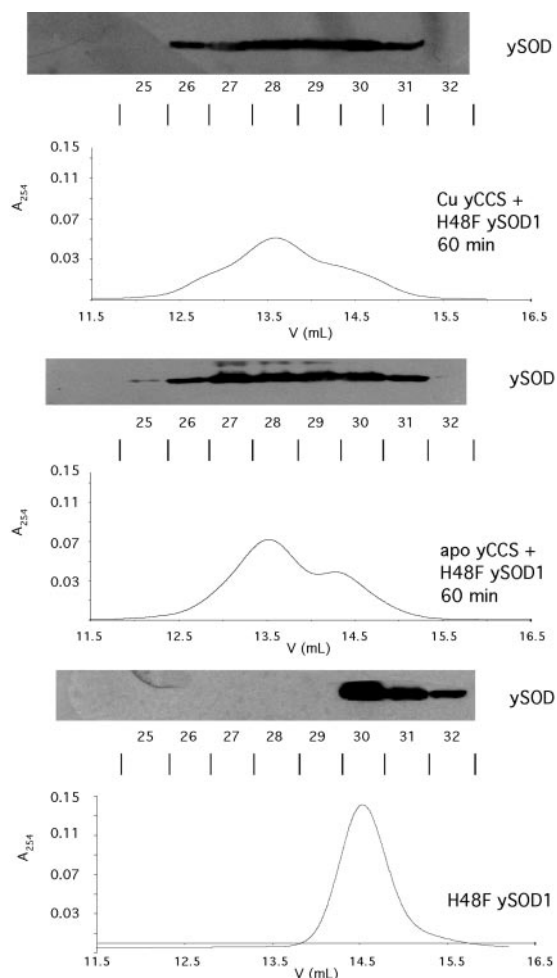


FIG. 3. Addition of Cu- or apo-yCCS to [H48F]ySOD1 shifts the elution volume of mutant SOD1. Gel filtration profiles and Western blots using ySOD1 primary antibody of all protein-containing gel filtration fractions from a 60-min incubation of apo- and Cu-yCCS with [H48F]ySOD1 (fractions 25–32) and from [H48F]ySOD1 alone. Heterodimer-containing fractions were primarily found in fractions 27–29. A trace amount of oxidized heterodimer remained intact in the apo-yCCS/[H48F]ySOD1 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]ySOD1 are detected beyond fraction 26, and no protein is detected in earlier fractions by Bradford assay.

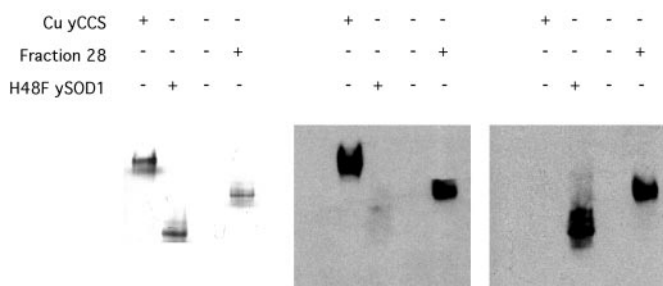


FIG. 4. Isolation and characterization of the heterodimer of yCCS and [H48F]ySOD1. Coomassie-stained nondenaturing 12% Tris-HCl gel of isolated heterodimer, Cu-yCCS and [H48F]ySOD1 (left) and Western blots using yCCS (middle) and ySOD1 (right) primary antibodies. Isolated heterocomplex from gel filtration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

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.

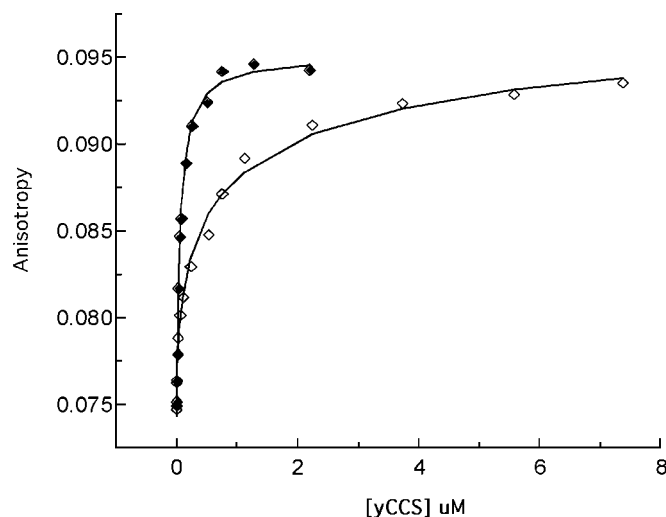


FIG. 5. Fluorescence anisotropy titration of CPM-labeled [H48F]ySOD1 with apo- and Cu-yCCS. Apo-CCS (open diamonds), Cu-CCS (filled diamonds) generated *in situ* by adding CuSO_4 to the strongly reducing buffer (1 mM dithiothreitol/25 μM TCEP) as described. The solid lines are the best fit theoretical curves with the following refined values $A_f = 0.074 \pm 0.0005$, $A_b = 0.094 \pm 0.00063$, and $K_{\text{eq,obs}} = 2.0 (\pm 0.4) \times 10^6$ for apo-CCS; and with $A_f = 0.074 \pm 0.0006$, $A_b = 0.095 \pm 0.0005$, and $K_{\text{eq,obs}} = 1.8 (\pm 0.3) \times 10^7$ for Cu-CCS.

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-yCCS 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 yCCS and ySOD1. Two key arguments against the heterodimer model have been cited: 1) the ySOD1 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-ySOD1 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 yCCS (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 yCCS determined by Rosenzweig and co-workers (20) as a

TABLE I
Summary of fluorescence anisotropy titration results

Metal ^a	$K_{eq, obs}$	A_r	A_b	χ^2
—	$2.0 (\pm 0.4) \times 10^6$	0.074 ± 0.0005	0.094 ± 0.0006	8.3×10^{-7}
87 nM ZnSO ₄	$3.4 (\pm 1.2) \times 10^6$	0.075 ± 0.0006	0.092 ± 0.0008	1.6×10^{-6}
87 nM CuSO ₄	$1.8 (\pm 0.3) \times 10^7$	0.074 ± 0.0006	0.095 ± 0.0005	5.0×10^{-7}

^a The concentration of labeled [H48F]SOD1 is 10–12 nM.

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 those 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 proteins must be briefly exposed to oxygen before complex formation is observed suggests that oxidation of cysteine to cystine is required in either SOD1 or CCS.

In a recent study of [H48F]ySOD1 and yCCS (33), we provided other biophysical evidence for a SOD1/CCS heterodimer and showed that added ZnSO₄ is required. There is little effect of added Zn(II) in the current study because the E,Zn-SOD1 form is employed here whereas the E,E-SOD1 form was used in the previous study. The addition of ZnSO₄ to the latter produces the E,Zn-SOD1, which most likely stabilizes the structure of SOD1 enough to allow docking with CCS. Thus the new data presented here lead to the same conclusion but now reveal a significant energetic effect of copper on the formation of the SOD1-CCS heterodimer.

Finally, it is becoming apparent that the functions of CCS may extend beyond partner recognition and copper transfer. Recent studies in the hCCS/hSOD1 system reveal that the human copper chaperone may also serve a folding function in

the activation of hSOD1 (12). The fact that yCCS binds tightly to a mutant ySOD1 raises the possibility that the chaperone will interact with damaged or other mutated forms of the wild-type protein and thereby will play a protective role in the cell. Mutations in SOD1, including one in a copper-binding side chain, lead to severe neurological disorders in patients afflicted with familial amyotrophic lateral sclerosis (FALS) and in mouse model systems (42, 38). If the cellular activity or aggregation state of the chaperones are adversely affected by mutations in SOD1, this entire branch of the copper trafficking network may be altered. Such mechanisms should be considered while the molecular basis of this disease is explored.

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