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Thomas J. Lyons · Aram Nersissian · Huijuan Huang
Hyeyeong Yeom · Clinton R. Nishida
Janet A. Graden · Edith Butler Gralla
Joan Selverstone Valentine

The metal binding properties of the zinc site of yeast copper-zinc superoxide dismutase: implications for amyotrophic lateral sclerosis

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Abstract We have investigated factors that influence the properties of the zinc binding site in yeast copper-zinc superoxide dismutase (CuZnSOD). The properties of yeast CuZnSOD are essentially invariant from pH 5 to pH 9. However, below this pH range there is a change in the nature of the zinc binding site which can be interpreted as either (1) a change in metal binding affinity from strong to weak, (2) the expulsion of the metal bound at this site, or (3) a transition from a normal distorted tetrahedral ligand orientation to a more symmetric arrangement of ligands. This change is strongly reminiscent of a similar pH-induced transition seen for the bovine protein and, based on the data presented herein, is proposed to be a property that is conserved among CuZnSODs. The transition demonstrated for the yeast protein is not only sensitive to the pH of the buffering solution but also to the occupancy and redox status of the adjacent copper binding site. Furthermore, we have investigated the effect of single site mutations on the pH- and redox-sensitivity of Co^{2+} binding at the zinc site. Each of the mutants H46R, H48Q, H63A, H63E, H80C, G85R, and D83H is capable of binding Co^{2+} to a zinc site with a distorted tetrahedral geometry similar to that of wild-type. However, they do so only if Cu^{+} is bound at the copper site or if the pH in raised to near physiological levels, indicating that the change at the zinc binding site seen in the wild-type is conserved in the mutants, albeit with an altered pK_a. The mutants H71C and D83A did not bind Co^{2+} in a wild-type-like fashion under any of the conditions tested. This study reveals that the zinc binding site is exquisitely sensitive to changes in the protein environment. Since three of the mutant yeast proteins investigated here contain mutations analogous to those that cause ALS (amyotrophic lateral sclerosis) in humans, this finding implicates improper metal binding as a mechanism by which CuZnSOD mutants exert their toxic gain of function.

Key words Copper · Zinc · Superoxide dismutase · Amyotrophic lateral sclerosis · Yeast

T.J. Lyons
Nutritional Sciences Program, Department of Molecular Biology, 217 Gwynn Hall, University of Missouri-Columbia, Columbia, MO 65211, USA

A. Nersissian · H. Yeom † · E.B. Gralla · J.S. Valentine (✉)
Department of Chemistry and Biochemistry,
University of California, Los Angeles, 607 Circle Drive South, Box 951569, Los Angeles, CA 90095-1569, USA
Tel.: +1-310-8259835
Fax: +1-310-2067197
e-mail: jsc@chem.ucla.edu

H. Huang
Department of Computer Science, University of California, Los Angeles, 405 Hilgard, Los Angeles, CA 90095-1569, USA

C.R. Nishida
School of Pharmacy, University of California, San Fransisco, 513 Parnassus Avenue, Box 0446, Room S-1134, San Fransisco, CA 94143 USA

J.A. Graden
Kelly Scientific Resources, 1710 Walton Road, Suite 301, Bluebell, PA 19420, USA

Introduction

Copper-zinc superoxide dismutase (CuZnSOD) is a 32 kD homodimeric metalloenzyme that catalyzes the disproportionation of superoxide anion (O_2^-) to give hydrogen peroxide (H_2O_2) and dioxygen (O_2) [1]. Each subunit of the dimer contains one catalytic copper ion and one zinc ion [2, 3]. In the oxidized form of the enzyme, Cu^{2+} is bound to the protein via four histidine residues (His46, His48, His63, and His120, using the yeast CuZnSOD numbering system) arranged in a distorted square planar geometry. A weakly bound water molecule assumes an axial position, making the copper pentacoordinate. The Zn^{2+} ion is coordinated in a distorted tetrahedral arrangement by three histidine residues (His63, His71, and

His80) and one aspartate residue (Asp83). The doubly deprotonated His63 side-chain couples the Cu^{2+} and Zn^{2+} ions via a bridging imidazolate ligand [4, 5]. In solution, reduction of Cu^{2+} to Cu^{+} is accompanied by the rupture of this bridge [6]. While a recent crystal structure of reduced yeast CuZnSOD confirms this rearrangement of the active site upon reduction, showing Cu^{+} in a trigonal planar geometry with the copper ion detached from His63 [7], another crystal structure of reduced bovine CuZnSOD shows an intact bridge [8]. The two metal binding sites are also bridged by a hydrogen bond network that connects His46 in the copper site with His71 in the zinc site via Asp124 [9] (see Fig. 1).

Both the spectroscopic properties and the enzymatic activity of bovine CuZnSOD are essentially invariant between pH 5 and 9, leading to the conclusion that the enzyme retains its structure over a wide pH range [2]. However, if the pH of the buffering solution falls below pH 4, a reversible and kinetically slow conformational change was shown to occur at the zinc binding site of the bovine protein that results in the possible expulsion of the metal ion bound at that site [10]. It was also shown that the pK_a of this transition did not change significantly with the nature of the metal bound at the zinc site; viz., the transition occurred at roughly the same pH when either Zn^{2+}, Co^{2+}, or Cu^{2+} were bound there. This observation suggested that simple competition for the metal ligands by protons was not the only cause of the phenomenon. Rather, a pH-dependent conformational change of the protein was implicated [11].

CuZnSOD isolated from bovine blood has been the subject of extensive investigation since the discovery of its catalytic function in 1969 [12], while considerably less information is available about the enzyme from other species. Using bovine CuZnSOD as a flagship enzyme, detailed structural information about the CuZnSOD family has been provided over the years by a plethora of analytical techniques. In this paper we demonstrate that this low pH transition is not only a property of the bovine enzyme but that it occurs in the yeast and human enzymes as well. In addition, we report the investigation of the structural parameters governing the properties of the zinc binding site using the yeast wild-type protein and several site-directed mutants. The ability of the yeast protein to form a native-like zinc binding site is not only sensitive to the pH of the solution but it is also sensitive to the status of the adjacent copper binding site and to a diverse set of mutations in the protein matrix.

Little is known about why CuZnSOD evolved to contain zinc. The prevailing theory is that it plays a structural role. However, there is ample evidence that zinc can also modulate the reactivity of the enzyme (see Discussion). This idea becomes important in light of the discovery in 1993 that point mutations in the human CuZnSOD gene result in an unknown gain of function that causes approximately 2% of cases of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disorder that strikes motor neurons, causing paralysis and ultimately death. Three of the mutants investigated in this paper are analogous to mutations in the human enzyme that cause ALS [13,

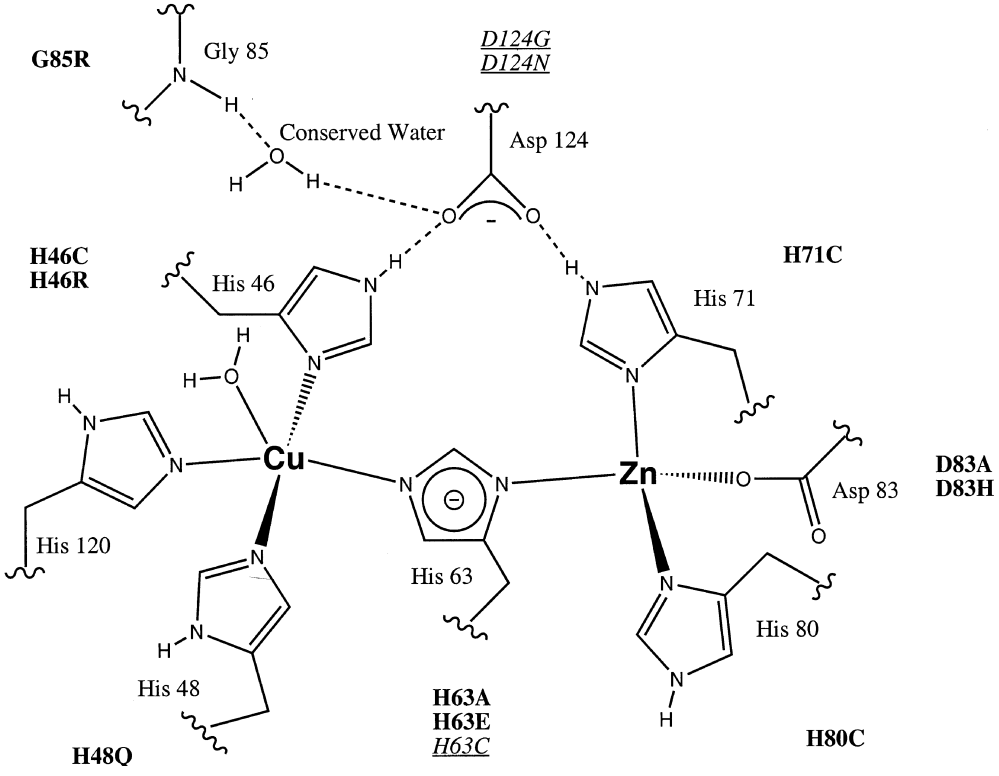


Fig. 1 Active site diagram of yeast CuZnSOD. Mutations that are the subject of this investigation are labeled with *bold print* (*underlined* and *italicized* mutations are merely discussed and are not the subject of this investigation)

14, 15], raising the possibility of a relationship between the ability of this protein to form a native zinc site and the presumed disease-causing gain of function.

Materials and methods

Lyophilized yeast CuZnSOD was purchased from Peptech (Denmark; batch no. 8997368, product no. 3007; mfg. date 10/17/89). Recombinant yeast CuZnSOD mutants were expressed in and isolated from BL21 *Escherichia coli* using the pET-3d expression plasmid. The mutants H63A and H63E (the bridging histidine at position 63 changed to alanine and glutamic acid, respectively), G85R (glycine at position 85 changed to arginine), and D83A and D83H (aspartic acid at position 83 changed to alanine and histidine, respectively) were prepared by oligonucleotide-directed mutagenesis and purified by published procedures [16, 17, 18]. H46R and H48Q were prepared by overlap extension PCR mutagenesis and purified by published procedures [17, 18]. H46C and H80C were prepared and purified by published procedures [19]; purity was confirmed by gel electrophoresis. Human wild-type CuZnSOD was purified via published procedures [20]. DEAE cellulose was purchased from Whatman and G-75 superfine Sephadex was purchased from Sigma.

We have found previously that the metal binding properties characteristic of apo bovine CuZnSOD can be reproduced for only one subunit of the yeast apoprotein dimer. One subunit is competent to bind metals in a bovine-like fashion and the other subunit (the "phantom" subunit) is not competent to do so and does not participate in metal titrations until the competent subunit has been filled and even then the metal binding properties of the phantom subunit are abnormal [20]. For this study we used a molar extinction coefficient at 280 nm of 3000 M^{-1} cm^{-1} per dimer to determine the concentration of apoprotein. Only 1 equiv per dimer of each metal is added so that the competent subunit is filled and the "phantom" subunit remains empty. For a more detailed description of the rationale for this assumption, see [20].

Apoproteins were obtained by dialysis against 100 mM acetate buffer, pH 3.8, in the presence of 10 mM EDTA (four times against 1 L each time). EDTA was then removed by dialysis against 100 mM acetate buffer, pH 3.8, in the presence of 100 mM NaCl (three times against 1 L each time) [2]. Metal content was determined by atomic absorption spectroscopy to confirm that the samples were indeed apo, containing less than 10% of residual metal ions. Experiments were carried out in 100 mM acetate buffer at pH 5.5 unless otherwise noted. Metal-substituted proteins were obtained by directly infusing solutions of the apoprotein with aliquots of either 10 mM or 100 mM stock solutions of the appropriate metal sulfate salt (Ag^+ was added as $AgNO_3$). Titration experiments required approximately 200 μL of 0.3 mM apoprotein solution (see figure legends for exact concentrations used in each experiment).

pH adjustments were made by addition of 100 mM or 1 M acid or base (NaOH or HCl) and pH was measured using an Orion Research Microprocessor Ionalyzer/901 (Boston, Mass.) with an Ingold microelectrode (Wilmington, Mass.).

UV-visible spectra from 900 to 240 nm were determined using a Cary 3 UV-visible spectrometer (Varian, Sunnyvale, Calif.). Samples were centrifuged at 14,000 rpm for 1 min before spectra were taken to remove trace turbidity. Reduced proteins were obtained by addition of a single small crystal of solid dithionite directly to the protein solutions.

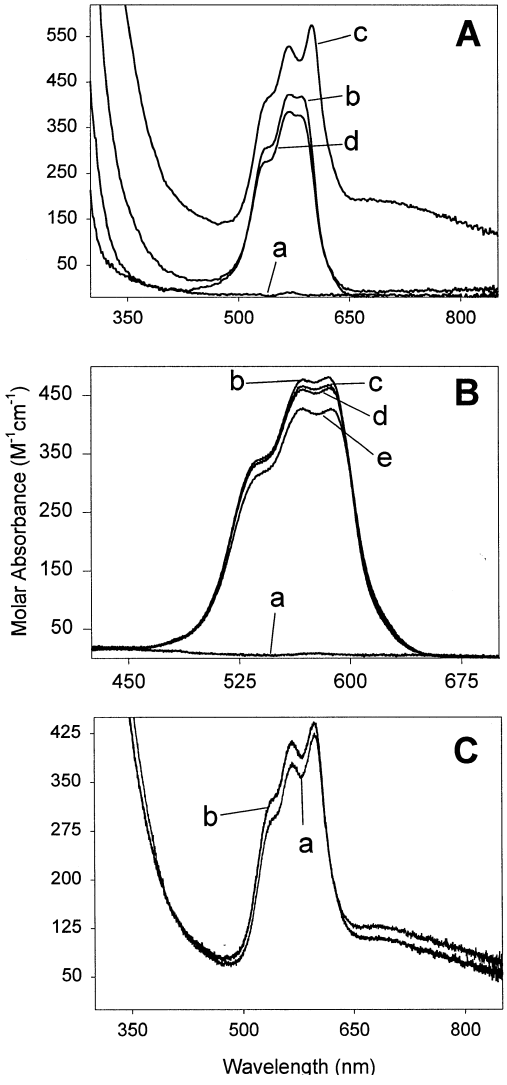
pK_a values were determined by plotting the change in absorption at a certain wavelength as a function of pH. Best fit sigmoidal plots of these data and first derivative graphs of the best fit curves were obtained to estimate the apparent pK_a values for these transitions.

Results

Addition of Co^{2+} and Cu^{2+} to apo wild-type yeast CuZnSOD

Figure 2 shows the characteristic visible spectra for the metal titration of apo yeast wild-type CuZnSOD in 100 mM acetate buffer, pH 5.5. The addition of

Fig. 2A–C Visible spectra of Co^{2+}-substituted wild-type yeast CuZnSOD reveal a conservation of spectroscopic properties from pH 5 to pH 9. **A** Metal titration of 0.328 mM protein in 100 mM acetate buffer: *a* apoprotein; *b* apoprotein+1 equiv Co^{2+} per dimer; *c* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer; *d* same as *c* with the addition of dithionite to reduce Cu^{2+} to Cu^+. **B** pH titration of 0.632 mM wild-type CuZnSOD in 100 mM acetate buffer: *a* apoprotein at pH 5.5; *b* apoprotein+1 equiv Co^{2+} per dimer, pH 5.5; *c* apaprotein+1 equiv Co^{2+} per dimer, pH 6.5; *d* apoprotein+1 equiv Co^{2+} per dimer, pH 8.2; *e* apoprotein+1 equiv Co^{2+} per dimer, pH 9.4. The slight decrease in the spectral intensity at higher pH is due to dilution of the sample by the addition of NaOH. **C** pH titration of 0.346 mM wild-type CuZnSOD in 100 mM acetate buffer to which 1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer have been added: *a* pH 5.5; *b* pH 9.3. Molar absorbance units are M^{-1} cm^{-1} per protein dimer



1 equiv of Co^{2+} per dimer resulted in the appearance of three relatively intense overlapping bands at 525 nm, 565 nm, and 585 nm due to Co^{2+} binding to the zinc site (Fig. 2Ab). These bands were essentially invariant from pH 5.50 to pH 9.4, as can be seen in Fig. 2Ba–e.

The further addition of Cu^{2+} to the above Co^{2+}-substituted species at pH 5.5 resulted in the superposition of the Co^{2+} bands on top of a 670 nm Cu^{2+} band, as well as a slight red shift of the 585 nm Co^{2+} band to 595 nm (see Fig. 2Ac). This red shift has been attributed to the formation of the imidazolate bridge in the case of the bovine enzyme [21]. The spectra of the copper and cobalt-substituted derivative were found to be relatively invariant from pH 5.5 to pH 9 (shown in Fig. 2Ca,b). Reduction of Cu^{2+} to Cu^{+} at pH 5.5 resulted in the disappearance of the 670 nm Cu^{2+} band and Co^{2+} bands that are essentially identical to the Co^{2+} bands seen in the absence of copper (see Fig. 2Ad and compare with Fig. 2Ab).

pH titration of as-isolated and Co^{2+}-substituted derivative of wild-type protein

Wild-type CuZnSOD isolated from yeast contains a full complement of two copper ions and two zinc ions. When the pH of the buffering solution was dropped from 5.5 to 3.0, there was a concomitant change in the visible spectrum at 450 nm. A small shoulder at this wavelength, attributed to a copper imidazolate charge transfer, disappeared at low pH. The loss of this band as a function of pH is graphed in Fig. 3 (closed squares) and the pK_a of the spectral change was calculated to be 3.8, a value that is identical to the low pH transition found in the bovine enzyme.

When a solution containing apo wild-type CuZnSOD to which 1 equiv Co^{2+} per dimer was titrated down in pH, the high-intensity Co^{2+} bands also disappeared (see Fig. 3, open squares), much like the 450 nm charge transfer band mentioned above. Interestingly, the pK_a of the loss of these bands was 4.7, not 3.8, suggesting either a marked difference between the as-isolated and half-reconstituted proteins or a greatly altered affinity of the yeast CuZnSOD zinc binding site for Co^{2+} relative to that of bovine CuZnSOD. Note that in the case of the bovine protein the pK_a for the low pH transition is the same for the as-isolated protein as it is for the protein reconstituted with either Zn^{2+}, Co^{2+}, or Cu^{2+} [11]. Also note that the pH titration of human wild-type CuZnSOD to which 2 equiv Co^{2+} per dimer have been added (see Fig. 3, inverted triangles) revealed that the pK_a of the change at the zinc site in the human enzyme is roughly the same as it is for the bovine enzyme (pK_a = 4.0).

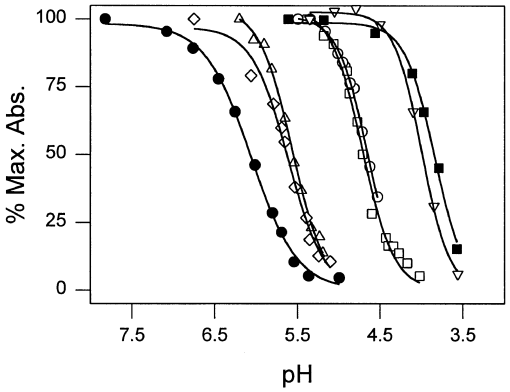


Fig. 3 pH titrations of wild-type yeast CuZnSOD and mutants demonstrate pH-dependent metal binding to the zinc site. The data represented by closed squares (■) are the pH titration of the yeast wild-type protein as isolated from yeast (0.568 mM) containing 2 equiv Cu^{2+} and 2 equiv Zn^{2+} per dimer. The low pH transition in this case was monitored by the change in absorbance of the 450 nm copper-imidazolate charge transfer band. The other data are for samples to which 1 equiv Co^{2+} was added per dimer (2 equiv Co^{2+} per dimer in the case of apo human CuZnSOD). The graph monitors the absorbance of the Co^{2+} chromophore as a function of pH and normalizes the values to the maximum value obtained during the titration. Other symbols: □ 0.273 mM yeast wild-type, monitored at 585 nm; ○ 0.521 mM H46C, at 585 nm; ● 0.320 mM H46R, at 585 nm; △ 0.119 mM H48Q, at 585 nm; ◇ 0.437 mM H80C, at 625 nm; ▽ 0.427 mM human wild-type, at 585 nm

Kinetics of the loss of zinc site integrity at low pH for wild-type CuZnSOD

Co^{2+} bound selectively to the zinc site when only 1 equiv Co^{2+} per dimer was added to the wild-type apoprotein at pH 5.5, as judged by the appearance of the characteristic d-d band at 585 nm. Figure 4A shows that upon a decrease in pH from 5.5 to 4.5 there was an immediate reduction in the intensity of the Co^{2+} bands, reaching equilibrium within the time it took to measure the pH and record a spectrum (less than 5 min). However, if Cu^{2+} was present in the adjacent copper site, the change in Co^{2+} binding upon the same drop in pH was slower and equilibrium was not reached until 105 min later (see Fig. 4B).

Effect of the copper site on Co^{2+} binding for wild-type CuZnSOD

In order to investigate further the role the copper site plays in influencing the nature of the zinc site, we obtained visible spectra at both pH 5.5 and pH 4.5 of a sample of wild-type apoprotein to which 1 equiv Co^{2+} per dimer has been added (see Fig. 5a). As expected from the data in Fig. 4, significantly less absorptivity due to Co^{2+} was seen at the lower pH (Fig. 5b). This pH 4.5 sample was then divided into two aliquots. To the first aliquot, 1 equiv Cu^{2+} per dimer was added. This addition resulted in the appear-

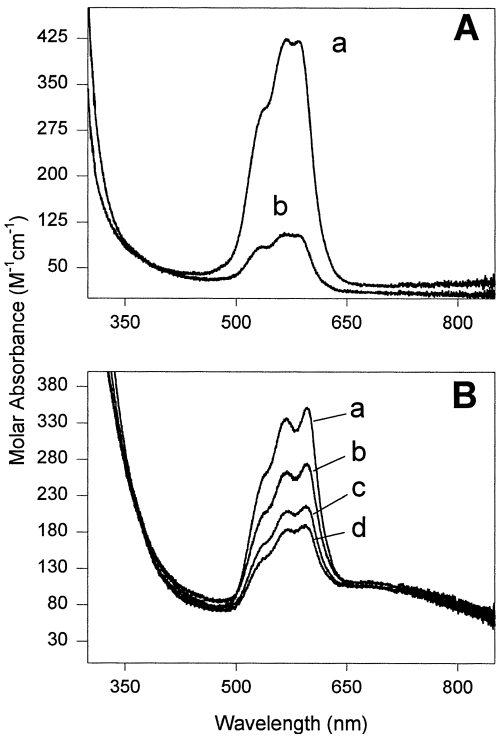
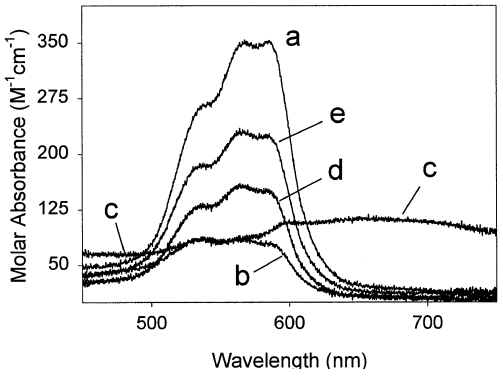


Fig. 4A,B Kinetics of the disappearance of Co^{2+} peaks in response to a drop in pH. **A** 0.273 mM apo yeast wild-type in 100 mM acetate buffer to which 1 equiv Co^{2+} per dimer has been added. The spectrum was recorded at pH 5.5 (*a*) and the pH was dropped from 5.5 to 4.5 quickly and a new spectrum (*b*) was recorded as soon as possible (5 min). **B** Same experiment as **A** except 1 equiv Co^{2+} and 1 equiv Cu^{2+} have been added and the decrease in Co^{2+} absorptivity is monitored over time: *a* pH 5.5 before pH was decreased; *b* pH 4.5, 15 min after; *c* pH 4.5, 60 min after; *d* pH 4.5, 105 min after (low pH spectra for both **A** and **B** did not change even after 2 days, indicating equilibrium had been reached). Molar absorbance units are M^{-1} cm^{-1} per protein dimer

Fig. 5 The occupancy of the copper site strongly affects the extent of conversion of the zinc site at low pH. Metal titration of 0.175 mM apo wild-type CuZnSOD in 100 mM acetate buffer: *a* apoprotein+1 equiv Co^{2+} per dimer, pH 5.5; *b* apoprotein+1 equiv Co^{2+} per dimer, pH 4.5; *c* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer, pH 4.5; *d* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{+} per dimer, pH 4.5 (Cu^{2+} reduced to Cu^{+} with dithionite); *e* apoprotein+1 equiv Co^{2+} and 1 equiv Ag^{+} per dimer, pH 4.5. Molar absorbance units are M^{-1} cm^{-1} per protein dimer



ance of a Cu^{2+} band at 670 nm and the further decrease of the Co^{2+} bands (Fig. 5c). The subsequent reduction of Cu^{2+} to Cu^{+} by dithionite resulted in the return of the high-intensity Co^{2+} bands to a level greater than that found in the absence of copper (Fig. 5d). To the second aliquot, 1 equiv Ag^{+} per dimer was added (Ag^{+} binds to the copper site in a similar fashion as Cu^{+} [2]), resulting in an even greater return of the Co^{2+} band intensities than was seen for the Cu^{+} bound protein (Fig. 5e). The occupancies of the copper site can be ordered with respect to the beneficial effects each has on the ability of the wild-type zinc site to bind Co^{2+} normally: Ag^{+} bound at the copper site > Cu^{+} bound > empty copper site > Cu^{2+} bound.

Co^{2+}-substituted derivatives of several metal ligand mutants of CuZnSOD also undergo a pH-induced change in the Co^{2+}-binding affinity at the zinc site

When 1 equiv Co^{2+} per dimer was added to metal-free preparations of the site-directed mutants H46C, H46R, H48Q, and H80C, all were found to bind Co^{2+} in a relatively normal fashion to their zinc binding sites. However, this binding was pH dependent; the pH profiles of Co^{2+} binding for each of the aforementioned proteins are shown in Fig. 3 (H46C, open circles; H46R, closed circles; H48Q, open triangles; H80C, open diamonds). Following the absorbance at 585 nm (625 nm for H80C), we monitored the change in Co^{2+} binding as a function of pH. The apparent pK_a of the loss of Co^{2+} binding for the wild-type protein is 4.7. The apparent pK_a values of this transition for the mutant proteins are as follows: H46C, 4.7; H46R, 6.0; H48Q, 5.6; and H80C, 5.6.

H48Q converts the zinc site from a strong binding site to a weak binding site

Titration of apo H48Q with Co^{2+} at pH 5.5 resulted in Co^{2+} bands that appeared normal in shape and position but were drastically reduced in intensity. Furthermore, a careful titration with Co^{2+} demonstrated that the Co^{2+} spectrum did not saturate until greater than 4 equiv of Co^{2+} per dimer had been added to the solution, revealing weak zinc site binding behavior (Fig. 6). The inset graph in Fig. 6 is a plot of the intensity of Co^{2+} bands as a function of equivalents Co^{2+} added to the apoprotein. While Fig. 6 demonstrates that the zinc site in H48Q is apparently normal, albeit with a lowered metal affinity, Fig. 3 (open triangles) demonstrates that this weak binding site is converted to a strong binding site at higher pH values.

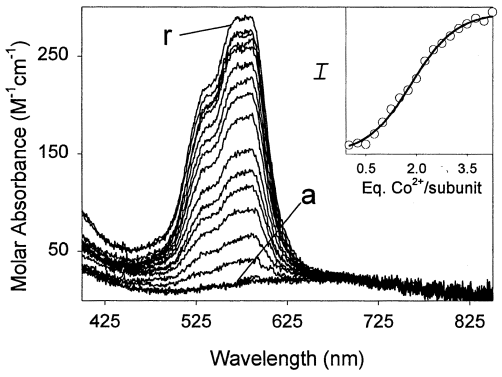


Fig. 6 The mutation H48Q converts the zinc binding site from a strong binding site to a weak one at pH 5.5. Co^{2+} titration of 0.217 mM apo yeast H48Q in 100 mM acetate buffer pH 5.5: *a* is a spectrum of the apo protein and *b*–*r* are spectra of sequential additions of 0.25 equiv Co^{2+} per dimer up to 4.25 equiv Co^{2+} per dimer. *Inset* is a plot of the intensity of the Co^{2+} absorptivity at 585 nm as a function of equiv of Co^{2+} added as well as a best fit sigmoidal curve of the data. Molar absorbance units are M^{-1} cm^{-1} per protein dimer

Co^{2+} binding affinity at pH 5.5 for the zinc site of the mutants G85R, D83H, and H63E can be improved if Cu^{+} is bound at the copper site; this is not true for the mutants D83A and H63A

While some of the metal binding behavior of the mutants G85R (analogous to the human ALS mutant hG85R), H63E, and H63A have been previously reported [16, 17, 18], those studies incorrectly determined protein concentration by a factor of two and therefore those results must be reinterpreted [20]. Here we reinterpret and expand upon those studies.

As was reported previously, addition of 1 equiv of Co^{2+} to apo G85R at pH 5.5 in acetate buffer resulted in the appearance of a non-wild-type-like Co^{2+} band of moderate intensity centered at roughly 550 nm (Fig. 7Aa). The fine structure and lowered intensity of the Co^{2+} band suggest that the Co^{2+} may be pentacoordinate [22] and therefore that it is probably not bound at the zinc site, but rather at the copper site. Further addition of Cu^{2+} to this species resulted in the

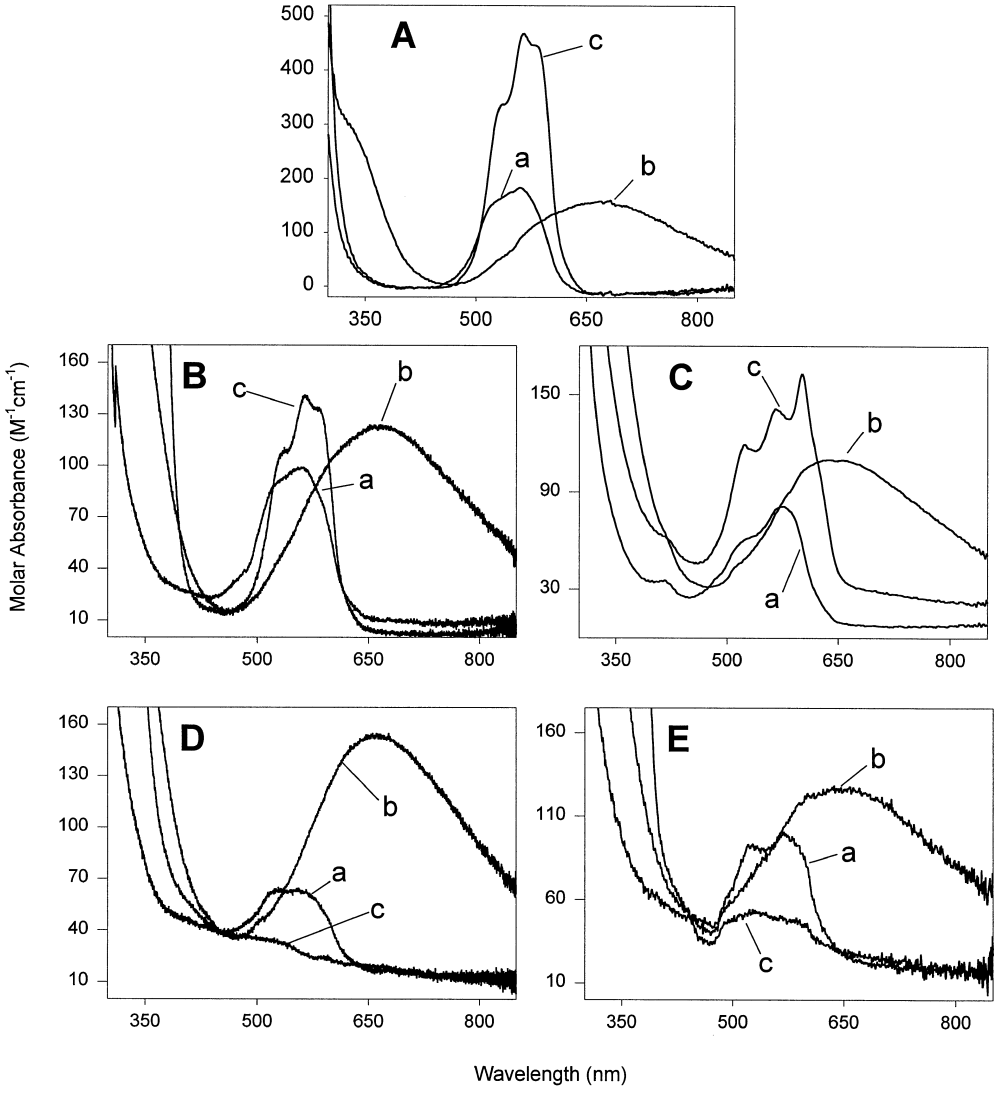


Fig. 7A–E The Co^{2+} binding affinity at pH 5.5 of the zinc site is strongly influenced by the occupancy of the copper site in several mutants. Metal titrations in 100 mM acetate buffer at pH 5.5 for **A** 0.300 mM apo yeast G85R; **B** 0.312 mM apo yeast D83H; **C** 0.862 mM apo yeast H63E; **D** 0.600 mM apo yeast D83A; and **E** 0.202 mM apo yeast H63A. For all figures: *a* apoprotein+1 equiv Co^{2+} per dimer; *b* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer; *c* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{+} per dimer (Cu^{2+} reduced to Cu^{+} with dithionite). Molar absorbance units are M^{-1} cm^{-1} per protein dimer

complete disappearance of this Co^{2+} band and the appearance of a 670 nm Cu^{2+} peak typical of Cu^{2+} at the copper site (Fig. 7Ab). These data suggest that Co^{2+} and Cu^{2+} are competing for the same binding site at pH 5.5, i.e., that Co^{2+} binds to the copper site and is then displaced by the Cu^{2+}. Reduction of Cu^{2+} resulted in the rebinding of Co^{2+}, this time to the zinc site, as judged by the appearance of a normal wild-type-like Co^{2+} spectrum (Fig. 7Ac).

The titration of apo D83H (zinc ligand Asp83 mutated to histidine) proceeded very similarly to the titration of apo G85R. The addition of 1 equiv Co^{2+} per dimer to the apoprotein yielded a broad visible band identical to the one seen for the similarly metallated G85R (Fig. 7Ba). The addition of 1 equiv Cu^{2+} to this derivative caused the disappearance of the Co^{2+} band and the appearance of a 670 nm Cu^{2+} band (Fig. 7Bb) and reduction of Cu^{2+} then caused the partial return of wild-type-like Co^{2+} bands (Fig. 7Bc). The fact that the return of wild-type-like Co bands in D83H is not as complete as for G85R may reflect the more drastic nature of the D83H mutation (D83 being a zinc binding ligand).

Despite some differences in the shape and position of the relevant bands, the titration of apo H63E (the histidine that acts as a bridging ligand between copper and zinc, H63, replaced with a glutamate) also proceeds very similarly to that of apo G85R. Addition of 1 equiv Co^{2+} per dimer yielded Co^{2+} bands of relatively low intensity and of poor structural definition (Fig. 7Ca). This again would suggest Co^{2+} bound at the copper site. The addition of Cu^{2+} to this derivative resulted in the loss of Co^{2+} bands and the appearance of a 670 nm Cu^{2+} band, reflecting competition by the two metal ions for the same site (Fig. 7Cb). The reduction of Cu^{2+} to Cu^{+} resulted in the reappearance of high-intensity, albeit not wild-type-like, Co^{2+} bands. The Co^{2+} bands in this last derivative were of lower intensity than those observed for wild-type and were red-shifted (Fig. 7Cc). Such bands are, however, indicative of tetrahedral binding and the fact that they are not similar to those of wild-type may reflect the different liganding properties of the glutamate side chain that has replaced His63 [22].

Both mutants D83A (zinc ligand Asp83 mutated to alanine) and H63A (bridging histidine, H63, replaced with alanine) present examples of mutants that are not capable of binding Co^{2+} to the zinc site at pH 5.5 even in the presence of Cu^{+} bound to the copper site. If 1 equiv Co^{2+} per dimer is added to the apo derivative of either mutant, an amorphous band of low intensity appears centered around 550 nm (Fig. 7Da and Ea). As before, these bands strongly suggest that the Co^{2+} has bound to the copper site. The addition of Cu^{2+} displaces the Co^{2+} and results in the appearance of a 670 nm Cu^{2+} band (Fig. 7Db and Eb). However, the reduction of Cu does not result in the formation of a zinc site with a tetrahedral geometry as was the case with G85R, D83H, and H63E. Instead, the Co^{2+} remains unbound or weakly bound to a low-affinity zinc site (Fig. 7Dc and Ec). It is apparent that at this pH a liganding amino acid is necessary at both position 63 and position 83 for a strong binding zinc site to form.

The Co^{2+} binding affinity of the zinc sites of G85R, D83H, H63E, and H63A can be improved if the pH is increased; D83A was not observed to form a wild-type-like zinc site at any pH

As stated above, if 1 equiv Cu^{2+} and 1 equiv Co^{2+} per dimer is added to each of the apo derivatives of G85R, D83H, H63E, and H63A, the resulting visible spectrum shows only a 670 nm Cu^{2+} band (Fig. 8Aa, Ba, Ca, and Da, respectively). However, if the pH of these derivatives is increased, wild-type-like Co^{2+} bands emerge in the spectra of all four mutants while the copper band remains and is relatively unperturbed. The inset graphs in Fig. 8 show the increase in absorbance at 595 nm due to Co^{2+} as a function of pH for each mutant, and from these graphs we can approximate the pK_a values for the emergence of the Co^{2+} bands. While it was not possible to obtain an exact pK_a of these transitions for all the mutants owing to a blue shift of the Cu^{2+} band at pH values above 7, we can state with some certainty that their pK_a values have all been shifted to much higher values (the values are as follows: G85R, $pK_a >> 5$; D83H, $pK_a \approx 5.8$; H63E, $pK_a \approx 7.3$; and H63A $pK_a \approx 6.2$). No Co^{2+} bands emerged when the pH was increased for the D83A mutant (Fig. 8Ea–e), suggesting that even at high pH a liganding amino acid is critical at position 83 in the sequence.

Metal binding properties of the secondary bridge mutants H71C and H46C

We also investigated the role that the secondary bridge (His46-Asp124-His71; see Fig. 1) plays in formation of the zinc site. Mutation of the zinc ligand His71 to cysteine resulted in a mutant that was not capable of binding Co^{2+} normally. Addition of 1 equiv of Co^{2+} per dimer to the apoprotein resulted in the appearance of Co^{2+} bands of relatively low intensity (Fig. 9Ab), indicative of penta- or hexacoordination [22]. These bands did not significantly change upon increasing the pH of the buffering solution from 5.5 to 7 (Fig. 9Ac). The small increase in Co^{2+} intensity seen at pH 7 was also seen in the UV region (see inset of Fig. 9A) and cannot be eliminated by centrifugation, facts which indicate that this increase is most likely caused by increased light scattering due to protein aggregation at the higher pH and not because there is a change in the affinity of the zinc site for Co^{2+}.

At first glance, mutation of the copper ligand His46 to cysteine had seemingly negligible effects on the

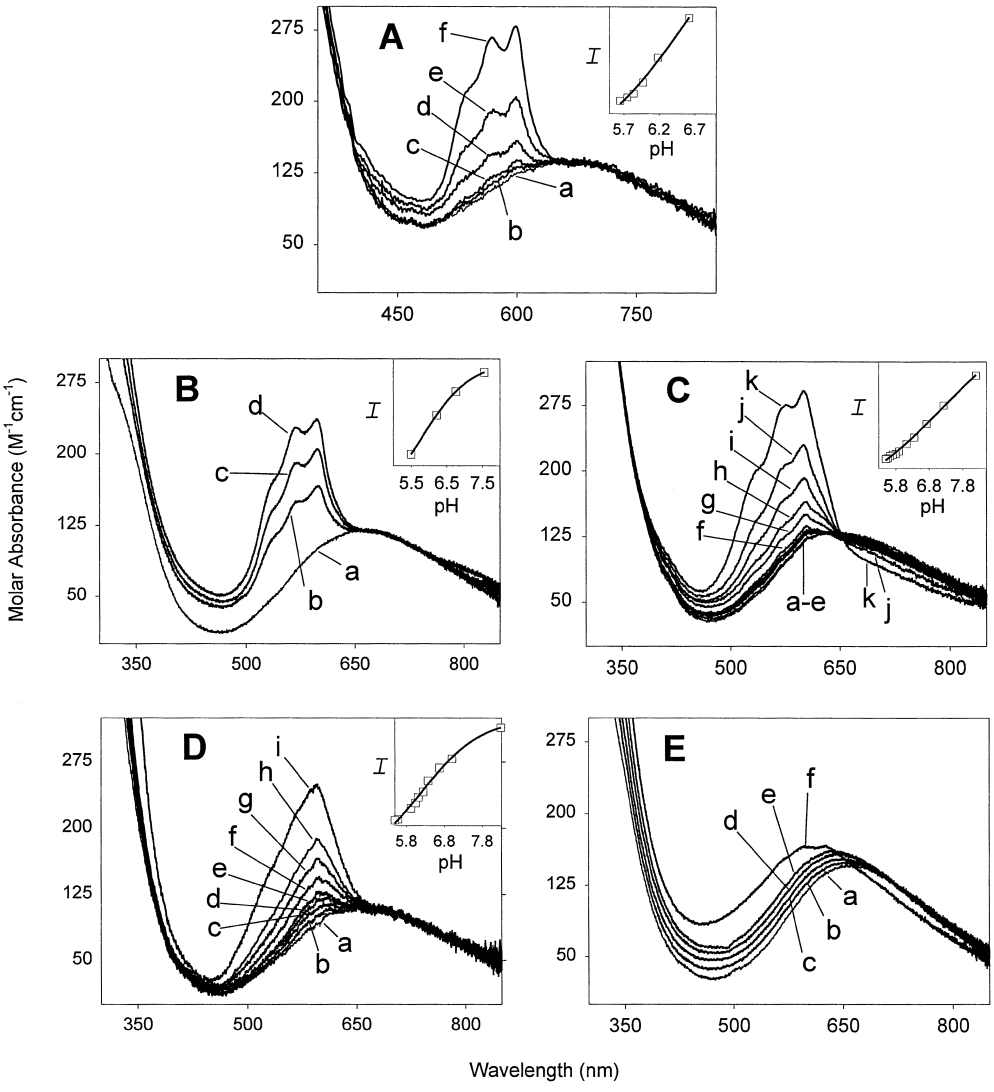


Fig. 8A – E The Co^{2+} binding affinity of the zinc site can also be increased at higher pH. pH titrations in 100 mM acetate buffer of various mutants to which 1 equiv Co^{2+} and 1 equiv Cu^{2+} have been added. **A** 0.230 mM G85R at *a* pH 5.65; *b* pH 5.85; *c* pH 5.84; *d* pH 5.97; *e* pH 6.18; *f* pH 6.62. **B** 0.686 mM yeast D83H at *a* pH 5.50; *b* pH 6.22; *c* pH 6.76; *d* pH 7.56. **C** 0.548 mM yeast H63E at *a* pH 5.51; *b* pH 5.57; *c* pH 5.63; *d* pH 5.71; *e* pH 5.80; *f* pH 5.90; *g* pH 6.12; *h* pH 6.35; *i* pH 6.72; *j* pH 7.22; *k* pH 8.19. **D** 0.294 mM apo yeast H63A at *a* pH 5.58; *b* pH 5.92; *c* pH 6.04; *d* pH 6.11; *e* pH 6.24; *f* pH 6.37; *g* pH 6.67; *h* pH 7.00; *i* pH 8.28. **E** 0.600 mM apo yeast D83A at *a* pH 5.50; *b* pH 6.00; *c* pH 7.00; *d* pH 7.65; *e* pH 8.49. *Insets:* plot of the intensity of Co^{2+} absorptivity at 595 nm as a function of pH as well as a best fit sigmoidal curve of the data. Molar absorbance units are M^{-1} cm^{-1} per protein dimer

zinc binding site (Fig. 9B). Upon lowering the pH of the 1 equiv Co^{2+} per dimer derivative (Fig. 9Ba) of this mutant from 5.5 to 4.5 there was, as expected, a decrease in absorbance at 585 nm due to Co^{2+} with an apparent pK_a of 4.7 (Fig. 9Bb; also see Fig. 3, open circles). However, if 1 equiv Cu^{2+} per dimer was then added to this low pH species, there was little noticeable change in the Co^{2+} bands (Fig. 9Bc) (although the Co^{2+} bands were mostly obscured by an intense absorption which is assigned to a Cu^{2+}-thiolate charge transfer band; see inset Fig. 9B). In addition, reduction of Cu^{2+} to Cu^{+} in this low pH species did not result in the return of high-intensity Co^{2+} bands (Fig. 9Bd), a result that differs from results of similar experiments for the wild-type protein (compare Figs. 5 and 7B).

Discussion

Cobalt as a probe of zinc binding sites

The significance of these experiments cannot be evaluated without first providing an explanation of the benefits of using cobalt to probe the zinc binding site. Zn^{2+} is a d^{10} metal ion and as such has completely filled d-orbitals and is therefore spectroscopically silent. While there are methods to monitor the binding of zinc to metalloproteins [23], it is often easier to replace the zinc with a more spectroscopically versatile metal ion and hope that the protein retains its properties.

Co^{2+} is an excellent mimic of Zn^{2+}, having a similar ionic radius and geometric preferences [22]. Co^{2+} is beneficial in that its visible spectroscopy is extremely sensitive to the nature of the ligands and their geometrical arrangement around Co^{2+}. For instance, Co^{2+} in an octahedral ligand field has minimal absorbance in the visible region (ε of approximately 5 M^{-1} cm^{-1})

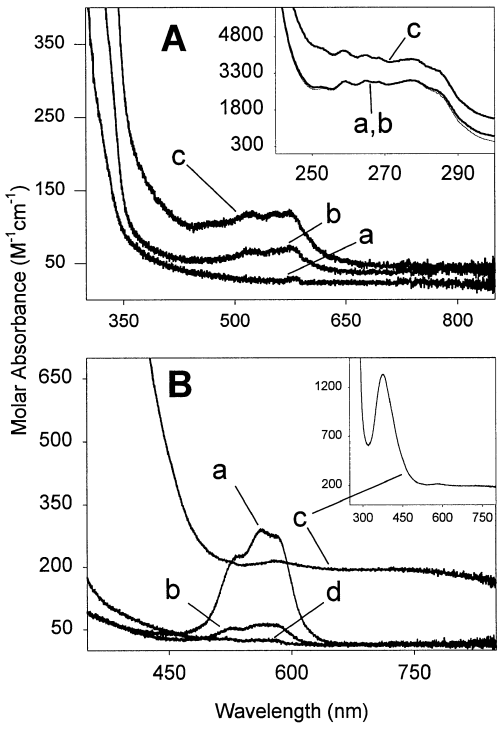


Fig. 9A,B Metal binding properties of the secondary bridge mutants H71C and H46C. **A** pH titration of 0.246 mM apo yeast H71C in 100 mM acetate buffer: *a* apoprotein, pH 5.5; *b* apoprotein+1 equiv Co^{2+} per dimer, pH 5.5; *c* apoprotein+1 equiv Co^{2+} per dimer, pH 7.0. *Inset* in **A** shows the UV region of the same spectra. **B** Visible spectra of metal-substituted derivatives at various pH values for apo yeast H46C in 100 mM acetate buffer: *a* apoprotein+1 equiv Co^{2+} per dimer, pH 5.5; *b* apoprotein+1 equiv Co^{2+} per dimer, pH 4.5; *c* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer, pH 4.5; *d* apoprotein+1 equiv Co^{2+} and 1 equiv Cu^{+} per dimer, pH 4.5 (Cu^{2+} reduced to Cu^{+} with dithionite). *Inset* in **B** expands the range for trace *c*, showing the strong copper-thiolate charge transfer band that dominates the spectrum. Molar absorbance units are $M^{-1}\,cm^{-1}$ per protein dimer

while Co^{2+} in a tetrahedral geometry yields bands that are approximately 100-fold more intense [22]. Consequently, the intensities of bands in the visible spectrum are superb gauges of the structure of the zinc site. Co^{2+} bands are also sensitive to the properties of the ligands that are bound to it. For example, Co^{2+} bound to the zinc site of copper-free CuZnSOD binds two types of ligands, three imidazole nitrogens and one carboxylate oxygen, and this ligand set results in a very unique visible spectrum. However, when copper binds, the imidazolate bridge forms and changes one of the imidazole ligands to an imidazolate. This electronic change causes a shift of one of the Co^{2+} bands to a higher wavelength (red shift) [21]. Thus the fine structure of Co^{2+} bands can be used as a fingerprint of a certain ligand set [22].

Co^{2+} can substitute for Zn^{2+} in wild-type bovine CuZnSOD without appreciably affecting the dismutase activity or the crystal structure [2, 24] and has been repeatedly used as a reporter of the structural and metal binding properties of CuZnSOD. This study makes use of the descriptive spectroscopy of Co^{2+} to investigate the factors that govern the proper formation of the zinc site in yeast CuZnSOD. The information gleaned from these studies can be compared with similar studies on the bovine enzyme in order to interpret its significance.

pH-dependent conformational changes in bovine CuZnSOD

Several studies have shown that there is a reversible conformational change at low pH in bovine CuZnSOD. This transition is accompanied by small changes in the properties of the bound Cu^{2+} ion. These changes were found to include the loss of the strong shoulder at 450 nm found in the visible spectrum and an increase in the $A_{\|}$ value in the EPR spectrum [10]. Similar changes were seen at high pH upon removal of the Zn^{2+} ion from the protein, suggesting that this conformational change is a result of a change at the zinc site [2].

A later study characterized this transition using the Co^{2+}-substituted enzyme (Cu_2Co_2SOD). In this case, Co^{2+} replaced Zn^{2+} in the zinc site, and a decrease in pH from 5.5 to 3 resulted in a decrease in Co^{2+} band intensity in the visible spectrum without significant changes in the copper chromophore. The change in Co^{2+} spectroscopy supported the hypothesis that a conformational change was occurring at the zinc site, resulting in an alteration of the tetrahedral geometry of this site. This idea was further supported by EPR studies which showed that, while Cu_2Co_2SOD is EPR-silent at high pH owing to the coupling of the Cu^{2+} and Co^{2+} spins across the imidazolate bridge, lowering the pH to 3 resulted in the appearance of a Cu^{2+} EPR signal that was, again, very similar to the signal obtained for the Zn^{2+}-free enzyme at pH 7 [25, 26]. Therefore, the data suggested that imidazolate bridge breakage and gross geometrical change occurred at the zinc site upon a decrease in pH, at least in the bovine enzyme.

Another study demonstrated that the metal bound at the zinc site was most likely expelled from the protein at low pH, or at least the metal became weakly bound and could be easily removed from the protein by dialysis [11]. This study also demonstrated that the apparent pK_a of this pH-dependent binding was the same regardless of which metal was bound at the zinc site; that is to say, the pK_a did not change whether Zn^{2+}, Co^{2+}, or Cu^{2+} was bound. The implication of this finding is that the phenomenon was probably not a simple competition by protons for the metal binding ligands; rather it was more likely the result of a conformational change in the protein structure that modulates the affinity or integrity of the zinc site. Regardless, all three of the aforementioned studies showed

that this low pH transition has an apparent pK_a of approximately 3.8.

Low pH conformational change at the zinc site of yeast CuZnSOD

We have investigated the possibility that this low pH transition is a general property of CuZnSODs using the enzymes from humans and *Saccharomyces cerevisiae*. Indeed, Fig. 3 shows that for native yeast CuZnSOD (which contains two Cu^{2+} and two Zn^{2+} per dimer) there is a pH-dependent conformational change that results in uncoupling of the copper and zinc sites at low pH. The pK_a of this transition is approximately 3.8, indicating a remarkable degree of conservation from the yeast to bovine proteins. In addition, the human protein reconstituted with 2 equiv Co^{2+} per dimer also retains this pH-dependent change at the zinc site with approximately the same pK_a (4.0).

Using Co^{2+} as a probe of the integrity of the zinc site, we studied the ability of the wild-type yeast enzyme and several site-directed mutants to form the distorted tetrahedral zinc site at various pH values and under different redox conditions. Figure 2 shows that the zinc site of the yeast enzyme is capable of binding at least one Co^{2+} at pH 5.5 (the second subunit of yeast CuZnSOD has been shown previously to be incapable of binding Co^{2+} under any of the conditions tested [20]). The intensity of the Co^{2+} bands ($\varepsilon = 400\ M^{-1}\ cm^{-1}$) confirms that the ligands in this case are oriented about the Co^{2+} in a distorted tetrahedral geometry [22]. The shape and intensity of these bands are invariant from pH 5.5 to 9.4, a result that is reminiscent of studies on the bovine enzyme [2].

Figure 3 demonstrates that, for the wild-type protein below pH 5.5, there is loss of Co^{2+} absorptivity with an apparent pK_a of 4.7. Identical experiments for the site-directed mutants H46C (p$K_a = 4.7$), H46R (p$K_a = 6.0$), H48Q (p$K_a = 5.6$), and H80C (p$K_a = 5.6$) are also plotted in Fig. 3, demonstrating that while the zinc site geometry and the low pH transition are conserved in yeast wild-type and some site-directed mutants, the pK_a of the transition is markedly sensitive to changes in the protein structure. It should be noted that the pK_a of this transition for reconstituted wild-type yeast (half the subunits filled with Co^{2+}) is significantly higher than the pK_a for the as-isolated yeast wild-type, reconstituted human, and reconstituted and as-isolated bovine proteins. The source of this discrepancy is most likely to be the fact that the apo yeast wild-type CuZnSOD can only be reconstituted with copper and cobalt in one subunit [20]. Thus, unlike the as-isolated yeast protein, which is composed of two fully metallated and equivalent subunits, the half-reconstituted protein may have different structural features that, while retaining pH dependence, alter the pK_a of the transition.

Effect of copper on the integrity of the zinc site at low pH

Since these experiments were done in the absence of metal bound at the copper site, it was necessary in addition to investigate the effect the copper site has on the zinc site. Figure 2 shows that binding of Cu^{2+} to the copper site at pH 5.5 causes a slight decrease in intensity of the Co^{2+} bands as well as a small red shift that is most likely due to the anionic nature of the imidazolate ligand [21]. However, reduction of the Cu^{2+} to Cu^{+} in this last species results in loss of the Cu^{2+} band and a reversion of the Co^{2+} bands to an appearance identical to that seen in the absence of any metal bound at the copper site. Thus, the reduced wild-type and copper-free wild-type proteins have nearly identical zinc sites at pH 5.5. Based on these data, copper has seemingly small effects on the structure of the zinc site at high pH values.

However, copper does affect the low pH transition, as demonstrated by the experiment shown in Fig. 4. This transition occurs if Cu^{2+} is bound at the copper site, as can be seen by the decrease in absorptivity due to Co^{2+} at pH 4.5. However, unlike the copper-free protein, which equilibrates rapidly at low pH, the Cu^{2+}-replete protein takes nearly 2 h to reach equilibrium at pH 4.5. Thus, the presence of metal bound at the copper site affects the kinetics of reorganization of the zinc site at low pH.

The nature of the metal bound at the copper site clearly affects the extent of reorganization at low pH. The Co^{2+}-substituted protein (copper site empty) has an absorptivity of approximately 400 $M^{-1}\ cm^{-1}$ at 585 nm at pH 5.5. If the pH is decreased to 4.5, this absorptivity decreases by roughly 75% (shown in Fig. 5). (Since the fine structure of these peaks does not change significantly, this is interpreted to mean that the site has been converted from a strong binding site to a weak binding one, although we cannot rule out definitively the possibility that all of the Co^{2+} remains bound in an alternative configuration at low pH.)

If Cu^{2+} is added to the Co^{2+}-substituted protein at pH 4.5, there is a further decrease in the Co^{2+} absorptivity, indicating that Cu^{2+} causes an even higher percentage of zinc sites to change their configuration. In other words, an empty copper site seems to be more conducive to proper zinc site formation at this pH than is a copper site filled with Cu^{2+}. If the Cu^{2+} in this species is reduced to Cu^{+}, an increase in Co^{2+} absorptivity is seen. In fact, the Co^{2+} absorptivity in the Cu^{+} species is larger than that seen in the absence of metal bound at the copper site, indicating that Cu^{+} is yet more conducive to zinc site formation at this pH than is an empty copper site. The binding of Ag^{+} at pH 4.5 elicits a further increase in Co^{2+} absorptivity, suggesting that Ag^{+} is even better still at promoting native zinc site formation than is Cu^{+}.

Since reduction of wild-type bovine CuZnSOD has been shown to result in conformational changes in the

protein [27] and may also result in increased protein stability [28], it is not surprising that Cu^+ and Ag^+ binding have such a profound and beneficial effect on the zinc site. The magnitude of their effects may result from their different Lewis acidities, which would modify the dynamics of any hydrogen bond networks influenced by the copper site.

The redox status of the copper site is influential at higher pH in certain site-directed mutants

The major effects that copper has on the metal binding properties of a wild-type zinc site do not manifest themselves at physiological pH. However, if the protein architecture is perturbed by point mutations, this influence can be readily seen at pH values above 5. Of the mutants G85R, D83H, and H63E, none is capable of binding Co^{2+} in a native-like fashion if the copper site is empty. In fact all the evidence supports the conclusion that Co^{2+} does not bind to the zinc site at all but to the copper site (which is expected to have a more symmetrical arrangement of ligands and therefore Co^{2+} absorption bands of lower intensity and less structural definition [22]). Although it is possible that, at this pH, the Co^{2+} is bound to a weak binding zinc site and as such has lowered intensity, the assignment of Co^{2+} bound to the copper site is supported by the fine structure and also by the fact that addition of copper at pH 5.5 results in the complete disappearance of Co^{2+} bands [strongly suggesting the presence of hexaaquocobalt(II) and hence the expulsion of Co^{2+} from the protein]. If Cu^{2+} is then reduced to Cu^+, high-intensity wild-type-like Co^{2+} bands return in the spectrum, suggesting that Co^{2+} can now bind to a native-like zinc site (see Eq. 1):

$$Apo \overset{+Co^{2+}}{\longrightarrow} Co^{2+}E \overset{+Cu^{2+}}{\longrightarrow} Cu^{2+}E + Co^{2+}_{aq} \overset{+e^-}{\longrightarrow} Cu^{+}Co^{2+} \quad (1)$$

where E = empty zinc site

The mutants D83A and H63A behave similarly to the others up to a point. Adding Co^{2+} to these apoproteins also results in what is probably Co^{2+} bound at the copper site. The addition of Cu^{2+} results in the expulsion of Co^{2+} from the copper site. However, these mutants are distinctly different in that the reduction of Cu^{2+} to Cu^+ does not yield a native-like zinc site. In fact there is no evidence whatsoever that at pH 5.5 the zinc sites of these mutants are capable of binding metals. Equation 2 summarizes the proposed metal binding scheme of these two mutants at pH 5.5:

$$Apo \overset{+Co^{2+}}{\longrightarrow} Co^{2+}E \overset{+Cu^{2+}}{\longrightarrow} Cu^{2+}E + Co^{2+}_{aq} \overset{+e^-}{\longrightarrow} Cu^{+}E + Co^{2+}_{aq} \quad (2)$$

where E = empty zinc site

Increasing the pH to near-physiological levels substantially enhances the affinity of the zinc site for Co^{2+} in most site-directed mutants

As we have shown, the ability of the mutants G85R, D83H, D83A, H63E, and H63A to bind Co^{2+} to a native-like zinc site is impaired at pH 5.5 if either the copper site remains empty or Cu^{2+} is bound to that site. For all the mutants except D83A, this can be changed if the pH is increased to physiological levels. The 1 equiv Co^{2+}, 1 equiv Cu^{2+} derivatives of these proteins show no appreciable Co^{2+} binding at pH 5.5, but if the pH is increased then Co^{2+} bands begin to appear superimposed over the Cu^{2+} band. The pK_a of the return of these bands varies from mutant to mutant and is much higher than wild-type. These bands appear normal in shape and intensity and suggest that at higher pH the zinc site is converted back to a high-affinity site. The metal binding scheme of all of these mutants is summarized in Eq. 3:

$$Apo \overset{+Co^{2+}+Cu^{2+}}{\longrightarrow} Cu^{2+}E + Co^{2+}_{aq} \longrightarrow Cu^{2+}Co^{2+} \quad (3)$$

where E = empty zinc site

The above data suggest that the copper site communicates with the zinc site and helps govern its proper formation. One obvious way in which this can occur is via the imidazolate bridge. The ability of His63 to coordinate at the zinc site may be a factor in determining the integrity of the site. This may help explain why Cu^+ (and by corollary Ag^+, which is a mimic of Cu^+) is more conducive to formation of a native zinc site at low pH, as the imidazolate bridge detaches from the copper site when Cu^+ is bound [6, 7], perhaps facilitating the formation of a bond with the metal ion in the zinc site.

The site-directed mutants H63E and H63A, in which the bridging histidine is mutated to a glutamic acid and an alanine, respectively, seem to support this idea. Both mutants have profound effects of the pK_a of the zinc site transition. Neither mutant apoprotein is capable of binding Co^{2+} normally at pH 5.5 if the copper site is vacant or has Cu^{2+} bound to it. While reduction of the 1 equiv Cu^{2+}, 1 equiv Co^{2+} derivative of H63E causes intense and highly red-shifted Co^{2+} bands to appear, the same does not occur in the same derivative of H63A. One possible explanation for these observations is that, in the case of H63E, reduction liberates Glu63 from binding to the copper site and allows it to bind to the ion in the zinc site through its carboxyl group. Coordination of this unorthodox carboxyl group to Co^{2+} might then be the source of the red-shifted bands in the visible spectrum [19]. Reduction would not have such an effect on the H63A mutant because at this pH a liganding amino acid is needed at position 63, and alanine cannot function in this capacity.

At higher pH values, both of these mutants (H63A, H63E) behave more like each other. In both cases, if the pH is increased, high-intensity Co^{2+} bands appear superimposed on the Cu^{2+} band at 670 nm. The pK_a values of Co^{2+} binding for H63E and H63A are roughly 7.3 and 6.2, respectively. Thus, at higher pH, it appears that a more native-like zinc site can be formed for both of these mutants and that a liganding amino acid at position 63 is not necessary for zinc site formation. As a brief aside, it should be mentioned that it has been reported that mutation of His63 to cysteine in the human enzyme was found to yield a protein that bound a substoichiometric amount of Co^{2+} in a tetrahedral manner at pH 5.5, although higher pH values were not investigated [29].

Influence of the secondary bridge on the zinc site

Although the imidazolate bridge is clearly important for zinc site formation, its existence does not completely govern the conformational change seen at the zinc site in response to pH and copper site occupancy. This is made obvious by the fact that H63A still retains this transition and does not retain any semblance of a bridge. Another obvious way in which the copper site could modulate the integrity of the zinc site is via the secondary hydrogen bond bridge formed by residues His46, Asp124, and His71. This network is similar to the hydrogen bond networks seen in the serine-histidine-aspartate triads of serine proteases. The pK_a of this type of bond can vary widely and is sensitive to environmental effects [30] (see Fig. 10). Mutants in human CuZnSOD that are missing this network (Asp124Gly, Asp124Gln) were isolated as zinc-deficient proteins [9], perhaps indicating that formation of this network is crucial to the formation of the zinc site.

We have investigated the effect that mutations in this network have on the integrity of the zinc site. The His46 to cysteine mutant is presumably no longer capable of participating in this hydrogen bond network. Ironically, if the copper site is vacant, there seems to be no effect on the low pH conformational change at the zinc site. The pK_a of this transition is 4.7, which is essentially identical to that of wild-type. However, if Cu^{2+} is added to the 1 equiv Co^{2+} derivative at pH 4.5, there is relatively little change in the Co^{2+} bands and reduction of Cu^{2+} to Cu^{+} at pH 4.5 does not result in the increase in Co^{2+} band intensity, as is seen in the wild-type protein. Both findings indicate that the communication between the two sites has been partially severed.

Mutation of residues at the other end of this network is more critical to the zinc site. The His71 to cysteine mutation results in a protein that is not capable of binding Co^{2+} in a native fashion at any pH or under any condition tested. Again, while formation of this hydrogen bond network is important for zinc site integrity, it does not entirely determine the dynamics of zinc site structure.

It seems clear that neither the imidazolate bridge nor the secondary bridge can independently account for the low pH conformational change in CuZnSOD and it is possible that the conformational change at the zinc site is mediated via another, as yet unknown,

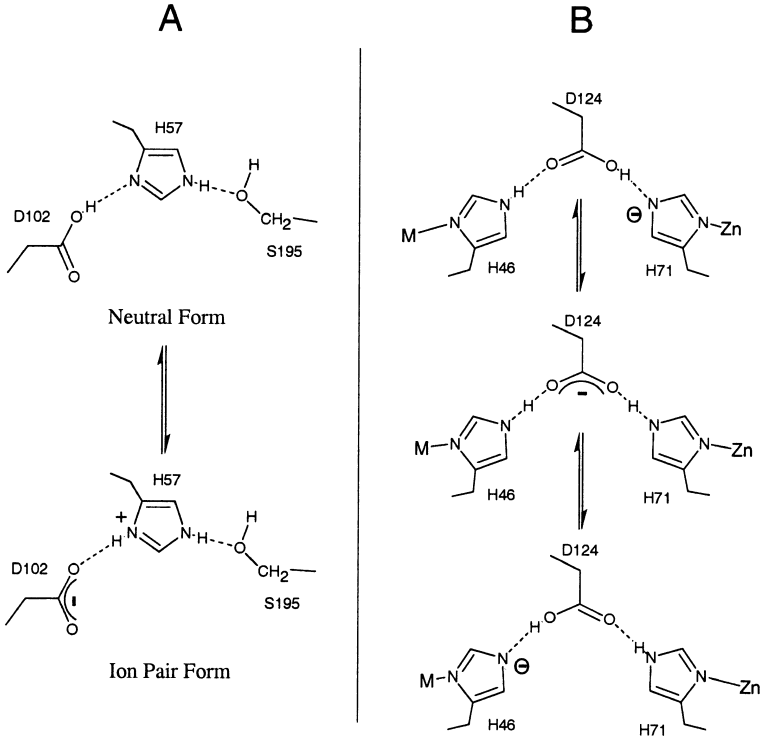


Fig. 10A,B Schematic diagram of hydrogen bonds networks. **A** Serine-histidine-aspartate triad of the serine protease bovine pancreatic β-trypsin (adapted from [27]). **B** Secondary bridge hydrogen bond network in CuZnSOD

mechanism. What is evident is that the pH and redox dependence of this interconversion is sensitive to the environment of the protein matrix.

The role of the zinc site in the structure and function of CuZnSOD

To assess appropriately the importance of these findings, it is first necessary to analyze the role that the zinc site plays in CuZnSOD. CuZnSOD was first isolated in 1939 and determined to be a copper protein of unknown function [32]. It was not until 1970, a year after the SOD function was discovered, that zinc was proven to be a cofactor of the enzyme [33]. Zinc can be replaced with Co^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, or VO^{2+} with only modest effects on the catalytic function of the enzyme [2, 34]. Even the zinc-free enzyme is fully active at physiological pH. However, while the activity of the holo enzyme is relatively constant over a wide pH range, the activity of the zinc free enzyme is markedly pH dependent and falls off rapidly above pH 7 [35].

Zinc can be thought of as playing a structural role in CuZnSOD, a role that is supported by data which demonstrated that zinc binding to the bovine enzyme enhances stability towards denaturation by SDS [36, 37] and guanidinium chloride [38], and towards degradation by proteolysis [39]. In addition, zinc has been demonstrated to be a major contributing factor to the unusual thermal stability in both the bovine [40] and human [41] proteins. Various techniques have also shown that the binding of zinc alone to the apoprotein is sufficient to induce a native fold on the protein [36, 40, 41, 42]. Recent results from our laboratory indicate that the binding of one zinc per dimer is sufficient to induce a native fold for the entire protein (H. Zhu, J. S. Valentine, unpublished results).

Zinc also has important effects on the structure and reactivity of the copper site. It is thought that zinc binding pre-forms the copper site [23] and enhances the affinity of the copper site for metal ions [43]. Along with the aforementioned effects on catalytic activity, zinc deficiency also results in several other changes in copper site reactivity. For instance, zinc-deficient yeast CuZnSOD is more rapidly reduced by ascorbate than is the holoprotein [17] and zinc-deficient human CuZnSOD reacts more readily with peroxynitrite to nitrate tyrosine residues [31]. Zinc-deficient bovine CuZnSOD, as well as derivatives in which the zinc has been replaced by Cu^{2+} and Co^{2+}, are more readily inactivated by peroxide than is the holoprotein [34, 44].

At low pH, where the copper and zinc sites are uncoupled, the copper ion can no longer be reduced by ferrocyanide as is possible at higher pH [45]. In addition, there are gross changes in the electrochemistry of the copper site of bovine CuZnSOD at low pH, making the site more accessible to the electrode [46] as well as changing the redox potential [47].

This study adds to our understanding of the factors that govern the formation of the zinc site in yeast CuZnSOD, and yet our understanding is still far from complete. Based on these studies, a histidine at position 71 seems to be absolutely critical to zinc site formation. Liganding amino acids at positions 63 and 83 also appear to be very important. While disruption of the secondary bridge on one end can be tolerated (H46C, H46R, and G85R), disconnection of this network from the other end is calamitous for the zinc site (H71C, D124G, D124N). Table 1 summarizes the results discussed in this paper.

Table 1. Summary of zinc site status for individual proteins[a]

Protein	Zinc site status	pK_a of transition
Human wild-type reconstituted with Co^{2+}	Td at pH 5.5	4.0
Bovine wild-type as-isolated and reconstituted with Zn^{2+}, Co^{2+}, or Cu^{2+}	Td at pH 4–9	3.8
Yeast wild-type as-isolated	Td at pH 4–9	3.8
Yeast wild-type reconstituted with Co^{2+}	Td at pH 5–9	4.7[c]
Yeast H46C[b]	Like yeast wild-type at high pH	4.66
Yeast H46R[b]	Td at high pH	6.05[c]
Yeast H48Q[b]	Td at high pH	5.58
Yeast H63A[b]	Td at high pH	6.2
Yeast H63E[b]	Td at high pH and at pH 5.5 if Cu^{2+} is reduced to Cu^{+}	7.3
Yeast H71C[b]	Not Td under any condition tested	NA
Yeast H80C[b]	Td at high pH	5.60[c]
Yeast D83A[b]	Not Td under any condition tested	NA
Yeast D83H[b]	Td at high pH and at pH 5.5 if Cu^{2+} is reduced to Cu^{+}	5.8
Yeast G85R[b]	Td at high pH and at pH 5.5 if Cu^{2+} is reduced to Cu^{+}	>5

[a] Td = zinc site with ligands arranged in a distorted tetrahedral geometry
[b] Using the visible spectroscopy of Co^{2+} to monitor the geometry of the zinc site
[c] Value determined with no metal ion bound at the copper site

Conclusions

Understanding the structural parameters of the zinc site is important for the study of mutations that cause ALS [14], as several mutants have been found to have a lowered affinity for zinc [31] and others have been shown to display abnormal zinc site spectroscopy [17]. These changes in zinc site properties may be related to the changes that occur in the wild-type at low pH.

The zinc site seems to be able to exist in two states: one state binds Co^{2+} to a strong binding site with tetrahedral geometry and another forms a weak binding zinc site. The conversion from one to the other is regulated by pH and by the metal ion bound at the copper site. It is also apparent that mutations all over the protein impact this transition, generally raising the pK_a to values closer to physiologically relevant ones. This finding may help explain a recent discovery by this group that yeast CuZnSOD that has been demetallated is composed of two inequivalent subunits, one which is competent to bind metals normally and another that is not [20]. Apo yeast CuZnSOD may contain one subunit that is "frozen" in the wrong configuration. The ramifications of these findings are far reaching, especially concerning the relationship between CuZnSOD and ALS.

In this paper we discuss three yeast mutants that are analogous to mutations that cause ALS. All three ALS mutations investigated appear to have a pH-induced transition similar to that of the wild-type, but with a pK_a that has been shifted up to near physiological pH. This suggests that it is a real possibility that, at physiological pH, these proteins might be expected to contain a substantial portion of zinc sites that have been converted to the weak Co^{2+} binding configuration, thus making this phenomenon potentially relevant for the etiology of ALS.

It is possible that the ALS-causing mutations result in a protein that does not bind zinc as tightly as the wild-type and is therefore less stable and more prone to aggregation and denaturation. It is also possible that the less-stable zinc site allows for undesirable reactivity to occur at an "unprotected" copper site. An even more interesting possibility is that the ALS mutants have modified zinc sites that allow other metals besides zinc to bind at this site and participate in novel chemical reactions. The zinc site in the wild-type bovine CuZnSOD does indeed have a naturally high affinity for copper [48]. This high affinity apparently increases if the pH is raised from 7 and there is no metal bound at the zinc site [48, 49]. It is possible that the ALS mutants possess zinc sites which, while retaining a high affinity for zinc, have increased affinities for other metals.

Since it is now generally accepted that mutations in human CuZnSOD cause ALS via a gain of function [50], it is imperative that we uncover the intricate mechanisms by which this enzyme achieves its remarkable stability. It may be through understanding how this protein folds correctly that we stumble upon the trait or traits that contribute to the toxicity of its mutants. However, we must stress that, while there are many similarities between the yeast and human enzymes, there are distinct differences in their biophysical properties [20, 51]. While it is possible that these differences may simply reflect a greater propensity of the yeast enzyme to undergo certain changes that are characteristic of all CuZnSODs, the fact that they exist must temper any conclusions we draw. It is also quite possible that the yeast protein is an aberration, a fact that diminishes its importance for those researching ALS.

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