ORIGINAL ARTICLE

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Metal ion reconstitution studies of yeast copper-zinc superoxide dismutase: the "phantom" subunit and the possible role of Lys7p

Abstract Using a corrected molar extinction coefficient for yeast apo copper-zinc superoxide dismutase (CuZnSOD), we have confirmed that the metal binding properties of this protein in vitro differ greatly from those of the bovine and human CuZnSOD enzymes. Thus yeast apo CuZnSOD was found to bind only one Co^{2+} per protein dimer under the conditions in which the bovine and human CuZnSOD apoenzymes readily bind two per dimer. The spectroscopic properties characteristic of the two Cu^{2+} plus two Co^{2+} per dimer or four Cu^{2+} per dimer metal-substituted bovine apo CuZnSOD derivatives were obtained for the yeast apoprotein but by the addition of only half of the appropriate metals, i.e., one Cu^{2+} plus one Co^{2+} per dimer or two Cu^{2+} per dimer. This half-metallated yeast CuZnSOD has been characterized by UV-visible and EPR spectroscopy as well as by native polyacrylamide gel electrophoresis. We conclude that yeast apo CuZnSOD, unlike the bovine and human apoproteins, cannot be reconstituted fully with metal ions under the same conditions. Instead, only one subunit of the homodimer, the "normal" subunit, can be remetalled in a fashion reminiscent of the well-characterized bovine protein. The other "phantom" subunit is not competent to bind metals in this fashion. Furthermore, we have shown that CuZnSOD protein isolated from *Saccharomyces cerevisiae* that lacks the gene coding for the copper chaperone, Lys7p, contains only one metal ion, Zn^{2+}, per protein dimer. The possibility that yeast CuZnSOD can exist in multiple conformational states may represent an increased propensity of the yeast protein to undergo changes that can occur in all CuZnSODs, and may have implications for amyotrophic lateral sclerosis.

Introduction

Cytosolic copper-zinc superoxide dismutase (CuZn-SOD) is a homodimeric enzyme containing one copper and one zinc ion per subunit. The two subunits are held together by extensive hydrophobic contacts that involve 9% (50 Å^2) of the total surface of each subunit [1]. In the oxidized form of the enzyme, each Cu^{2+} ion is bound by four histidines (H46, H48, H63, and H120 by the yeast numbering) in a distorted square planar arrangement [1, 2]. There is also a water molecule coordinated in an axial position on the copper ion in the oxidized enzyme [2]. The zinc ion is tetrahedrally coordinated by three histidines (H63, H71, H80) and one aspartate (D83) [2, 3]; H63 serves as the imidazolate bridge between the copper and zinc sites [2] (see Fig. 1). In the reduced state, in solution, H63 detaches
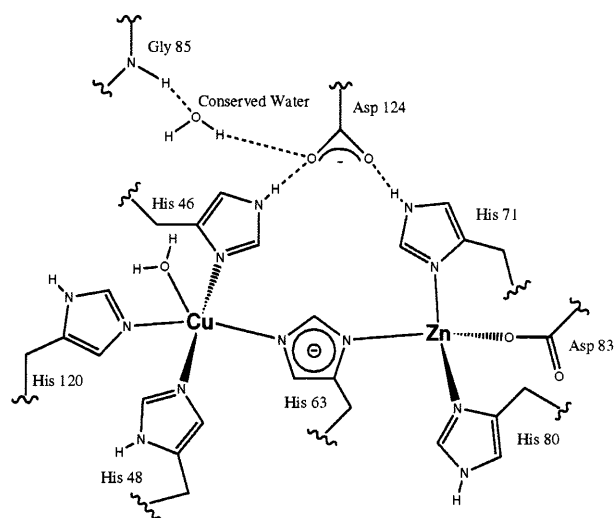

Fig. 1 Active site diagram of CuZnSOD

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and the Cu$^+$ ion is trigonal planar [4]. However, conflicting studies have described the histidine bridge as either intact [5] or broken [3] in the crystalline state.

CuZnSODs are a primary line of defense against the hazards of aerobic life [6], and, as such, they have remained remarkably conserved throughout the evolutionary process [7]. The periplasmic enzyme from bacteria, the cytosolic and plastidic enzymes from eukaryotes, and the extracellular enzyme from mammals all possess significant sequence and structural homology [7–11].

Despite their similarities, CuZnSODs display a wide range of variation with respect to their biophysical characteristics. Some of these differences are strikingly obvious. For example, the cytosolic CuZnSOD is, with one exception [12], a homodimeric enzyme [13], while the extracellular enzyme from humans is tetrameric [14], and the periplasmic enzyme from *E. coli* is monomeric [15]. Other differences are more subtle, as will become apparent from the results of the present study.

Here we demonstrate that cytosolic CuZnSOD apoprotein from the simple yeast *Saccharomyces cerevisiae* (yeast CuZnSOD) displays a marked difference in its metal ion binding behavior as compared to the cytosolic CuZnSODs from higher organisms, notably the bovine (bovine CuZnSOD) and human (human CuZnSOD) enzymes, and we confirm and expand upon the conclusions of previous reports of asymmetric metal ion binding properties of the yeast CuZnSOD apoprotein subunits [16, 17]. This startling difference between the yeast and mammalian enzymes has been obscured by a historical problem involving the extinction coefficient of apo yeast CuZnSOD and its consequent use for protein concentration determination. The biological significance of this asymmetry in the mechanism of in vivo metallation and possible roles for the copper chaperone Lys7p are also discussed.

An asymmetric structure for the bovine CuZnSOD dimer has been proposed to exist during catalysis [18] and there is ample evidence to suggest that the properties of one subunit can affect the properties of the other in an anti-cooperative fashion [19, 20]. Asymmetry has also been noted in the structure of the human amyotrophic lateral sclerosis (ALS) mutant G37R CuZnSOD. These observations are discussed in the light of our findings and we hypothesize that the properties of the yeast protein disclosed herein may be extreme examples of characteristics that are common to all CuZnSODs.

Materials and methods

Lyophilized yeast CuZnSOD was purchased from Peptech (Denmark, batch no. 8997368, product no. 3007; mfg. date 10/17/89) and lyophilized bovine CuZnSOD was purchased from Oxis International (Portland, Ore. lot no. 298-47P). Recombinant human CuZnSOD was isolated from yeast using a modification of a published procedure [21]. The procedure was modified by the addition of a single purification step after gel filtration using Source 15Q ion exchange media (Pharmacia). Purity was confirmed by gel electrophoresis. DEAE-cellulose was purchased from Whatman, and G-75 superfine Sephadex was purchased from Sigma.

K396-11b, a *lys7Δ* strain of *S. cerevisiae*, was generously provided by Dr. George Sprague [22]. YEP600, a multicopy plasmid containing yeast CuZnSOD gene under the control of its own promoter, was transformed into K396-11b and grown in modified synthetic media with 2% dextrose and without added leucine (SD -leu) to select for transformants [23, 24]. For large-scale preparations, transformed *lys7Δ*/YEP600 cells were first grown overnight in SD -leu media and then inoculated into 10 L of YPD. These cultures were grown for 50 h at 30 °C and at 175 rpm to minimize aeration.

Yeast CuZnSOD was purified to >99% purity from large-scale preparations of *lys7Δ*/YEP600 by a two-step process. Yeast were lysed using glass beads in the presence of 0.5 mM PMSF. After centrifugation at 10000 rpm for 15 min to remove insoluble debris, the lysate was dialyzed overnight versus 2.5 mM phosphate buffer, pH 7.0. Purification involved DEAE-cellulose ion exchange chromatography of the lysate, followed by Sephadex G75 superfine size exclusion chromatography. Purity was estimated by SDS-PAGE using Coomassie stain.

Metal concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS) at the Caltech Environmental Analysis Center. Samples were dissolved in 0.1% HNO$_3$. Amino acid analysis by acid hydrolysis was done by the UCLA Microsequencing Facility.

Electrospray ionization mass spectrometry was performed at the UCLA Center for Molecular and Medical Sciences Mass Spectrometry using a Perkin-Elmer/Sciex API III equipped with an Ion Spray source. Protein was dissolved in 50:50:0.1 acetonitrile:water:formic acid and a 10 μL aliquot was directly injected into the electrospray source. Conditions were as follows: *m/z* range, 600–2000; mass step, 0.3; dwell time, 1.2 msec; scan speed, 5.84 sec/scar; flow rate, 10 μL/min.; orifice voltage 90 V. Protein concentrations were roughly 20 μM.

UV-visible spectra from 900 to 240 nm were determined using a Cary 3 UV-visible spectrometer (Varian; Sunnyvale, Calif.). Samples were centrifuged for 1 min before spectra were taken to remove turbidity. Analysis of UV spectra in 6 M guanidine-HCl was performed by mixing 50 μL protein solution in 100 mM acetate buffer, pH 5.5, with 50 μL 12 M guanidine-HCl (dissolved by heating). Spectra were then taken from 240 to 300 nm. EPR spectra were obtained with a Bruker ER200d-SRC X-band EPR spectrometer (Billerica, Mass.) equipped with an ER43MRD microwave bridge, an ER4111VT variable temperature unit, and an EIP 548 microwave frequency counter. Parameters for 90 K spectra are as follows: field center, 3000 G; sweep width, 1600 G; frequency, 9.54 GHz; modulation amplitude, 5.0 G; power, 20.7 mW; time constant, 640 ms; receiver gain 5.0×10^5; sweep time 200 s.

Metal-free proteins were obtained by published procedures [25]. The holo protein is first dialyzed against 100 mM acetate buffer at pH 3.8 in the presence of 10 mM EDTA (four times against 1 L each time). EDTA is 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). Metal-substituted proteins were obtained by directly infusing solutions of the apoprotein with the appropriate metal sulfate salt (Ag$^+$ was added as AgNO$_3$). Stock metal solutions were either 10 mM or 100 mM depending on concentration and volume of protein, which was generally 200 μL of approximately 0.3 mM. For bovine CuZnSOD and human CuZnSOD the second equivalent Co^{2+}/dimer bound slowly, so a period of 24 h. was allowed for the solutions to equilibrate.

Metal titrations were carried out in 100 mM acetate buffer at pH 5.5. The pH was adjusted by addition of 100 mM NaOH and was measured using an Orion Research Microprocessor Ionalyzer/901 (Boston, Mass.) with an Ingold microelectrode (Wilmington, Mass.).

Native gel electrophoresis using 9% acrylamide was performed in accordance with published procedures at 4 °C [26].

Results

Protein concentration determination

The historical problem with the extinction coefficient of apo yeast CuZnSOD that was alluded to above involved a wide variation in the values that have been reported in the literature; thus it was critical that we redetermine this value. If a protein sequence is known and there are no cofactors present that absorb in the UV, one of the most reliable methods to estimate the molar extinction coefficient is the Edelhoch method [27], which utilizes the unique UV features of aromatic amino acid residues. According to this method, the molar extinction coefficient at 280 nm was determined using the following equation:

$$\varepsilon_{280} = (5500)(\#trp) + (1490)(\#tyr) + (125)(\#cystine) \text{ M}^{-1}\text{ cm}^{-1}$$

Yeast apo CuZnSOD has no tryptophan residues, one tyrosine residue, and one disulfide bond per subunit, resulting in a calculated ε_{280} of 3230 M^{-1} cm^{-1}/dimer. Bovine apo CuZnSOD has identical numbers of tryptophan, tyrosine, and cystine residues [7], and therefore it is expected to have the same extinction coefficient at 280 nm, i.e., 3230 M^{-1} cm^{-1}/dimer, a value that agrees well with the experimentally determined value of 2920 M^{-1} cm^{-1}/dimer for the bovine protein. Human CuZnSOD contains one tryptophan residue, no tyrosine residues, and one cystine per subunit and has a calculated ε_{280} of 11500 M^{-1} cm^{-1}/dimer, which agrees well with the experimentally determined value of 10800 M^{-1} cm^{-1}/dimer.

Protein concentration can also be estimated by absorbance at 258 nm, which is due primarily to phenylalanine residues. One phenylalanine residue has an extinction coefficient of 200 M^{-1} cm^{-1} [28]. The absorbance of the six phenylalanines per subunit in yeast CuZnSOD plus the small residual absorbance from tyrosine at this wavelength should give an ε_{258} of at least 2400 M^{-1} cm^{-1}/dimer and therefore an OD$_{258}$:OD$_{280}$ ratio of approximately 1:1. Examination of the UV spectrum of apo yeast CuZnSOD indicates that this is indeed the case (see Fig. 2). Bovine CuZnSOD has four phenylalanines per subunit [7] and therefore should have an OD$_{258}$:OD$_{280}$ ratio of slightly less than 1:1, as is in fact observed (see Fig. 2).

Protein concentration was also estimated using amino acid analysis and the tyrosinate difference spectral method. From amino acid analysis data, the concentration of each amino acid was divided by the number of times that amino acid appears in the CuZnSOD sequence, and the resulting values were averaged to obtain the final protein concentration. The tyrosinate method uses the differential absorbance of tyrosine (pH 6) and tyrosinate (pH 11) at 295 nm [28]. Both amino acid analysis and the tyrosinate difference spectral method agree with the extinction coefficient calculated
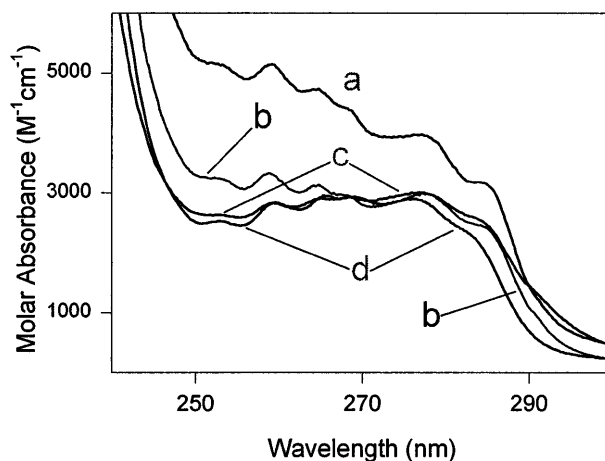

Fig. 2 Ultraviolet spectra from 240 to 300 nm for protein samples in 100 mM acetate buffer, pH 5.5. *a*, 0.258 mM yeast CuZnSOD reconstituted with 1 equiv Cu^{2+} per dimer; *b* 0.280 mM apo yeast CuZnSOD; *c*, 0.243 mM yeast CuZnSOD isolated from *lys7Δ* yeast; *d* 0.155 mM apo bovine CuZnSOD. Molar absorbance units are M^{-1} cm^{-1} per protein dimer

by the Edelhoch method with values of 3300 M^{-1} cm^{-1}/dimer and 3600 M^{-1} cm^{-1}/dimer, respectively. Since the Edelhoch method for molar extinction coefficient determination agrees well with the experimentally determined value (based on metal titrations), we have used the latter value to determine protein concentrations in this study (3000 M^{-1} cm^{-1}) since this value allows us to properly metallate one subunit of the yeast CuZnSOD dimer.

Copper has significant UV absorption and therefore can interfere with protein concentration determination. The very intense UV absorbance peak for Cu^{2+} centered at approximately 240 nm interferes with the 258 nm protein peak to a greater extent than with the 278 nm peak (see Fig. 2). The OD$_{258}$:OD$_{280}$ ratio can be used to estimate crudely the residual copper content in metal ion depleted protein. By contrast, Zn^{2+} and Co^{2+} ions do not interfere with the UV spectra of CuZnSOD (data not shown), as expected from previous reports on the bovine enzyme [29, 30].

Metal content determination

Since metal contamination can lead to erroneous titration results, we determined the concentrations of copper and zinc in our protein samples. Apoprotein preparations used in this study were found to contain <10% residual metal ions by ICP-MS, although actual values varied slightly for each preparation. Yeast CuZnSOD isolated from *lys7Δ* yeast was found to contain 0.92 equiv Zn^{2+} and 0.14 equiv Cu^{2+} per dimer.

Mass spectrometry

To rule out the possibility of damaged protein, we obtained a mass spectrum of apo yeast CuZnSOD which shows a major peak of molecular mass 15723.33 ± 1.31, corresponding to the metal-free protein, which represents the vast majority of the sample. A minor peak with a molecular weight that is 39 Da higher is also present in the mass spectrum (see Fig. 3).

Metal ion binding studies: Co^{2+}

Co^{2+} is known to bind selectively to the zinc sites in each subunit of apo bovine CuZnSOD in acetate buffer, pH 5.5, resulting in three intense and overlapping bands in the visible spectrum [25], thus making Co^{2+} an excellent probe of the metal binding properties of CuZnSOD. As expected, Co^{2+} binds to apo yeast CuZnSOD in an apparently similar fashion under the same conditions, but the endpoint of the titration corresponds to 0.55 Co^{2+} per subunit, i.e., 1.1 Co^{2+} per protein dimer (see Fig. 4). The observed extinction coefficient of 425 M^{-1} cm^{-1} per Co^{2+} is characteristic of tetrahedrally bound Co^{2+} [31]. Identical titrations for apo bovine CuZnSOD and human CuZnSOD gave endpoints at approximately 2 equiv. Co^{2+} per dimer; they are shown in Fig. 4B.

Figure 5A and 5B shows optical spectra of apo yeast CuZnSOD solutions to which 2 equiv of Co^{2+} per dimer have been added, at two different pH values. The difference spectra represent the contribution of the second equivalent of Co^{2+} plus any changes in the absorptivity of the first equivalent of Co^{2+}.

Metal ion binding studies: Cu^{2+}

The results of the titration of apo yeast CuZnSOD with Cu^{2+}, in acetate buffer, pH 5.5, can be seen in Fig. 6.
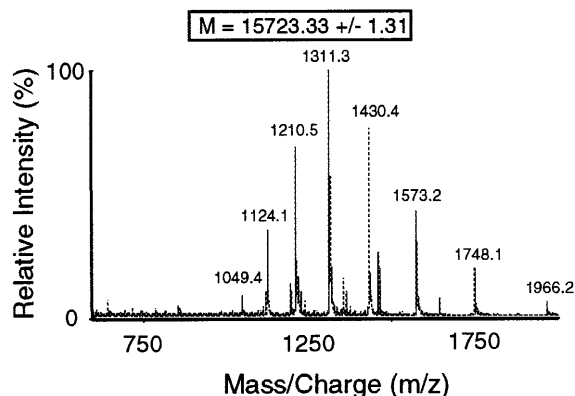

Fig. 3. Electrospray ionization mass spectrum of apo yeast CuZnSOD. Protein was dissolved in 50:50:0.1 acetonitrile:water:formic acid. Protein concentration was approximately 20 μM and the parameters are listed in the Materials and methods section
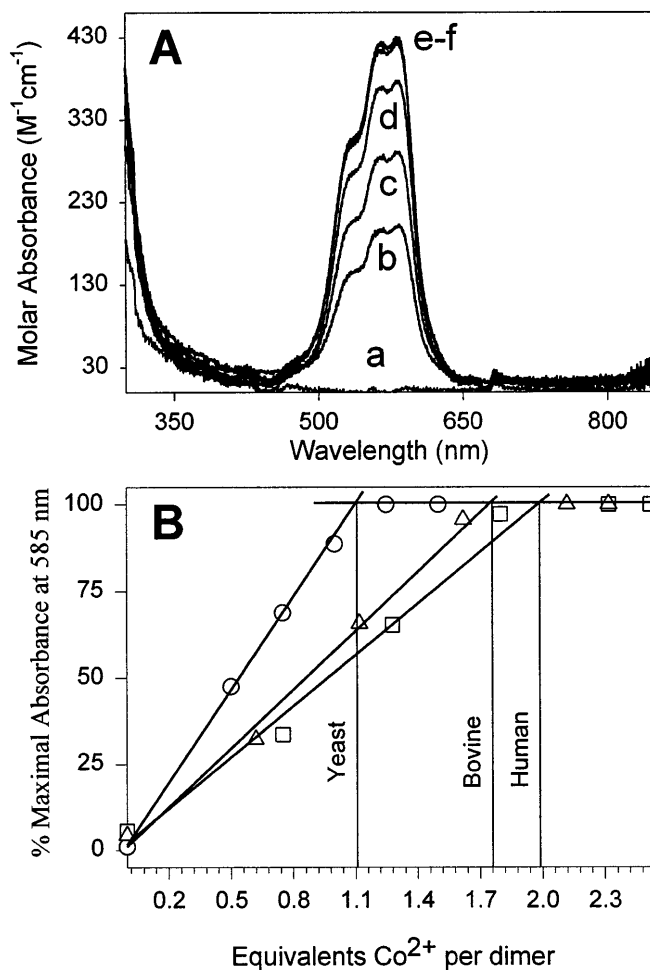

Fig. 4 A, B Co^{2+} titration of apo yeast CuZnSOD, bovine CuZnSOD, and human CuZnSOD in 100 mM acetate buffer, pH 5.5. **A** Co^{2+} titration of 0.340 mM apo yeast CuZnSOD monitored by visible absorption spectroscopy at 300–850 nm. *a*, apo yeast CuZnSOD; *b*, 0.5 equiv Co^{2+} per dimer; *c*, 0.75 equiv Co^{2+} per dimer; *d*, 1.00 equiv Co^{2+} per dimer; *e*, 1.25 equiv Co^{2+} per dimer; *f* 1.50 equiv Co^{2+} per dimer. **B** Endpoints of Co^{2+} titrations determined by plotting molar absorptivity at 585 nm (where Co^{2+} has the greatest absorptivity) versus number of equivalents Co^{2+} added. ○, 0.340 mM apo yeast CuZnSOD; △, 0.308 mM apo bovine CuZnSOD; □, 0.302 mM apo human CuZnSOD. Molar absorbance units are M^{-1} cm^{-1} per protein dimer. The extinction coefficient per Co^{2+} for the yeast enzyme is 425 M^{-1} cm^{-1} while the extinction coefficient per Co^{2+} for both the human and bovine enzymes is 370 M^{-1} cm^{-1}

The first equivalent per dimer binds to a protein site with a λ_{max} of 670 nm. The second equivalent per dimer binds to a site with a peak maximum at >810 nm. The EPR spectrum (Fig. 8, below) of apo yeast CuZnSOD to which 2 equiv Cu^{2+} per dimer have been added is strikingly similar to the characteristic spectrum of bovine CuZnSOD with Cu^{2+} bound to all four metal sites per dimer, that is the result of magnetic coupling of the Cu^{2+} ions across the imidazolate bridge [32].
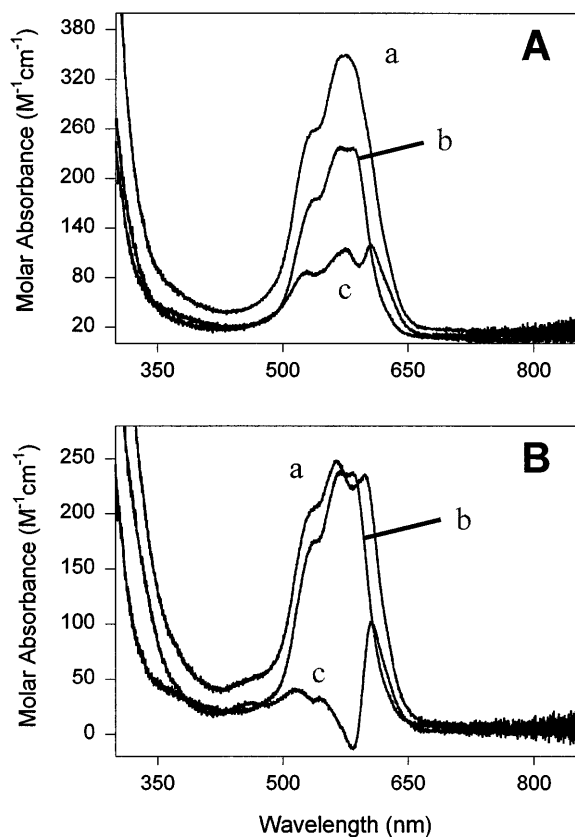
Fig. 5 A, B Visible spectra from 300 to 850 nm for 0.308 mM apo yeast CuZnSOD to which 2 equiv Co²⁺ per dimer have been added. Protein is dissolved in 100 mM acetate buffer. **A** *a*, derivative at pH 6.79; *b*, derivative at pH 5.71; *c*, difference *a–b*. **B** *a*, derivative at pH 8.90; *b*, derivative at pH 5.71; *c*, difference a–b. Molar absorbance units are M^{-1} cm^{-1} per protein dimer
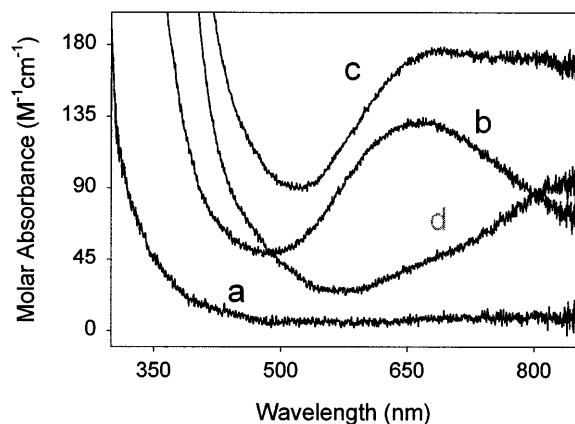

Fig. 6 Cu^{2+} titration monitored by visible spectroscopy from 300 to 850 nm for 0.333 mM apo yeast CuZnSOD in 100 mM acetate buffer, pH 5.5. *a*, apoprotein; *b*, 1 equiv Cu^{2+} per dimer; *c*, 2 equiv Cu^{2+} per dimer; *d* difference c–b. Molar absorbance units are M^{-1} cm^{-1} per protein dimer
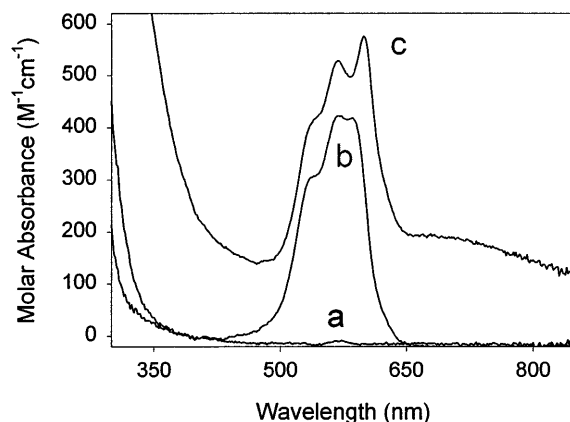

Fig. 7 Visible spectra from 300 to 850 nm for various derivatives of 0.328 mM yeast CuZnSOD in 100 mM acetate buffer, pH 5.5. *a*, apo; *b*, 1 equiv Co^{2+} per dimer; *c* 1 equiv Co^{2+} and 1 equiv Cu^{2+} per dimer. Molar absorbance units are M^{-1} cm^{-1} per protein dimer

Metal ion binding studies: Co^{2+} plus Cu^{2+}

In an attempt to make the copper and cobalt substituted derivative of yeast CuZnSOD, we first added 1 equiv Co^{2+} per dimer to the apoprotein and obtained a spectrum with the characteristic peaks for tetrahedral Co^{2+} (see Fig. 7). Subsequent addition of 1 equiv Cu^{2+} per dimer resulted in the appearance of the 670 nm copper-in-the-copper site band and the red shifting of the 585 nm cobalt band that is believed to result from the formation of the imidazolate bridge [33]. The EPR spectrum of this half-metallated species shows the disappearance of the Cu^{2+} EPR signal that is attributed to antiferromagnetic coupling of the two paramagnetic metals across the bridge (see Fig. 8). The EPR spectrum after addition of 1 equiv per dimer each of Zn^{2+}

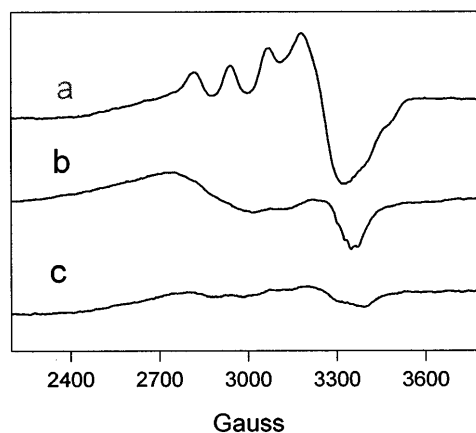**Fig. 8.** EPR spectra of yeast CuZnSOD taken at 90 K in 100 mM acetate buffer, pH 5.5. *a*, apoprotein + 1 equiv Cu^{2+} and 1 equiv Zn^{2+} per dimer; *b*, apoprotein + 2 equiv Cu^{2+} per dimer; *c*, apoprotein + 1 equiv Cu^{2+} and 1 equiv Co^{2+} per dimer. Protein concentrations are 0.230 mM and the EPR parameters are listed in the Materials and methods section
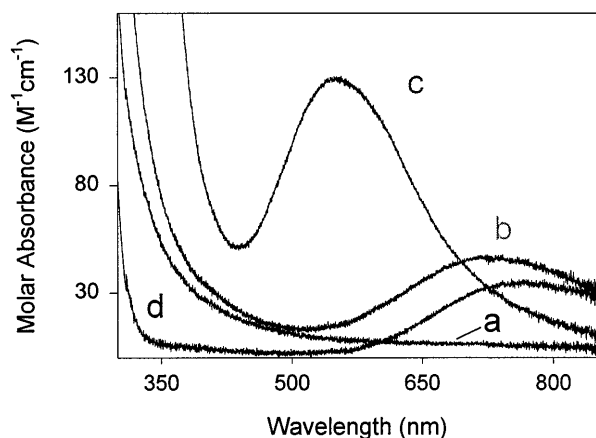
Fig. 9 Visible spectra from 300 to 850 nm for various derivatives of 0.500 mM yeast CuZnSOD in 100 mM acetate buffer. *a* apoprotein + 1 equiv Zn^{2+} and 1 equiv Ag^+ per dimer, pH 5.5; *b*, apoprotein + 1 equiv Zn^{2+}, 1 equiv Ag^+, and 1 equiv Cu^{2+} per dimer pH 5.5; *c*, same as *b* at pH 9.0; *d*, Cu^{2+} in 100 mM acetate buffer, pH 5.5. Molar absorbance units are $M^{-1} cm^{-1}$ per protein dimer

and Cu^{2+} to apo yeast CuZnSOD in acetate buffer, pH 5.5, is also given for comparison in Fig. 8.

Metal ion binding studies: Zn^{2+} plus Ag^+ plus Cu^{2+}

To probe metal binding of the "phantom subunit", sequential additions of 1 equiv per dimer of Zn^{2+}, then 1 equiv Ag^+, then 1 equiv Cu^{2+} were made to apo yeast CuZnSOD in acetate buffer, pH 5.5. Addition of Zn^{2+} and Ag^+ produced no appreciable changes in the UV-visible spectrum (see Fig. 9). The subsequent addition of Cu^{2+} resulted in the appearance of a band at 710 nm. (The spectrum of Cu^{2+} in acetate buffer is also shown for comparison in Fig. 9.)

Increasing the pH from 5.5 to 9.0 resulted in a blue shift of the λ_{max} and an increase in intensity of the copper band (Fig. 9). The further addition of Co^{2+} or
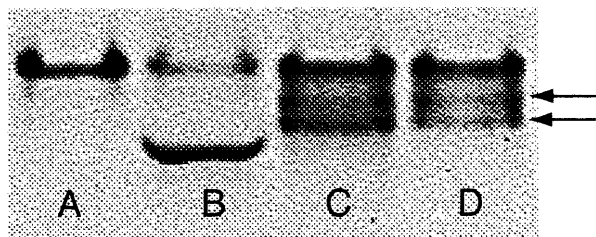

Fig. 10 Native gel electrophoresis of yeast CuZnSOD derivatives with 9% acrylamide at 4 °C. Lane *A*, purchased holo yeast CuZnSOD. Lane *B*, the apo derivative of the purchased yeast CuZnSOD. Lane *C*, apo purchased yeast CuZnSOD + 1 equiv Zn^{2+} per dimer. Lane *D*, yeast CuZnSOD isolated from *lys7Δ* yeast. *Arrows* indicate minor bands in lanes *C* and *D*. Samples are 10× diluted with water from approximately 0.3 mM solutions in 100 mM acetate buffer, pH 5.5. 5 μL of the 10× diluted protein solutions was run in each lane

more Zn^{2+} did not affect the spectrum (data not shown).

Native gel electrophoresis

Holo and apo yeast CuZnSODs migrated differently on native gels, with the apo derivative migrating faster (see Fig. 10). This difference allowed us to monitor changes in protein structure upon metal binding. The addition of 1 equiv of Zn^{2+} per dimer to the apoprotein induced a change in mobility, resulting in three bands: the major band with a mobility similar to that of the holoprotein and two others with faster mobilities not corresponding to the apoprotein. The CuZnSOD protein isolated from *lys7Δ* yeast, which contained 1 Zn^{2+} per dimer (see above), migrates similarly to the sample of apoprotein remetallated with 1 Zn^{2+} per dimer.

Metallation of protein isolated from *lys7Δ* yeast

The purified yeast CuZnSOD from *lys7Δ*/YEP600 was found to contain 0.14 equiv Cu^{2+} per dimer and 0.92 equiv Zn^{2+} per dimer. Since this protein is metal deficient we investigated its ability to bind metals. Addition of 1 equiv Cu^{2+} per dimer to this as-isolated protein resulted in a band in the visible spectrum at 670 nm. Addition of a second equivalent of Cu^{2+} per dimer gave an increase in absorbance at 750 nm, which we attribute to unbound Cu^{2+}. Addition to the as-isolated protein of up to 1.5 equiv Co^{2+} per dimer had no effect on the visible spectrum (see Fig. 11 A and 11B).

Discussion

Determination of extinction coefficient

Since an estimation of protein concentration is critical to the proper interpretation of this data, it was necessary to determine a reliable extinction coefficient for apo yeast CuZnSOD. Table 1 summarizes the reported extinction coefficients dating back to 1982 [16, 25, 34–39] as well as those determined in this study. As should be evident from the table, there is a wide variation in the reported values as well as a discrepancy between these values and the value calculated by the Edelhoch method.

In a recent paper from this laboratory, we reported a value for the extinction coefficient that was based on metal titration studies and which is almost twice the calculated extinction coefficient [37]. We now know that value to be incorrect, but it was this two-fold difference that alerted us to fact that there might indeed be an asymmetric dimer in the yeast protein, as was reported previously [16, 17].

Since an extinction coefficient that is too high by a factor of two results in a protein concentration that is
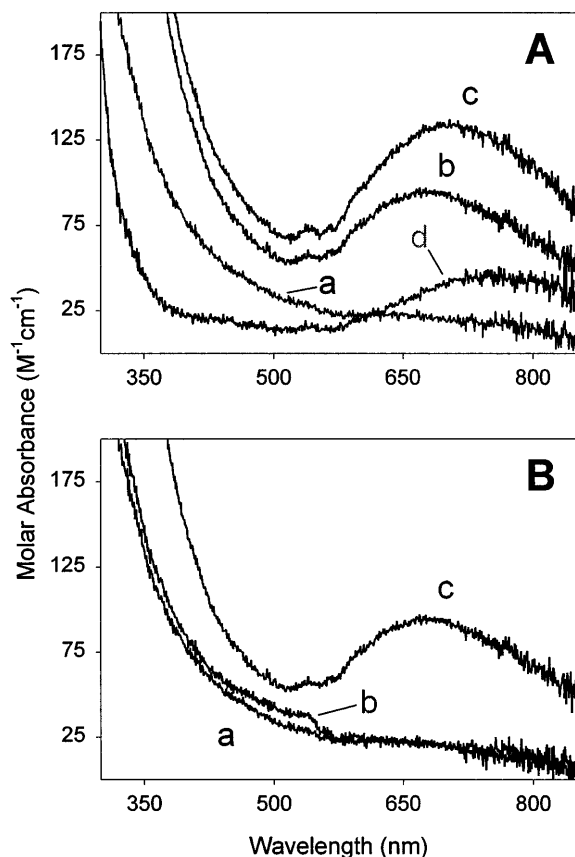
Fig. 11 A, B Visible spectra from 300 to 850 nm of various derivatives of yeast CuZnSOD isolated from *lys7Δ* yeast in 100 mM acetate buffer, pH 5.5. **A** Cu^{2+} titration of the as-isolated protein *a* as-isolated protein; *b* as-isolated protein + 1 equiv Cu^{2+} per dimer; *c*, as-isolated protein + 2 equiv Cu^{2+} per dimer; *d*, difference *c–b*. **B** Co^{2+} and Cu^{2+} titration of the as-isolated protein: *a* as-isolated protein; *b*, as-isolated protein + 1.5 equiv Co^{2+} per dimer; *c*, as-isolated protein + 1 equiv Cu^{2+} per dimer. Molar absorbance units are M^{-1} cm^{-1} per protein dimer

Edelhoch method is correct, only one subunit per dimer of the apoprotein is binding metal ions, and the concentration of yeast CuZnSOD has been systematically underestimated by a factor of two.

Two recent papers supporting the Edelhoch method have demonstrated that this method is accurate for a vast number of different proteins [27, 40]. The margin of error is generally within 10%, but it increases slightly if there is no tryptophan, which is the case with yeast CuZnSOD and bovine CuZnSOD. The tyrosinate difference spectral method also agrees with the Edelhoch method; however, both of these results rely on a single tyrosine residue. For independent validation of the value, we used amino acid analysis.

The OD_{258}:OD_{280} ratio for yeast CuZnSOD provides very convincing evidence for the validity of the Edelhoch method. The fact that this ratio is, as predicted, approximately 1:1 supports the use of calculations to determine the extinction coefficient as described above (see Results and Fig. 2).

Perhaps more important evidence is the fact that both the calculated and experimental extinction coefficients for apo bovine CuZnSOD are in agreement and that both subunits of the dimeric bovine enzyme can be fully metallated using this value (see Fig. 4B). Since bovine CuZnSOD and yeast CuZnSOD have similar aromatic makeup, they are expected to have approximately the same absorptivity at 280 nm.

The fact that the tyrosines in these two proteins are not in the same position does allow for the possibility of differential environmental effects on the nature of tyrosine absorptivities, and, indeed, visualization of the protein structures from the X-ray structural coordinates revealed that the tyrosine residue in yeast CuZnSOD is mostly buried inside the β-barrel [2], while the tyrosine residue in bovine CuZnSOD is mostly solvent exposed [1]. However, we have compared the UV spectra of apo yeast CuZnSOD in acetate buffer and in 6 M guanidine-HCl and have found that while there is a slight change in the shape (less sharp) and position (slight blue shift) of the tyrosine absorptivity upon guanidinium treatment, there is almost no change in the intensi-

correspondingly low, our metal titration results could be explained by three theories: (1) the Edelhoch method cannot be applied to this protein; (2) half the protein was modified or damaged in some way; or (3) the

Table 1 Summary of reported molar extinction coefficients for apo yeast CuZnSOD

Source	Molar extinction coefficient per dimer ($\varepsilon_{278\,nm}$ M^{-1} cm^{-1})	Method
Dunbar et al. 1982 [34]	4800[a]	Amino acid analysis
Dunbar et al. 1984 [16]	4160	Amino acid analysis
Ming et al. 1988 [38]	4480	Lowry assay
Kofod et al. 1991 [35]	4900[b]	Based on Dunbar et al. 1982
Nishida et al. [39]	4900	Bradford assay
Lu et al. 1996 [36]	4720	Lowry assay
Lyons et al. 1997 [37]	5800	Metal titration
This study (experimental)	3000	Co^{2+} titration
This study (calculated)	3230	Edelhoch method
Bovine apo [25]	2920[b]	Lowry assay
Bovine apo (calculated)	3230	Edelhoch method

[a] Approximated from Fig. 4 in [34]
[b] Extinction coefficient at 258 nm instead of 278 nm

ty at 280 nm (data not shown). This finding is consistent with denaturation studies of albumin [28]. Seeing that there is little difference in tyrosine intensity between the native and guanidinium-treated proteins, we conclude that the two-fold difference seen for the calculated and experimental extinction coefficients is not due to the local environment of the tyrosine. Thus we are confident that the Edelhoch method has given us a reasonable approximate value for the molar extinction coefficient. Since the Edelhoch value (3230 M^{-1} cm^{-1}) and the experimental value based on metal titrations (3000 M^{-1} cm^{-1}) are within 10% of each other, we have assumed that the experimental value to be the correct one.

Has the apoprotein been modified or partially denatured?

To remove the metals, the protein is subjected to low pH (3.8) for a significant period of time, and it is possible that undesirable chemistry occurs at this pH, leaving the protein covalently modified. Alternatively, the protein might have been irreversibly denatured at this pH. To address these possibilities, we present mass spectrometric data that demonstrate a peak with the correct molecular weight (see Fig. 3). While there is a minor peak with a slightly larger molecular weight, it does not represent a significant portion of total protein. In addition, it is unlikely that damaged or misfolded protein is the cause of the aberrant metal binding characteristics as it is improbable that every preparation would result in the exact same proportions of unmodified dimers/properly folded and damaged/misfolded dimers (that being 1:1).

The subunits in the yeast CuZnSOD dimer have drastically different metal binding characteristics

If we assume that the extinction coefficient calculated by the Edelhoch method is correct, then the simplest explanation of the results obtained by previous investigators is that the subunits in the protein dimer are inequivalent. For this reason, we reexamined the metal binding properties of apo yeast CuZnSOD.

Titration of the apo yeast CuZnSOD with Co^{2+}, shown in Fig. 4, clearly shows binding of only one tetrahedral Co^{2+} per dimer. This result is in sharp contrast to those for bovine and human CuZnSOD apoproteins which each bind two tetrahedral Co^{2+} ions per dimer [38, 41]. Under these same conditions, the second equivalent per dimer of Co^{2+} either does not bind to yeast CuZnSOD at all or does so in an octahedral orientation, which would result in minimal absorbance in the visible region [31].

The second equivalent of Co^{2+} will bind if the pH of the buffering solution is raised to 7 or higher (see Fig. 5A and 5B). However, comparison of the spectra of these high pH derivatives with published spectra of fully cobalt-substituted bovine CuZnSOD (bovine Co-CoSOD) indicate that the second equivalent of cobalt is binding at a copper site [29, 38] and not in the zinc site of the second subunit. Thus the two subunits in the yeast CuZnSOD dimer are not behaving equivalently as they would in bovine CuZnSOD and human CuZnSOD.

It could be argued that results based on Co^{2+} binding are suspect since Co^{2+} is not the native metal. However, Co^{2+} is an excellent spectroscopic probe for the zinc site of CuZnSOD. Cobalt-substituted bovine CuZnSOD (bovine CuCoSOD) is fully functional as a SOD [25] and a crystal structure of bovine CuCoSOD shows that there are no perturbations in the structure [42].

Cu^{2+} can also be used as a probe of the binding sites of CuZnSOD. Copper-substituted bovine CuZnSOD (bovine CuCuSOD) is fully active [32], but no X-ray crystal data have been published yet for this derivative. For apo bovine CuZnSOD in acetate buffer, at pH 5.5, Cu^{2+} binds first to the two copper sites of the dimer, with a broad visible band at 670 nm ($\varepsilon = 150$ M^{-1} cm^{-1}), and then to the two zinc sites, with another broad visible band centered at 810 nm with roughly the same extinction coefficient [25]. Figure 6 shows the analogous titration for yeast CuZnSOD. The first equivalent per dimer binds at 670 nm, indicative of binding at the copper site of one subunit. The second equivalent of Cu^{2+} does not then bind to the copper site of the second subunit as it would in bovine CuZnSOD. Instead it binds at 810 nm, indicative of binding at the zinc site. EPR confirms that both equivalents of Cu^{2+} have bound to the same subunit.

EPR and visible spectroscopy have also confirmed that we can make what appears to be the fully copper- and cobalt-substituted proteins, based on comparison with the spectra of bovine Cu_2Co_2SOD and Cu_2Zn_2SOD, after addition of only half the metals required to fill all four metal ion binding sites per dimer (see Figs. 7 and 8). In other words, we can completely reconstitute the spectroscopic properties catalogued for bovine Cu_2Zn_2SOD, Cu_2Cu_2SOD, and Cu_2Co_2SOD with only half the appropriate metals added to apo yeast CuZnSOD. This observation indicates that only one subunit of the yeast CuZnSOD dimer is competent to bind metals in a normal bovine- or human-like fashion [38, 41].

The "phantom" subunit is capable of binding copper, but the spectroscopic properties are abnormal

The fact that there is asymmetry in yeast CuZnSOD dimers that have been stripped of their metals in this admittedly artificial manner explains the historical problem of the extinction coefficient; which is to say that one subunit behaved as though it was not present during previous metal reconstitution experiments.
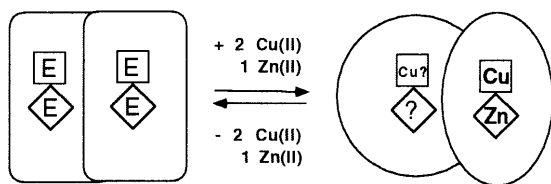
Scheme 1 Scheme for asymmetric metallation of apo yeast CuZnSOD; E = empty site

What remains to be proven is the existence of the unmetallated, or "phantom", subunit.

To visualize the visible spectroscopy of metal ions binding to the phantom subunit, we blocked the sites of the "normal" subunit with spectroscopically silent d^{10} metal ions. One equivalent of Ag^+ and one equivalent of Zn^{2+} per dimer were added to apo yeast CuZnSOD. These two metal ions have higher affinities than Cu^{2+} for the copper and zinc binding sites, respectively, and thus block the normal sites from further metal binding [43, 44]. Subsequent addition of 1 equiv per dimer of Cu^{2+} to this species yielded a band in the visible region at 710 nm with an extinction coefficient of 50 M^{-1} cm^{-1} (see Fig. 9). This band is not due to free Cu^{2+} in acetate buffer, which absorbs instead at 750 nm. The 710 nm band is due to protein-bound copper which is probably bound, albeit abnormally, to the copper site of the phantom subunit. This assignment is supported by the fact that there is a blue shift of the copper peak accompanied by an increase in the intensity upon raising the pH of this silver-zinc-copper substituted derivative (see Fig. 9). This behavior is observed for Cu^{2+} in the copper site of CuZnSOD when it is uncoupled with the zinc ion in the derivative His63Ala CuZnSOD in which the bridging histidine has been replaced by alanine [45].

The preceding observation is taken as evidence that the second subunit of yeast CuZnSOD is capable of binding copper, but only after both metal ion binding sites of the first subunit are full. Spectroscopic evidence for either Co^{2+} or Zn^{2+} binding to the phantom subunit could not be obtained under any of the conditions assayed (including heat and urea denaturation, addition of reducing agents, or excess metal). Scheme 1 details a model of the metal binding characteristics of yeast CuZnSOD.

Other evidence for subunit asymmetry

Asymmetry in yeast CuZnSOD was proposed previously in a series of papers starting with reports of data from ^{111}Cd PAC (perturbed angular correlation) spectroscopy [46] and extended X-ray absorption fine structure spectroscopy [47] which suggested that the two subunits in cadmium-substituted yeast CuZnSOD (cadmium substituted for zinc) were either inequivalent or equivalent yet slowly exchanging between two states, although these data were later challenged by the same

group using ^{113}Cd NMR [35]. Later it was shown by metal dissociation kinetics [34] and by cobalt binding [16, 17] that the two subunits were not identical and that only one of them could be reconstituted with Co^{2+}. Although they arrived at the correct conclusion, this group did not use the correct extinction coefficient, so that their results were later challenged [38]. In hindsight, it is not surprising that the properties of yeast CuZnSOD were the subject of so much controversy since one subunit of the dimer did not participate in these metal titration experiments.

Evidence supporting asymmetry in the yeast CuZnSOD dimer also includes urea denaturation studies showing that, while both bovine CuZnSOD and yeast CuZnSOD were reversibly inactivated by incubation in 8 M urea, dilution of urea-inactivated yeast CuZnSOD resulted in immediate recovery to 50% normal levels followed by slower recovery to full activity [48]. This biphasic renaturation implied some sort of subunit cooperativity in the refolding pathway. Also, not all of the copper in yeast CuZnSOD could be detected by EPR, perhaps indicating that one subunit preferred to be reduced while the other preferred to be oxidized [49]. Some recent theoretical studies on bovine CuZnSOD [50] and a crystal structure of a mutant form of human CuZnSOD [51] also suggest that the subunits in other CuZnSODs may not always be equivalent.

Is this asymmetry a distinct property of yeast CuZnSOD?

It is clear that yeast CuZnSOD is different from CuZnSOD from other organisms. For instance, when NMR spectra of bovine CuZnSOD, human CuZnSOD, and swordfish CuZnSOD were compared to the spectrum of yeast CuZnSOD, they were nearly identical except for one zinc binding ligand resonance that was shifted in the yeast protein [52]. Also, yeast CuZnSOD was shown to interact with lipid membranes to a much greater degree than did bovine CuZnSOD, human CuZnSOD, swordfish CuZnSOD, or canine CuZnSOD, suggesting different surface features [53]. Yeast CuZnSOD also has a relatively low melting temperature compared to enzymes from other species [54]. These differences may reflect deviant properties of the yeast enzyme or they may represent a greater propensity of the yeast enzyme to undergo changes that are universal for CuZnSODs. It is interesting in this regard that, although the human and bovine apoproteins bind two equivalents of Co^{2+}, the second equivalent binds much more slowly than the first (data not shown, see Materials and methods section). This observation has led us to postulate that slow binding to the second subunit in the mammalian proteins is related to the metal binding properties of the yeast protein and that perhaps the binding of metals to the phantom subunit in yeast CuZnSOD is an extremely slow kinetic process (months to years). Other investigators have also docu-

mented anti-cooperative metal binding in the bovine protein, showing that the complete reconstitution of one subunit slows down the reconstitution of [19] and decreases the copper binding affinity of the other subunit [20].

Are there multiple conformations of yeast apo CuZnSOD?

It is possible that the protein unfolds during the demetallation procedure in such a way that one subunit remains in the native conformation and the other subunit is converted to an alternative, yet stable, metal binding-incompetent conformation. It is also possible that both subunits in the apoprotein are equivalent (or that the subunits are dissociated) and that the binding of one zinc per dimer results in the aforementioned conformational change.

Results of thermal denaturation studies may support the idea of multiple states. It was demonstrated that yeast CuZnSOD could be reversibly denatured, and the thermograms consisted of two species with different melting points. While the majority of the protein denatured at the higher temperature, the proportion of the lower melting band increased upon each remelting [55].

Data supporting multiple conformations are also shown in Fig. 10. The holo enzyme and the apo enzyme migrate differently on native gels. However, apoprotein + 1 equiv Zn^{2+} per dimer and the protein isolated from *lys7Δ* yeast (also containing 1 equiv Zn^{2+} per dimer) have a major band that migrates with the holo enzyme. Thus, one zinc per dimer seems to be sufficient to induce a more native-like structure. It is also clear from the native gel that apoprotein plus 1 equiv Zn^{2+} per dimer has multiple charge isomers, resulting in three bands on the gel. These bands may be the result of multiple conformations.

Conformational changes induced by binding of Zn^{2+}

Large conformational changes accompany the binding of Zn^{2+} to apo CuZnSOD. This conclusion has been drawn from NMR studies [56], second-derivative UV spectroscopy of phenylalanine and tyrosine residues [57], IR spectroscopy [58], and SDS denaturation studies [59]. Anti-cooperative metal binding has also been demonstrated for CuZnSOD, meaning that the binding of metal to one subunit affects metal affinity of the adjacent subunit [60, 61]. Lippard et al. [56] even showed evidence suggesting that the addition of only 1 equiv Zn^{2+} per dimer to bovine CuZnSOD was sufficient to induce a native-like fold.

Asymmetrically metallated protein in vivo

The question must be asked whether or not the above results represent an in vitro artifact or a biologically relevant phenomenon. Is this asymmetry a result of misfolding due to the artificial procedure for metal depletion, or is there a genuine intersubunit communication with in vivo relevance? If the latter is true, how then do yeast cells properly metallate the protein? The answer to this may well lie with the newly identified copper chaperone Lys7p. Lys7p has been shown by genetic analysis to be involved in the proper insertion of copper into CuZnSOD in vivo [62, 63]. Unlike the other copper chaperones known to date, which are small proteins consisting only of the copper-shuttling domain, Lys7p has a domain that has significant homology to its target (see Fig. 12). The idea that Lys7p could act as a traditional protein chaperone, specific for CuZnSOD, as well as a copper shuttle, is enticing.

Our data presented here support the idea that an asymmetric dimer containing a phantom subunit is the conformation of the protein inside *lys7Δ* cells. When yeast CuZnSOD was isolated from *lys7Δ* yeast, it was found to be deficient in copper as expected but it also contained only 1 Zn^{2+} per subunit. The addition of 1 equiv Cu^{2+} per dimer resulted in a 670 nm band indicative of copper binding to the copper site. However, the second equivalent per subunit did not bind in a similar site as it should. Instead, a band with a λ_{max} of 750 nm appeared that is characteristic of free copper in acetate buffer (see Fig. 12A and Fig. 9, trace d for comparison). Similar results were obtained when Co^{2+} was titrated into this protein, i.e. no evidence of binding to the zinc site of the phantom subunit. Therefore, metal-deficient protein that is synthesized inside *lys7Δ* cells behaves similarly to artificially demetallated protein, that is to say it appears to contain one normal and one phantom subunit.

Presence of apo yeast CuZnSOD in vivo

Still the biological relevance of this discovery is yet to be proven. After all, the absence of Lys7p is an artificial condition as well. Does apo CuZnSOD ever exist within a normal yeast cell? Several studies do provide evidence that there is a pool of metal-deficient CuZnSOD inside of functioning cells. Recently, CuZnSOD was purified from copper-depleted rat liver cells and was shown to be deficient in both copper and zinc [64]. Copper-deficient CuZnSOD was also seen in human lymphoblasts exposed to ^{64}Cu in the presence of cycloheximide [65], and undifferentiated (but not differentiated) human K562 cells [66].

More importantly, a substantial amount of inactive enzyme was also seen in *S. cerevisiae* grown anaerobically or under conditions of glucose repression that could be reactivated in whole cell lysates by the addition of copper [67]. Two other studies have also shown

```
Lys7   .....MTTNDTYEATYAIPMHCENCVNDIKACLKNVPGINSLNFDIEQQIMSVESSVAP
ySOD1  ................................................................
Ccs    MASDSGNQGTLCTLEFAVQMTCQSCVDAVRKSLQGVAGVQDVEVHLEDQMVLVHTTL-P
hSOD1  ................................................................
                          *   *     *
Lys7   S-TIINTLRNCGKDAIIRGAGKP----NSSAVAILETFQKYTIDQKKDTAVRGLARIVQ
ySOD1  ..........................VQAVAVL----------KGDAGVSGVVKFEQ
Ccs    SQEVQALLEGTGRQAVLKGMGSGQLQNLGAAVAIL----------GGPGTVQGVVRFLQ
hSOD1  ..........................ATKAVCVL----------KGDGPVQGIINFEQ

Lys7   VGENKTLF---DITVNGV-PEAGNYHASIHEKGDVSKGVESTGKVWHKFDE----PIEC
ySOD1  ASESEPTTVSYE--IAGNSP-NAERGFHIHEFGDATNGCVSAGPHFNPFKKTHGAPTDE
Ccs    LTPERCLI---EGTIDGLEP--GLHGLHVHQYGDLTNNCNSCGNHFNPDGASHGGPQDS
hSOD1  KESNGPVKVWGS--IKGLT---GLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGGPKDE
                   *  *             s-s        *            *
Lys7   -FNESDLG--KNLYSGKTFLSAP-------LPTWQLIGRSFVI---SKSLNHPENEPSS
ySOD1  VRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTS-VVGRSVVIHAGQDDLGKGDTEESL
Ccs    DRHRGDLGNVRADADGRAIFRMEDE----QLKVWDVIGRSLIIDEGEDDLGRGGHPLSK
hSOD1  ERHVGDLGNVTADKDGVADVSIEDSVISLSGDHC-IIGRTLVVHEKADDLGKGGNEEST
          *  *                                   *     *
Lys7   VKDYSFL----GVIARSAGVWENNKQVCACTGKTVWEERKDALANNIK..........
ySOD1  KTGNAGPRPACGVIGLTN...............................
Ccs    ITGNSGERLACGIIARSAGLFQNPKQICSCDGLTIWEERGRPIAGKGRKESAQPPAHL
hSOD1  KTGNAGSRLACGVIGIAQ...............................
         s-s                        x   x
```

Fig. 12. Sequence alignment of yeast and human CuZn superoxide dismutase with Lys7 and its human homologue Ccs. *Shaded areas* denote positions that are identical in at least two sequences. (*) denotes residues known to be involved in metal binding in either SOD or Lys7. (s-s) denotes cysteines involved in disulfide bonds in the SOD sequences. (x) denotes a possible metal-binding motif

that CuZnSOD is capable of protecting yeast cells from toxic levels of both copper and silver ions [68, 69]. This metal buffering capacity is potentially due to the presence of a pool of copper-deficient protein.

Conclusions

We believe that the results presented here have in vivo significance. During growth on fermentable carbon sources or under anaerobic conditions, yeast synthesize large amounts of CuZnSOD mRNA but not proportional amounts of active enzyme [67]. During this stage of growth the demand for CuZnSOD activity is not great as there is little respiration, which accounts for the majority of superoxide production [6]. However, during a shift to oxidative metabolism the importance of active CuZnSOD increases [70]. Indeed, DNA microarray studies of yeast genome expression showed a three-fold increase in SOD1 message during the diauxic shift [71] (to view the results from this reference, see cmgm.stanford.edu/pbrown/explore/index.html). However, the presence of an inactive enzyme suggests that cells rely on the activation of inactive CuZnSOD as well as de novo synthesis. Activation might be achieved enzymatically with relatively small amounts of Lys7p. While no data are yet available regarding the translational or post-translational control of Lys7p, it is known

that the gene is evenly expressed throughout the growth phase, but expression decreases after entrance into the stationary phase [71].

Since apo CuZnSOD is much less stable than holo CuZnSOD [54], cells might need a means of stabilizing and maintaining an inactive pool of protein to prevent degradation or aggregation before activation. We propose that the insertion (possibly non-enzymatic) of one zinc per dimer could result in a stabilized native-like protein conformation that is primed for activation by Lys7p. Scheme 2 shows a proposed route for the activation of a metal-deficient pool of CuZnSOD in vivo. Why nature chose to insert only one zinc per dimer for enzyme stabilization is more difficult to rationalize. It is
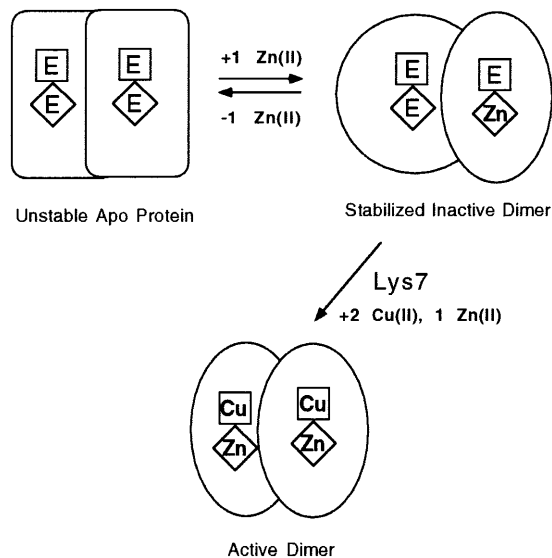
Scheme 2 Proposed scheme for Lys7p-mediated metallation of inactive CuZnSOD; E = empty site

possible that only one zinc per dimer is used for economical reasons, assuming zinc is needed for other cellular functions. This makes sense, seeing that the concentration of CuZnSOD inside cells is actually fairly high.

In conclusion, we propose that the metal-depleted form of yeast CuZnSOD exists in an alternative conformation which forms a dimer with one subunit that can bind metals and another that can not. Furthermore, we propose that Lys7p may be responsible for converting this conformation to a metal-binding-competent conformation as well as for delivery of the metal cofactors.

The subunit asymmetry that we report here for yeast CuZnSOD may reflect a general property of CuZn-SODs and as such may be related to the effects of the ALS-causing mutations in human CuZnSOD since it has recently been found that human mutant G37R contains structurally inequivalent subunits in the crystalline state [51]. G37R is one of the 60+ mutations in human CuZnSOD that are believed to cause the neurodegenerative disorder ALS (Lou Gehrig's disease) [72]. The mechanism by which these mutations cause this debilitating and ultimately fatal disorder is not known. The idea that CuZnSODs, which have a well conserved tertiary fold, could exist in multiple conformations provides an intriguing vantage point from which to view the structure/function problem.

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662

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