KINETICS OF METAL DISSOCIATION IN THE YEAST 
Cu₂,Zn₂-SUPEROXIDE DISMUTASE. 
APPARENT ASYMMETRY IN THE METAL BINDING SITES

by

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Keywords: Active site, asymmetry, copper, zinc, superoxide dismutase

The inactivation and metal depletion of the yeast Cu₂,Zn₂-superoxide dismutase have been examined in the presence of a number of chelating reagents. Total inactivation and metal depletion occurred only at pH ≤ 3.5. The loss of enzyme activity exhibited biphasic kinetics and was directly related to the rate of loss of copper from the protein. Two rate constants characterized also the dissociation of zinc from the native enzyme. At low pH, in the absence of chelating reagents, there is a fifty percent reduction in dismutase activity and a concomitant decrease in the metal concentration to one equivalent each of copper and zinc per mole dimer. It is apparent that the dissociation constants for the copper-protein and zinc-protein complexes differ between the subunits of the dimeric enzyme.

Comparison of the hydrodymanic and optical properties of the native and apo-SOD reveals changes in the structure and stability of the protein as a result of metal removal.
1. INTRODUCTION

Comparison of the physical, chemical and enzymatic properties of the holo- and the corresponding apo-enzyme provides an insight into the role of metal prosthetic groups in the structure and function of metalloenzymes (28). These studies, however, rely on the preparation of an apo-protein which may be reconstituted with the native metals to yield physical and chemical properties identical to those of the native enzyme.

In recent years, the metal binding sites in the bovine erythrocyte Cu2,Zn2-SOD have been the focus of a considerable number of investigations (3, 4, 8, 26, 27). SOD catalyses the dismutation of superoxide radical generated in those cellular reactions which proceed via the univalent reduction of oxygen (12). The protein is comprised of two subunits of identical amino acid sequence and the active site of each subunit accommodates one copper and one zinc atom (11). The enzyme activity is derived from the copper, which undergoes alternate oxidation and reduction during the dismutation of superoxide (11, 16). Zinc has been ascribed no direct role in catalysis but appears to enhance the stability of the protein and may also function in determining the conformation around the active site (5, 18). It has generally been considered that the two active sites of the dimeric enzyme have identical structures (21) and a proposed unique feature of the active site is the bridging histidine, coordinated to both the copper and zinc (9, 21). However, two recent studies of the active site structure of the yeast SOD are inconsistent with the bridging ligand hypothesis (2, 7).

The apo-SOD may be readily prepared and reconstitution with copper and zinc yields a derivative very similar to the native enzyme with respect to its EPR, absorption spectrum and enzyme activity (4). Preparation of the metal-free protein has previously been accomplished by use of EDTA as the chelating reagent (4, 8). However, this reagent exhibits an inherent capacity to bind the protein and its effective displacement necessitates exhaustive dialysis against high concentrations of salt (4). Prior to studying the metal binding sites in the yeast SOD, we investigated alternative procedures for the preparation of the apo enzyme. It became apparent that enzyme inhibition, in the presence of a number of chelating reagents, was a biphasic reaction which could be attributed directly to the dissociation of copper. Furthermore, at low pH in the absence of chelating reagents, the dissociation constants for the copper-protein and zinc-protein complexes differed between the two subunits.

2. MATERIALS AND METHODS

The Cu2,Zn2-SOD was isolated from S. cerevisiae by a new procedure (HASEMANN et al., in preparation). 1,10-phenanthroline, dipicolinic acid and EDTA were from Sigma Chemical Co. Glass distilled water was used throughout and buffers were rendered metal-free by extraction with dithizone (Merck) according to the procedure of THEIrS (24). Dialysis tubing was cleaned by heating to 80 °C in 3% acetic acid, followed by washing with 2 mM-EDTA and finally rinsed several times with distilled water. Column chromatography was performed on Pharmacia PD-10 columns packed with Sephadex G-25 (medium). Prior to use the columns were washed with 50 mM-HCl followed by distilled water and several column volumes of metal-free buffer.

Enzyme inactivation and metal depletion were examined during dialysis of the native protein against various chelating reagents. The Cu2,Zn2-SOD was dissolved to 10 mg·ml⁻¹ in 25 mM 1,10-phenanthroline, dipicolinic acid or citrate. The protein solution was adjusted to the required pH and dialysed against 100 volumes of the reagent. Aliquots were removed at various time intervals and chromatographed on Sephadex G-25 columns (1.5 × 5.5 cm) eluted with 5 mM-MES, pH 5.5. The eluted protein was assayed for enzyme activity and metal content.

Enzyme activity was estimated according to the procedure of MARKLUND and MARKLUND (19). Protein concentration was determined from amino acid analyses of the protein after hydrolysis in 6M-HCl, in vacuo, for 24 hours at 110 °C. The protein samples were assayed for copper and zinc on a Perkin-Elmer 603 atomic absorption spectrometer.

Reconstitution of the metal-free protein was carried out at pH 5.0 in 20 mM sodium acetate. An equimolar concentration of zinc-chloride was added slowly to the apo-SOD (1 to 10 mg·ml⁻¹) and the sample allowed to stand overnight at
room temperature. The Zn$_2$-SOD was subsequently reconstituted with a stoichiometric amount of copper which was added in a series of aliquots over a period of several hours. The Cu$_2$-SOD was prepared by the slow addition of two equivalents of copper to the apo-SOD, followed by incubation overnight at room temperature. Sedimentation velocity was determined in a Spinco Model E analytical ultracentrifuge at 20 °C and at a speed of 52,640 rpm. The sedintating boundary was monitored by Schlieren optics and the maximum ordinate of the Schlieren peak was taken as the position of the boundary. The limiting sedimentation coefficients were determined over a range of protein concentrations from 1 to 10 mg · ml$^{-1}$. Molecular weights were determined by long column meniscus depletion sedimentation equilibrium according to the method of Chervenka (6). Experiments were performed at a rotor speed of 25,980 rpm at 20 °C and a protein concentration of approximately 1 mg · ml$^{-1}$.

The UV circular dichroism was measured on a Cary 61 circular dichroic spectropolarimeter. Protein concentrations ranged from 1 to 10 mg · ml$^{-1}$ and the pathlength of the cuvettes from 0.02 to 1 cm. Ellipticity is expressed in terms of the molar ellipticity (deg. cm$^2$ (subunit)$^{-1}$).

3. RESULTS

3.1. Kinetics of metal removal

The yeast Cu$_2$;Zn$_2$-SOD is inactivated during dialysis against chelating reagents. The pH dependence and time course of inactivation in the presence of excess 1,10-phenanthroline are shown in Figure 1. Both the rate and the extent of inactivation are pH-dependent and total loss of activity was apparent only at pH < 3.5. A similar pH-dependence was observed for the inactivation of SOD by dipicolinic acid and citrate. In all cases, the specific enzyme activity of the dialysed protein was identical before and after removal of excess reagent by gel filtration. Furthermore, the absorption spectra of the chromatographed proteins revealed no anomalous ultraviolet absorption, indicating the absence of any protein-bound reagent. Enzyme inactivation therefore appears not to arise from the formation of a stable ternary complex or non-specific interaction between the reagent and metallo-protein.

Metal analyses revealed progressive loss of both copper and zinc from the protein concomitant with the inactivation of the enzyme. In the presence of phenanthroline the rates of dissociation of copper and zinc were equivalent and paralleled the rate of enzyme inactivation. However, the corresponding semi-log velocity plots for the loss of metals and enzyme activity were indicative of biphasic kinetics (Figure 2a). Resolution of the kinetics into the fast and slow reactions yielded the rate constants shown in Table I. Significantly, the intercept of the slow reaction corresponds to a 50% decrease in the enzyme activity and the loss of one equivalent each of copper and zinc per mole of dimer (Figure 2). Biphasic reactions were apparent not only at pH 3.2, where total inactivation and metal removal occurred, but were observed at all pH's below pH 5, where the dismutase activity and the concentration of protein-bound metals were reduced to less than 50% of the native enzyme.

The dissociation of the metals from the Cu$_2$;Zn$_2$-SOD and the inactivation of the enzyme induced by dipicolinic acid and citrate similarly displayed biphasic kinetics (Table I). Dialysis of the native enzyme against these reagents yielded a differential rate of loss of copper and zinc from the protein, in contrast to the equivalent rate of loss of the two metals in the presence of phenanthroline. In particular, the essentially copper-free protein obtained after 24 hours
Table I

Rate constants for metal depletion at pH 3.2.a)

<table>
<thead>
<tr>
<th>SOD</th>
<th>Reagent</th>
<th>$K_1$(min$^{-1}) \times 10^3$</th>
<th>$K_2$(min$^{-1}) \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>copper</td>
<td>zinc</td>
</tr>
<tr>
<td>Cu$_2$Zn$_2$</td>
<td>1,10-phenanthroline</td>
<td>13.4</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>dipicolinic acid</td>
<td>9.6</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>citrate</td>
<td>5.4</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Cu$_2$</td>
<td>HCl</td>
<td>10.3</td>
<td></td>
</tr>
</tbody>
</table>

The concentration of chelating reagents was 25mM and the initial protein concentration was 10 mg·ml$^{-1}$. Without chelating agents the pH of the SOD solutions were adjusted with 0.1 M-HCl.

a) The rate constants of enzyme inactivation were identical to the rate constants for copper depletion.

dialysis against citrate, pH 3.2, retained greater than 0.5 equivalents of zinc per mole of dimer (Figure 2b). Nonetheless, regardless of the chelating reagent employed, the rate of loss of enzyme activity was identical to the rate of loss of copper. The observed enzyme inactivation therefore, can

Figure 2. Inactivation and metal depletion of the yeast Cu$_2$Zn$_2$-SOD during dialysis against (a) 25 mM-1,10-phenanthroline, pH 3.2 and (b) 25 mM-citrate, pH 3.2.

Aliquots were taken at various time intervals, chromatographed to remove excess reagent and the protein assayed for enzyme activity, (▲), copper, (●), and zinc, (○). The slope and intercept were estimated by the unweighted least squares fit.
be attributed directly to the dissociation of copper from the protein.

Inactivation of the Cu₂,Zn₂-SOD also occurs at pH ≤ 3.5 in the absence of chelating reagents. The inactivation was coincident with a decrease in the metal content of the protein and there was no significant difference between the rate of loss of copper, zinc and enzyme activity (Figure 3). However, equilibrium corresponded to a 50% reduction in enzyme activity and the dissociation of one equivalent of copper and zinc per mole dimer. The reaction was consistent with only a single rate constant (Table 1). No changes were detected in either the enzyme activity or the metal content of the Cu₂,Zn₂-SOD at pH > 4 in the absence of chelating reagents.

Partial loss of copper also occurs from the Cu₂-SOD at low pH. Although the initial rate of copper depletion is very rapid, approximately four times faster than from the Cu₂,Zn₂-SOD under the same conditions (Table 1), the dissociation is limited to one equivalent copper per mole of dimer.

3.2. Characterisation of the apo-SOD

Comparison of the absorption spectra of the native and apo-SOD reveals that concomitant with metal depletion there is not only a loss of the copper visible absorption band at 680 nm, but also a decrease in the ultraviolet absorbance between 250 nm and 320 nm (Figure 4). The optical rotation of SOD is also modified by metal removal (Figure 5). Although both the native and apo-SOD exhibit a minimum of similar intensity at 215 nm in their CD spectrum, the optical activity of the two proteins differs significantly between 250 nm and 230 nm (Figure 5).

Structural differences between the holo- and apo-proteins were also apparent from their hydrodynamic properties. The limiting sedimentation coefficient of the native yeast SOD is 3.0 ± 0.05 S and independent of pH above pH 4.0 (Figure 6). Measurements at lower pH are

![Figure 3](image1.png)

**Figure 3.** Loss of enzyme activity, (▲), copper, (●), and zinc, (○), from the Cu₂,Zn₂-SOD at pH 3.2 in the absence of chelating reagents.

![Figure 4](image2.png)

**Figure 4.** Ultraviolet absorption spectra of the native yeast Cu₂,Zn₂-SOD (—) and the apo-SOD (- - - -), at pH 6.5.

The metal-free protein was obtained by 24 hour dialysis of the native enzyme against 1,10-phenanthroline, pH 3.2 and the excess reagent removed by gel filtration. The protein concentration is 1 mg·ml⁻¹.
unfeasible due to partial loss of metals from the protein under these conditions. In comparison the apo-enzyme has a sedimentation coefficient of 2.5 S at pH 7.0 which decreases to 1.15 S at pH 2.0 (Figure 6). Molecular weight estimates of the apo-SOD indicate that the enzyme is dissociated to the monomer at low pH. In addition, low pH appears to induce unfolding of the apo-SOD as evidenced by the disappearance of the CD band at 280 nm, reduction in the molar ellipticities at 257 nm and 265 nm and a shift in and enhancement of the intensity of the negative band near 200 nm (Figure 5).

The low pH dependent changes in the circular dichroic and hydrodynamic properties of the apo-SOD are reversible upon increasing the pH, while reconstitution of the apo-SOD with zinc restores the sedimentation coefficient to that of the native enzyme (Figure 6). Reconstitution with both metals, however, is required for regeneration of the native CD and UV-absorption spectra.

In the current experiments, the most effective removal of metals from yeast Cu₂Zn₂-SOD was facilitated by dialysis of the native enzyme against 1,10-phenanthroline at pH 3.2. The apo-SOD prepared under these conditions contained less than 0.02 equivalents of copper and zinc per dimer. Reconstitution of the apo-SOD with an equimolar concentration of copper and zinc yielded greater than 90% of the native enzyme activity.

4. DISCUSSION

The yeast Cu₂Zn₂-SOD exhibits a pH dependent loss of metals which is apparent both in the presence and absence of chelating reagents. The
pH-dependence and the dissociation of the metals induced by low pH alone presumably arises from protonation of the amino acid residues involved in metal co-ordination, although local protein conformational changes may also contribute to the decreased affinity of the metal binding sites at low pH.

A pH dependent loss of metals in the absence of chelating reagents has been described recently for the bovine Cu2,Zn2-SOD (20). In contrast to the equivalent and partial loss of both metals from the yeast SOD at low pH, PANTOLIANO et al. (20) reported total loss of zinc from the native bovine SOD at pH 3.6, in the absence of any significant changes in the copper content of the protein. These apparent discrepancies between the two reports may be rationalized by the marked increase in the lability of the protein copper complex as the pH is reduced below pH 3.5 (DUNBAR, unpublished observations) and the high concentration of phosphate used in the study of the bovine enzyme. The stability constant (log Kp) for the zinc phosphate complex is 12.4 (22) and thus it may be expected that not only the labile zinc atom in SOD, but also the zinc atom coordinated at the high affinity site may be removed under these conditions (vide infra).

Biphasic kinetics characterized the dissociation of copper and zinc from the yeast Cu2,Zn2-SOD in the presence of chelating reagents. The resolution of the reaction kinetics into two rate constants, corresponding to one equivalent each of Cu2,Zn2 per dimer, strongly suggests asymmetric binding sites for both copper and zinc. Different stabilities for the metal-protein complexes in the two subunits may be concluded also from the limited (50%) loss of metals which occurred at low pH in the absence of chelating reagents. It appears, then, that low pH alone is sufficient to induce the dissociation of the metals from one subunit, while the copper and zinc in the alternate subunit are more firmly bound and require the presence of a chelating reagent for effective removal. The more rapid loss of copper from the Cu2-SOD, in comparison to the Cu2,Zn2-SOD, suggests that the affinity of the copper binding sites is at least, in part, dependent upon the coordination of zinc to the protein. This observation is in accordance with the previously proposed role for zinc in stabilizing the tertiary structure of the protein and directing the conformation around the metal binding sites (5).

Nonetheless, the coordination of zinc alone does not appear to underlie the observed asymmetry in the copper binding sites as different stabilities were apparent for the two copper coordination complexes in the Cu2,Zn2-free SOD.

The apparent asymmetry in the active site structures of the Cu2,Zn2-SOD contrasts with previous considerations that the subunits of the dimeric enzyme are identical (21). The two constituent polypeptide chains have the same amino acid sequence (15) and no differences between the subunits were reported from the 3 Å X-ray crystal structure of the bovine SOD (21). However, results are accumulating from more recent investigations which cannot be interpreted in terms of equivalent active sites (17) or the previously proposed coordination geometry for the copper and zinc in the two subunits (2, 7).

The apparent asymmetry in the active site structure, detected in the current experiments, may now provide a rational basis for these observations.

There is a marked degree of sequence homology between the Cu2,Zn2-superoxide dis-
mutase isolated from a number of species (1, 14, 15, 23). High resolution $^1$H-NMR has provided strong evidence that the observed sequence homology is translated into secondary and tertiary structural similarities between the proteins (13). In accordance with these observations, the hydrodynamic and optical properties of the yeast Cu$_2$Zn$_2$-SOD and the changes in these properties upon metal removal closely resembled those of the bovine enzyme.

The loss of metals from the yeast Cu$_2$Zn$_2$-SOD is accompanied by changes in the ultraviolet absorption and circular dichroic spectra of the protein. These observations, however, appear to arise at least partly from the loss of copper per se, rather than from gross conformational changes. Accordingly, both the native and apo-SOD exhibited a UV circular dichroic spectrum characterized by a minimum near 215 nm and consistent with the $\beta$-sheet structure. Furthermore, the loss of optical activity at 280 nm in the apo-SOD was regenerated upon coordination of copper to the protein and optical activity in copper peptides between 260 nm and 320 nm has previously been ascribed to the copper transitions (25). Nonetheless, despite the overall similarity in the CD spectra of the native and apo-SOD, the conformations are sufficiently distinct to yield sedimentation coefficients of 3 S and 2.5 S, respectively. The structural variation reflected in the differing sedimentation coefficients may also underlie the differences in ellipticity which were observed near 230 nm.

More apparent, however, is the decreased stability of the apo-SOD in comparison to that of the Cu$_2$Zn$_2$-SOD or Zn$_2$-SOD and which was revealed by the pH-dependence of the sedimentation coefficients. At low pH, the apo-SOD dissociates and is at least partially unfolded. Consistent with these observations, sedimentation velocity of the native SOD at pH 3.2 yields an asymmetric boundary presumably arising from the partial loss of metals and dissociation of the enzyme. It is unknown, however, whether dissociation precedes or is a consequence of the loss of metals. If dissociation is indeed the primary event, then the strength of the subunit interaction may be an indirect determinant of the stability of the protein-metal complexes.

REFERENCES


