HYDROGEN-DEUTERIUM EXCHANGE IN YEAST Cu_2Zn_2 -SUPEROXIDE DISMUTASE AND APO-SUPEROXIDE DISMUTASE

by

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The role of copper and zinc for the structural properties of yeast Cu_2Zn_2 -SOD was studied by measuring the hydrogen-deuterium exchange in peptide groups of Cu_2Zn_2 -SOD, Cu_2E_2 -SOD, E_2Zn_2 -SOD and apo-SOD. Apo-SOD displays remarkably rapid exchange kinetics as compared to Cu_2Zn_2 -SOD, indicating a less stable structural organization after metal removal. At neutral and alkaline pD the exchange rates are in agreement with the EX2 mechanism of exchange; the exchange rates at low pD suggest a structural transition. In Cu_2Zn_2 -SOD this transition occurs at pD about 4.5, and in apo-SOD at pD about 6.2, 1.7 pD units higher. Binding of either copper or zinc to apo-SOD results in reductions of the exchange rate, which is further reduced after full reconstitution to Cu_2Zn_2 -SOD, demonstrating that copper and zinc are equally important for the structural stability of the protein molecule.

1. INTRODUCTION

Cu_2Zn_2 -SOD from yeast is a member of a highly conserved group of enzymes found in the cytosol of eucaryotic cells, believed to act as scavengers of toxic superoxide ions, O_2^- , formed during oxidative metabolism. Yeast Cu_2Zn_2 -SOD has a molecular weight of 31,900, and it is composed of two identical subunits each containing a bimetallic Cu-Zn-center, which is essential for the catalytic mechanism (8, 15, 22). The X-ray crystal structure of bovine erythrocyte Cu_2Zn_2 -SOD at 2 Å resolution shows that each subunit consists of an eight stranded β -bar-

rel of Greek key topology, suggesting a very rigid structure. The copper is liganded to four histidines in a distorted square plane, and the zinc is liganded to three histidines and an aspartic acid in a tetrahedral geometry (28). ¹H-NMR studies indicate close similarity in the metal binding sites of bovine erythrocyte and yeast Cu_2Zn_2 -SOD (8), although a metal bridging histidine in the bovine enzyme is apparently not liganded to zinc in the yeast enzyme (3).

It is now well established that copper is essential for the catalysis of superoxide dismutation, during which copper is alternately oxidized and

Abbreviations: Cu_2E_2 -SOD = di-copper superoxide dismutase; E_2Zn_2 -SOD = di-zinc superoxide dismutase; SOD = superoxide dismutase.

reduced (15), whereas zinc is believed to be responsible for correct conformation around the active site (9, 24).

The copper and zinc can be readily removed from $\text{Cu}_2\text{Zn}_2\text{-SOD}$ by chelating reagents. According to circular dichroism measurements and ultracentrifugation experiments the apo-enzyme retains a globular structure, although a slight decrease of the sedimentation coefficient suggests a partial unfolding (10). A further decrease of the sedimentation coefficient seen in acid solutions could arise from either a dissociation of the dimeric molecule to monomers, or to an unfolding to random coils.

In the present investigation hydrogen-deuterium exchange rates of $\text{Cu}_2\text{Zn}_2\text{-SOD}$, $\text{Cu}_2\text{E}_2\text{-SOD}$, $\text{E}_2\text{Zn}_2\text{-SOD}$ and apo-SOD have been measured in order to obtain additional information on the importance of copper and zinc for stabilization of the molecular structure.

The exchange rates of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ and apo-SOD were determined at a wide pH-range, in order to characterize the acid-induced structural transformations of these molecules.

2. MATERIALS

Yeast $\text{Cu}_2\text{Zn}_2\text{-SOD}$, purified to homogeneity, was a kind gift from Pharmacia, Denmark. Apo-SOD was prepared according to DUNBAR et al. (10) by 24 hours dialysis of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ against 25 mM-orthophenanthroline, pH 3.2. Buffers for subsequent desalting were rendered metal free after extraction with 0.01% dithizon in CCl_4 (29). The purity of apo-SOD was controlled from the ratio of absorption at 258 nm and 280 nm which is 1.1 in apo-SOD against 1.6 in $\text{Cu}_2\text{Zn}_2\text{-SOD}$, and from absence of enzyme activity as determined by the method of MARKLUND and MARKLUND (25).

Reconstitution of apo-SOD with either copper or zinc was achieved by stepwise addition, over several hours, of a 2:1 molar ratio of either copper or zinc chloride in 20 mM-sodium acetate buffer at pH 5.0 in accordance with DUNBAR et al. (10). Fully reconstituted $\text{Cu}_2\text{Zn}_2\text{-SOD}$ was prepared by subsequent reconstitution of the copper depleted $\text{E}_2\text{Zn}_2\text{-SOD}$ with copper, using the same procedure. UV-spectrophotometric properties and hydrogen-deuterium ex-

change rates of the fully reconstituted apo-SOD showed less than 10% deviations from the native $\text{Cu}_2\text{Zn}_2\text{-SOD}$. D_2O was 99.97% pure from Norsk Hydro (Norway). All other chemicals were analytical grade.

3. METHODS

Hydrogen-deuterium exchange was measured by the infrared spectroscopy method (17). Approx. 9 mg of lyophilized SOD was dissolved in 300 μl water, and pH was adjusted to a desired value between pH 3 and pH 10 by addition of 0.1 M-HCl or 0.1 M-NaOH. Buffers were avoided, since they might have secondary effects on the protein conformation (11, 27). After lyophilization in 1 ml glass vials, the enzyme was redissolved in 300 μl D_2O , and after approx. 2 min, when all was dissolved, the solution could be transferred to a 0.1 mm path length CaF_2 -cell. pH in the residual solution was measured using a Radiometer cup electrode, G2221C, and a Radiometer Model 26 pH-meter. The measured value was used for calculations after correction for the effect of deuterium by the formula of GLASOE and YOUNG: $\text{pD} = \text{pH meter reading} + 0.4$ (16).

Spectra were recorded between 1300 cm^{-1} and 1800 cm^{-1} on a Beckman IR 9 spectrophotometer, as previously described (18), using a cell holder thermostated at 25 °C. The absorbance was followed continuously at the amide II absorption at 1540 cm^{-1} , from 2-3 min until 20 min after dissolving the sample. Later values, until 6 hours, occasionally 24 hours, after dissolving the sample were obtained from scanning of whole spectra.

The background of the amide II band, corresponding to the absorption of fully deuterated SOD, was determined after 2-3 hours exchange at 60 °C. This method gave reproducible results for all pD values. The background of the amide I band was determined from the absorbance measured at 1800 cm^{-1} , which is close to zero.

The rate of exchange was measured by following the disappearance of the amide II N-H band, A_{NH} , at 1540 cm^{-1} , upon deuteration of the amide groups (4). The constant amide I band, A_{AI} , at 1645 cm^{-1} , which is associated with C=O stretching motions, was used to normalize the amide II

Figure 1. IR spectra of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ at pD 4.88, 25 °C, a) after 27 min of exchange, b) after 22 hours of exchange, and c) after heating at 60 °C for 3 hours. The insert shows a difference curve between the spectra around the amide II band.

absorbances (4, 17). All values of A_{AII} and A_{AI} have been corrected for background absorptions.

The initial amide II absorption of the fully hydrogenated enzyme could not be measured directly; it had to be estimated from experiments where the exchange process was slowed down by lowering the temperature of the exchanging protein solutions, and adjusting pD to approx. 3.4 where the exchange rate has its minimum for most proteins (6, 12, 13).

4. RESULTS

The IR-absorbance spectra of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ show the usual protein features including the amide I band at 1645 cm^{-1} and the carboxylate band at 1580 – 1590 cm^{-1} (Figure 1). At low pD the position of the amide I band is changed from 1643 cm^{-1} to 1649 cm^{-1} . Apo-SOD has a similar spectrum (not shown) and at low pD its amide I band changes from 1640 cm^{-1} to 1650 cm^{-1} . An isosbestic point at 1500 cm^{-1} (Figure 1) was a control for the absence of contaminating water after prolonged exchange. The position of the

unusually broad amide II band was determined by the difference spectra indicated in the insert in Figure 1, and it has a maximum about 1540 cm^{-1} .

The background value of the amide II band at 1540 cm^{-1} is greatly dependent on pH because it is overlapped by a strong absorption at 1590 cm^{-1} , due to the carboxylate groups, and during acid titration of the 20 carboxylate groups of each enzyme subunit (22) this band almost disappears, reducing the background ratio from 0.10 to 0.03 (Figure 2). The large effect of pH variation on the background complicates exchange rate measurements in the pH interval 3 to 5, where carboxylate groups titrate.

The initial ratio of $A_{\text{AII}}/A_{\text{AI}}$ for $\text{Cu}_2\text{Zn}_2\text{-SOD}$ and for apo-SOD, corrected for the background, was found to be 0.35, as determined at low pD and low temperature by extrapolation to time zero. This value is lower than estimates for most other proteins examined (4, 17), and might be explained by the unusually broad character of the amide II band (see Figure 1). The integrated value of the amide II band thus resembles the insulin (19) and serum albumin (18) amide II band areas (results not shown).

The fraction of unexchanged peptide hydrogens, X , is given by:

$$X(t) = \frac{(A_{\text{AII}}/A_{\text{AI}})_{t=t}}{(A_{\text{AII}}/A_{\text{AI}})_{t=0}}$$

and this expression is used in the representation of all data.

The exchange rates of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ and apo-SOD are compared in Figure 3A and Figure 3B. It is seen that apo-SOD exchanges more rapidly

Figure 2. Background of amide II band as a function of pD. Values were obtained for $\text{Cu}_2\text{Zn}_2\text{-SOD}$ (●) and apo-SOD (○) after heating at 60 °C for 3 hours.

at all pD values than $\text{Cu}_2\text{Zn}_2\text{-SOD}$. While $\text{Cu}_2\text{Zn}_2\text{-SOD}$ has a structure which retains more than 50% unexchanged amide protons after five minutes of exchange at pD 8, 25 °C, about 80%

of the amide protons in apo-SOD have exchanged under identical conditions during the first two minutes.

The exchange curves in Figure 3A also indi-

Figure 3. Hydrogen-deuterium exchange curves for yeast superoxide dismutase at 25 °C. A: $\text{Cu}_2\text{Zn}_2\text{-SOD}$ at pD 9.35 (●), pD 8.05 (▲), pD 6.05 (▼), pD 5.32 (■), pD 4.88 (◆), pD 4.45 (★), and pD 3.65 (◄). B: Apo-SOD at pD 9.82 (○), pD 8.40 (△), pD 7.21 (▽), pD 6.75 (□), pD 6.20 (◇), pD 4.70 (☆), and pD 3.83 (◄). Background of amide II band is subtracted. X is calculated as $A_{\text{II}}/(A_{\text{I}} \times 0.35)$, 0.35 being the initial ratio $(A_{\text{II}}/A_{\text{I}})_{t=0}$.

cate that the exchange rate of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ has a minimum at pD 4.9-5.3. In apo-SOD the pD of minimal exchange rate is even higher, about pD 6.8 (Figure 3B), as opposed to the exchange rate minimum at pD about 3.4 usually observed in randomly coiled polypeptides (12).

In $\text{Cu}_2\text{E}_2\text{-SOD}$ and in $\text{E}_2\text{Zn}_2\text{-SOD}$ hydrogen-deuterium exchange rates were measured at pD 4.8 and pD 5.2, respectively. As seen from Figure 4, partial reconstitution with either copper or zinc reduces the exchange rate to approximately the same degree, yielding a rate intermediate between the exchange rates of apo-SOD and fully reconstituted $\text{Cu}_2\text{Zn}_2\text{-SOD}$.

Figure 4. Hydrogen-deuterium exchange curves of apo-SOD at pD 4.7 (○), $\text{Cu}_2\text{E}_2\text{-SOD}$ at pD 4.8 (▲), $\text{E}_2\text{Zn}_2\text{-SOD}$ at pD 5.2 (▼), and $\text{Cu}_2\text{Zn}_2\text{-SOD}$ at pD 4.8 (●).

4. DISCUSSION

The exchange rates measured for Cu₂Zn₂-SOD and apo-SOD are treated with the assumption that the exchange of the *i*'th buried amide proton with deuterium can be described by the following reaction scheme (17):

N denotes a protein conformation where the amide proton is protected from solvent, and O denotes conformations which allow contact with solvent.

The rate of exchange, k_o , of solvent exposed peptide groups, has been estimated from the exchange rate of the random coil peptide, poly-D,L-alanine (6, 12, 17). The exchange is catalyzed by protons, hydroxyl ions and water, and has a minimum rate about pH 3.0. An estimate of this exchange rate in D₂O as a function of pD and temperature T, in degrees centigrade, is given by the expression (17)

$$k_o(\text{min}^{-1}) = (10^{0.3-pD+10pD-6.3})10^{0.5(T-20)}$$

which has been used in the present treatment.

The exchange rates of most proteins are slowed down by factors 10^{-3} to 10^{-7} , due to the low probability of solvent exposure of buried amide protons. Previous experience with amide proton exchange in proteins, under the present conditions, has shown that the exchange in most cases occur according to the so-called EX₂ mechanism, in which the rate of H-D exchange in the *i*'th peptide group, k_{ex}^i , is given by (17):

$$k_{ex}^i = \rho^i k_o, \text{ where } \rho^i = k_+^i / (k_+^i + k_-^i)$$

The total extent of exchange is expressed as a sum of first order reactions:

$$X(t) = \left(\sum_{i=1}^n \exp(-\rho^i k_o t) \right) / n$$

where n is the number of peptide hydrogens per protein molecule.

Figure 5 illustrates the fraction of unexchanged peptide hydrogens, X, in Cu₂Zn₂-SOD as a function of the variable $\log(k_o t)$, as suggested by WILLUMSEN (30). This representation facili-

tates direct comparison of values obtained at different pD values. Curves representing theoretical molecules possessing peptide groups with identical ρ values from 1 to 10^{-8} are included, as suggested by HVIDT (19).

The overlapping of the exchange curves in the neutral and alkaline regions seen in Figure 5 is consistent with the EX₂ mechanism of exchange, and the distribution of opening equilibria, ρ^i , is apparently unchanged, indicating no significant conformational change of the protein in this pD range.

In Cu₂Zn₂-SOD all of the amide protons have possibilities for productive solvent exposure, as represented by ρ values, lower than 10^{-2} , and the ρ values span a range of at least seven decades. The large number of amide protons with ρ values below 10^{-5} , approx. 60%, places Cu₂Zn₂-SOD among the very slowly exchanging proteins, like subtilisin (21), carbonic anhydrase (32) and myoglobin (1). This protection of the peptide hydrogens in the molecule against contact with solvent, is also reflected in the high stability of Cu₂Zn₂-SOD toward the denaturing influence of e.g. heat, alkali and urea (2, 23).

Removal of the copper and zinc ions leads to an apo-enzyme which exchanges peptide amide protons with highly increased rates. Above pD 6.8 the exchange curves in Figure 5 coincide again to a single curve, as expected from the EX₂ mechanism, and this exchange curve also spans a broad range of ρ values, from $\rho = 10^{-2}$ to $\rho = 10^{-7}$. The curve is, however, displaced horizontally from the curve of Cu₂Zn₂-SOD, with a distance between the two curves corresponding to a 10^3 to 10^4 fold faster exchange of the apo-enzyme. This indicates that copper and zinc are of crucial importance for the structural stability, not only close to the active site, but for a section involving at least one third of the peptide hydrogens of the Cu₂Zn₂-SOD molecule. It is also evident, that the apo-enzyme still has a distinct structure, that causes a significant proportion of the amide protons to exchange more slowly by several decades than the intrinsic exchange rate, k_o .

This information supplements the NMR studies of Cu₂Zn₂-SOD and apo-SOD (9, 14, 24), which only revealed considerable differences in the active site region, and the ultracentrifugation studies of yeast apo-SOD (10) which showed a

Figure 5. The fraction of unexchanged amide protons in $\text{Cu}_2\text{Zn}_2\text{-SOD}$ and apo-SOD, X , is illustrated against $\log(k_0t)$, as a test of the dependence of average structural fluctuations, or ρ , on pD. The S-shaped curves are theoretical curves for given values of ρ from 1 to 10^{-8} (18). Filled symbols refer to $\text{Cu}_2\text{Zn}_2\text{-SOD}$ and open symbols to apo-SOD: pD 9.35 (●), pD 8.05 (▲), pD 6.05 (▼), pD 5.32 (■), pD 4.88 (◆), pD 4.45 (★), pD 3.65 (◀), pD 9.82 (○), pD 8.40 (△), pD 7.21 (▽), pD 6.75 (□), pD 6.20 (◇), pD 4.70 (☆), and pD 3.83 (◁).

decrease of the sedimentation coefficient from 3.0 S to 2.5 S upon metal removal, suggesting a general loosening of the protein structure. This is consistent with the observation for bovine apo-SOD, that this protein denatures at a temperature 45 °C lower than the holo-enzyme (23).

In contrast to this behaviour of the metalloenzyme superoxide dismutase, another zinc containing metalloenzyme, human carbonic anhydrase, only revealed a slight decrease in structural stability upon metal removal, as seen from hydrogen-deuterium exchange studies (32), and zinc removal from alkaline phosphatase of *E. coli* caused a similar small effect on the hydrogen-tritium exchange rates (5).

When pD is reduced from 4.9 to 3.6 the exchange curves of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ in Figure 5, determined at different pD values, do not coincide, and this suggests that a structural transition occurs, which involves a loosening of the secondary and tertiary structure of the molecule. In apo-SOD a similar transition occurs, but it takes place at higher pD values, between pD 6.8 and 4.7. This indicates that the copper and zinc atoms stabilize the structure against acid unfolding between pD 4.9 and pD 6.2.

It should be kept in mind, that Figure 5 relies on the estimate of k_0 made for poly-D,L-alanine, which is an oversimplification, since both the

nature of amino acid side chains (26) and the protein charge (13, 20, 31) would have some effect on k_0 , the exchange rate of the unfolded peptide chain segments. The data representation is, however, useful for the evaluation of structural transitions which substantially affect the average opening equilibrium, ρ , of the peptide amides, as observed in the present case.

Investigations of the visible, ultraviolet and ESR spectra of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ from bovine erythrocytes as a function of pH (7, 27), demonstrated a corresponding structural transition when pH was lowered from 4.5 to 3.0, concomitant with the release of zinc. In yeast apo-SOD the sedimentation coefficient was reduced from 2.50 S to 1.90 S when pH was lowered from 7.0 to 5.0 (10); this observation is consistent with the partial unfolding suggested by the present exchange results.

The exchange curves of $\text{Cu}_2\text{Zn}_2\text{-SOD}$ below pD 3.7, and apo-SOD below pD 4.7 converge toward the theoretical curve for $\rho = 1$, which is the exchange curve for solvent exposed amide protons. In $\text{Cu}_2\text{Zn}_2\text{-SOD}$ the metal ions are probably no longer liganded to the protein molecule at pD 3.65.

The exchange experiments with yeast $\text{Cu}_2\text{E}_2\text{-SOD}$ and $\text{E}_2\text{Zn}_2\text{-SOD}$ at pD about 5 show that coordination of copper as well as zinc to the

apo-enzyme is able to partially revert the conformational changes which increase the rate of hydrogen-deuterium exchange, but the changes are not sufficient for restoration of the exchange behaviour of the fully reconstituted enzyme, as indicated in Figure 4. Apparently the copper atoms, beyond their catalytical role, are equally important as the zinc atoms for the structural stability of yeast $\text{Cu}_2\text{Zn}_2\text{-SOD}$. Conformational changes subsequent to coordination of both copper and zinc, are also suggested by visible absorption and MCD spectra of yeast $\text{E}_2\text{Co}_1\text{-SOD}$ and $\text{Cu}_1\text{Co}_1\text{-SOD}$ (11), and by an increase in thermal stability of 30 °C and 21 °C following partial reconstitution of bovine apo-SOD with copper and zinc, respectively (23). These values are consistent with the metal induced, very extended conformational changes demonstrated by the present exchange experiments.

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