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DIFFERENTIAL EFFECTS OF UREA ON YEAST AND BOVINE COPPER, ZINC SUPEROXIDE DISMUTASES, IN RELATION TO THE EXTENT OF ANALOGY OF PRIMARY STRUCTURES

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Summary. Incubation in 8M urea (pH 7.4) inactivated yeast Cu, Zn superoxide dismutase with biphasic first order kinetics (k for the decrease from 100% to 16% activity = 6.5×10^{-3} min^{-1}; k for the decrease from 16% to 0.1% activity = 2.5×10^{-3} min^{-1}). The inactivation was fully reversible on dilution with or dialysis against urea-free buffer. No inactivation was shown to occur in similar experiments with the bovine Cu, Zn enzyme. EPR spectra recorded immediately after addition of 8M urea showed a more axial line shape and a higher $A_{\parallel}$ of the copper signal typical of the native enzyme. In the case of the yeast enzyme, this change was more pronounced and further incubation led to a new type of copper signal, typical of the inactivated enzyme. All EPR changes were reversible. Comparative analysis of the amino acid sequence of the two enzymes showed substantial identity of the protein regions contributing the ligands to the metals and the disulfide bridge. Differential destabilization of active sites by urea should be due to replacements in other protein segments, such as the three C-terminal and some N-terminal residues.

In a recent report (1) we have presented evidence that dissociation into protomers by 8M urea occurs in copper, zinc superoxide dismutases from widely different organisms. This indicates that other properties, besides the spectral properties and catalytic efficiency are conserved in Cu, Zn superoxide dismutases, in particular the structural features determining the stability of the dimeric arrangement of this class of proteins. It is therefore interesting to compare the behavior , with respect to a same reagent, of Cu, Zn superoxide dismutases isolated from organisms that are widely distant in the phylogenetic tree, and to make structure--reactivity correlations on this basis. Along this line we have investigated the yeast and bovine enzymes with respect to reversible modifications of some active site-linked parameters brought about by treatment with 8M urea. The observed

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differences in sensitivity to urea were confronted with the degree of analogy of the amino acid sequences, particularly within regions of crucial importance to the stability of the metal binding sites.

MATERIALS AND METHODS.

Yeast (Saccharomyces cerevisiae) superoxide dismutase was prepared by the method of Goscin and Fridovich (2). The apoprotein was prepared incubating solutions of the enzyme in 70% formic acid at 0° for 2 hours, followed by extensive dialysis against water and lyophilization. Titration of sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) was performed according to Cavallini et al. (3). Reduction and alkylation with radioactive iodoacetic acid of the apoprotein (210 mg) in 6.0 M guanidine hydrochloride was performed according to a procedure proved to be effective for the human Cu, Zn superoxide dismutase (4). Digestion with 2.5 mg of Staphylococcus aureus V8 protease (Miles Biochemicals) was carried out in 0.1 M ammonium bicarbonate at 37°C for 24 h. Digestion was stopped by addition of acetic acid (10% final) and the peptide mixture, which was completely soluble in this medium, was fractionated on a Sephadex G-25 column (4x130 cm) equilibrated and eluted with the same solvent. Techniques employed for further purification and subsequent analysis of peptides were as reported in (4).
Bovine superoxide dismutase was purified according to McCord and Fridovich (5). Urea solutions were deionized before use with AG 501 X8D mixed-bed resin (Bio Rad). Superoxide dismutase activity was assayed according to Rigo et al. (6). EPR measurements were recorded with a Varian E-4 spectrometer at approximately 9.11 GHz and -150°C.

RESULTS AND DISCUSSION.

Effect of urea treatment on enzyme activity. Addition of 8M urea to buffered solutions (0.1 M phosphate buffer, pH 7.4) of either yeast or bovine Cu, Zn superoxide dismutase ($\simeq 10^{-6}$ M) led to immediate drop of enzyme activity down to 50% of that typical of the native enzyme. This decrease is approximately that predicted by taking into account effects on diffusion by 8M urea viscosity (7) and therefore strongly supports the hypothesis that the monomer is as active as the dimer (8). On incubation at 22°C of the enzyme solutions in 8M urea for 4 days, the bovine enzyme retained the activity value recorded immediately after addition of urea, while the yeast enzyme displayed a further activity fall. This inactivation had a biphasic time dependence, that is a first order activity decrease down to 16% of the initial value after two days ($k = 6.5 \times 10^{-3}$ min^{-1}) and a second slower first order decay ($k = 2.5 \times 10^{-3}$ min^{-1}) to a final value of 0.1% activity

after 4 days. Hundred-fold dilution with buffer of the fully inactivated enzyme solutions, led to an abrupt recovery of activity up to 50% of the value typical of the native enzyme within 1 min and to a 100% recovery of activity within 8 hours. Similar recovery was obtained by dialysis against buffer of the urea-inactivated enzyme.

EPR spectra. The copper EPR spectra of either bovine or yeast superoxide dismutase were affected by urea treatment, and the spectral changes were distinctive in the two enzymes (Fig. 1). Immediate changes were much more pronounced in the yeast than in the bovine dismutase (Fig. 1, curves b and b'). In both cases the spectral line shapes became more axial and the hyperfine splitting constant in the $g_{\parallel}$ regions increased. The urea effect on the EPR spectra was specific, as addition of sucrose at such a concentration as to give the same relative viscosity of 8M urea (7) resulted in much less significant modifications of the spectrum of both enzymes (Fig. 1, d shows the effect on the bovine enzyme). Therefore EPR evidence is in favor of specific modifications of the active site in urea even though attribution of the result to freezing effects can not be ruled out. Analogously to the effects on the enzyme activity, further incubation had practically no effect on the bovine enzyme, while it converted the EPR spectrum of the yeast enzyme into an entirely new form of signal (Fig. 1, curves c and c'). All effects were reversible after dialysis against urea-free buffer.

Sequence homologies of bovine and yeast enzymes. The rather dramatic difference in sensitivity to 8M urea of the most specific properties of the active site of the two Cu, Zn superoxide dismutases under study has no apparent explanation in the spectroscopic properties of the two enzymes, which are identical (2). Nevertheless structural bases for this differential stability are to be found in the primary chemical frame of the proteins, which is expected to be as conservative as to allow the formation of identical active sites but as variant as to create conditions for different stability of the same sites. To unravel such structural bases we first focussed our attention on the existence and subsequent characterization of disulfide bonds, i.e. a chemical feature which is proved, at least for the bovine enzyme, to be relevant for the compactness of the subunit and the maintenance of the dimeric arrangement of the molecule (9,11) and at the same time is amenable to be pin-pointed in a relatively easy manner.

The half-cystine content reported for the yeast enzyme is 3.5-4 residues per di-

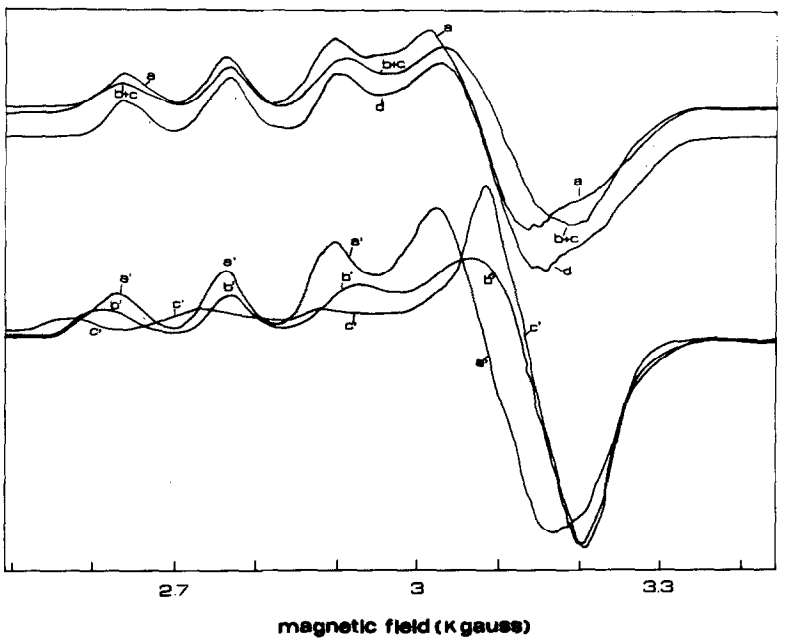

Fig. 1 - EPR spectra of bovine and yeast Cu, Zn superoxide dismutase. (a): bovine enzyme (0.8×10^{-4} M) in 0.1 M phosphate buffer, pH 7.4; (b) and (c): as (a), frozen immediately (b) or 24 hours (c) after addition of 8M urea at room temperature; (d): bovine enzyme (1×10^{-4} M) plus 0.5 M sucrose; (a') , (b') and (c') are the yeast enzyme (1.5×10^{-4} M) in the same conditions as (a) (b) and (c). Final concentrations of enzymes were obtained by addition of a few microliters of a concentrated stock solution of protein to relatively large volumes of either buffer alone or buffer containing 8M urea.

mer, as measured as cysteic acid after oxidation (2,12). We found a similar value (4.1) of carboxymethylcysteine content after reduction and treatment with iodoacetate of the apoenzyme under denaturing conditions (6.0 M guanidine hydrochloride). Titration with 5,5'-dithiobis(2-nitrobenzoic acid) gave 3.9 and 4.2 -SH groups per dimer in 8.0 M urea, after reduction with sodium borohydride, respectively for the apo- and holo-enzyme, while practically no -SH group was titrated under the same denaturing conditions but with omission of the reduction treatment. These results, together with the established dissociation of the enzyme in 8M urea (1), clearly indicate the presence of an intrasubunit disulfide bond per subunit, a feature common to the homologous protein from higher organisms (4,9, 11). From the digest with S.aureus protease of the reduced, carboxymathylated apoen

zyme, two radioactive peptides (SA-2 and SA-4) were isolated and sequenced. Their sequence is reported in fig. 2, aligned by homology along the polypeptide chain of the bovine enzyme (11), together with the sequence of two more peptides (SA-1 and SA-3) purified by the ion exchange chromatographies performed for the isolation of the radioactive fragments.

The two strectches of polypeptide chain reported in fig. 2 encompassing the two half-cystines, include 69 amino acid residues, thus accounting for about 46% of the entire subunit: the overall homology with the corresponding portions of the bovine enzyme is 68%. Of immediate interest is the observation that, beside the homology around the residues involved in the intrasubunit disulfide bond , also His 69, His 78 and Asp 81 are conserved, i.e. the four amino acid residues that in the bovine enzyme were proved to be ligands of the zinc atom. Conservation of three out of the four copper ligands of the bovine protein, histidines 44, 46 and 61 (no sequence information is up to now available on the fourth ligand , His 118) is also given in fig. 2. Such a high extent of conservation of the environment around the two metals is strongly related to the spectroscopic and catalytic similarities of the two proteins.

While this paper was in preparation we became aware of a report by a danish group concerning the determination of the first 54 residues of the amino terminal sequence of yeast superoxide dismutase (12). In fact during the course of the present work it has been possible to purify the whole array of S.aureus protease peptides from the N-terminal region of the molecule and their analysis fully confirmed the data from Petersen et al. (12). Comparison of this stretch of polypeptide chain with the bovine enzyme shows 42% homology; this value, though high enough to indicate an extent of stability of the molecule through evolution comparable to that of cytochrome c, is lower than that found for the polypeptide fragments reported here. In conclusion, the results presented above suggest substantial invariancy in Cu, Zn superoxide dismutases of protein regions likely to be essential to the maintenance of spectroscopic and catalytic properties, such as those peptides contributing the metal ligands and the disulfide bridge. On the other hand, a clue to explain the differential effects of urea on the active sites of the yeast and bovine enzymes can be found in substitutions observed in regions that are at the subunit interface, such as the last few C-terminal residues and part of the N-terminal segment (9).

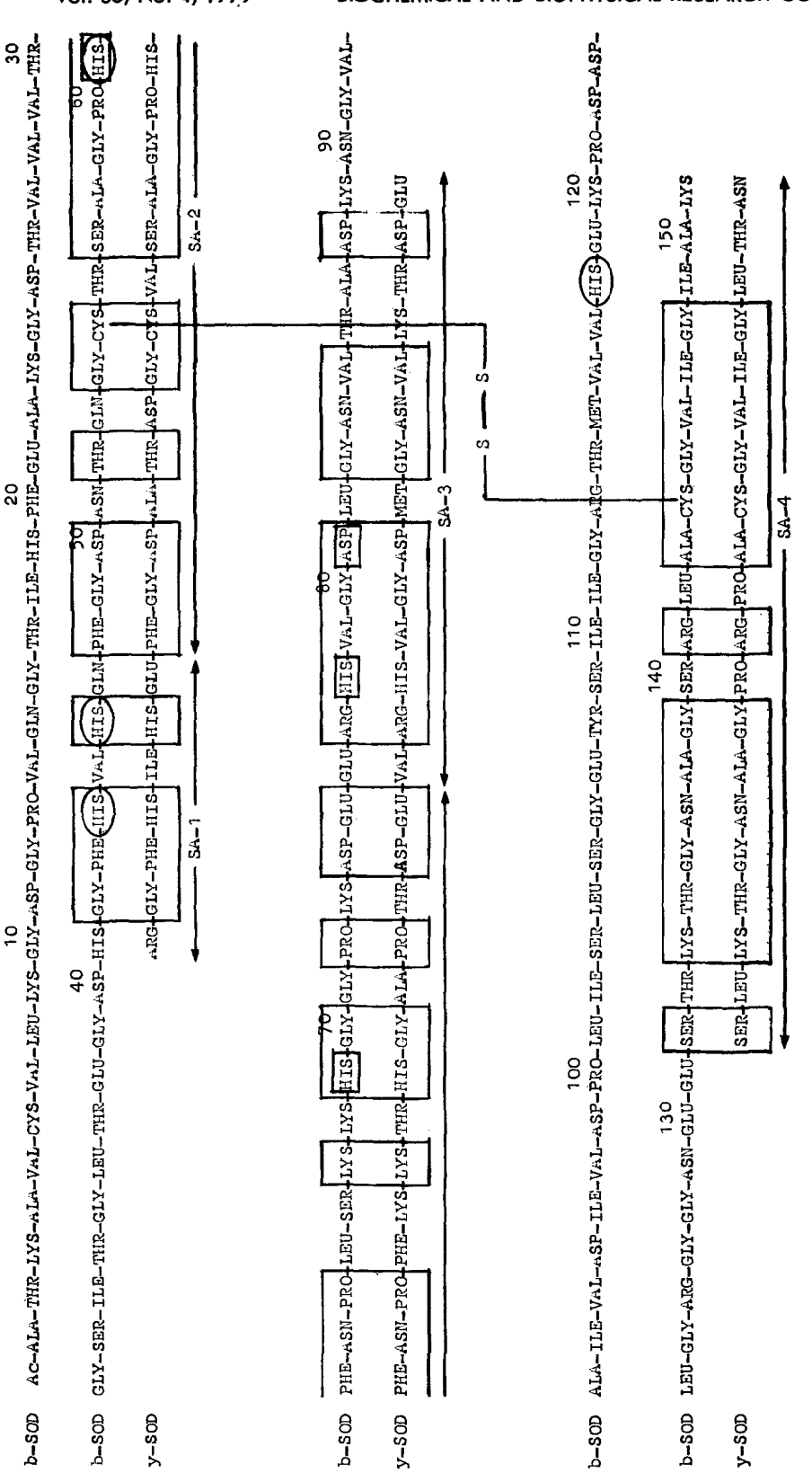

Fig. 2 - Comparison of the primary structure of bovine (b-SOD) and Saccharomyces cerevisiae (y-SOD) superoxide dismutases. Arrows under the yeast enzyme with symbols SA denote peptides derived from digestion of carboxymethylated protein with S.aureus protease. Boxes enclose residues identical in the two proteins. For the bovine enzyme residues that are ligands of the copper (circles) and the zinc (squares) atoms and the position of the disulfide bond are also indicated.

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