Primary structure of a cationic Cu,Zn superoxide dismutase

The sheep enzyme

M.E. Schininà, D. Barra, S. Gentilomo, F. Bossa, C. Capo, G. Rotilio+ and L. Calabrese*

Department of Biochemical Sciences, La Sapienza University of Rome and CNR Centre for Molecular Biology, 00185 Roma, and *+Department of Biology, Tor Vergata University of Rome, 00173 Roma, Italy*

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The complete amino acid sequence of Cu,Zn superoxide dismutase from sheep erythrocytes has been determined. The sequence is very similar to that of the bovine enzyme, having the same number of residues (151) and only two substitutions in the 'hypervariable' region (residues 17–30). The 5 overall substitutions confer a positive charge on the sheep enzyme at neutral pH (p$I \approx 8$). This charge is localized outside the active site region. The catalytic efficiency of the sheep enzyme is 15% less than that of the cow enzyme, confirming the hypothesis that the enzyme activity is related to the concentration of positive surface charge near the active site channel.

Superoxide dismutase (Sheep) Erythrocyte Amino acid sequence Electrostatic interaction

1. INTRODUCTION

The importance of electrostatic interactions in the mechanism of action of Cu,Zn superoxide dismutase (SOD) is well documented [1]. The anionic substrate (O_2^-) is directed to the site of dismutation, a catalytically active copper ion, by a channel that possesses a positive charge because of the presence of conserved basic amino acid residues [2]. In the case of the bovine enzyme, it was recently demonstrated that the rate constant of the catalytic reaction was entirely physically controlled and determined by the ionization of two residues with approximate pK values of 10 and 11, respectively [3]. This electrostatic facilitation is made more efficient by the negative charge on the molecular surface of SOD proteins characterized to date, which will repel O_2^- from uneffective collisions outside the active site region [4]. In view of these considerations, a special interest was developed in our laboratory in the characterization of SODs with higher pI than those of the most studied enzymes from cow, man and yeast, which all have pI values around pH 5, approximately. The pig enzyme has a pI of 6.5 and displays an unusual pH dependence of the activity [5]. However its sequence was shown to contain no amino acid substitutions, with respect to the bovine enzyme, in the segment including residues from 110 to 150, which forms the active site channel [6]. This result supports the idea that the net protein charge may play a role in subtle differences of catalytic efficiency and prompted us to search for more cationic SODs. This paper reports the primary structure of sheep SOD, the first case for an SOD with positive protein charge at neutral pH.

2. MATERIALS AND METHODS

SOD was isolated from sheep erythrocytes according to the standard procedure [7] with modifications to the last chromatographic column, in view of the high isoelectric point of the sheep en-

* To whom correspondence should be addressed

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zyme. The isolation procedure will be published in detail elsewhere.

The catalytic constants for enzyme dismutation were assayed at pH 9.6 by the polarographic method [8] and referred to the copper content of the sample. This was measured by either EPR, which is specific for the SOD copper, or chemical methods. The value was the same by either procedure. The precision of the polarographic method is ± 3% [8]. The isoelectric point was determined by isoelectric focusing on 4% polyacrylamide gels containing 3% ampholine spanning the ranges pH 3.5–9.5 and pH 7–10.

Procedures for preparation of the apoprotein and subsequent carboxymethylation were as described for the porcine enzyme [6]. *S*-Carboxymethylated apoprotein (two samples, 7 mg and 15 mg, respectively) was digested with trypsin or *Staphylococcus aureus* protease [9]. Peptides were isolated by high performance liquid chromatography (HPLC) on macroporous reverse-phase columns (Brownlee Labs, 10 μm) with gradients of 0–70% acetonitrile in 0.2% trifluoroacetic acid. Elution of peptides was monitored on a Beckman 165 spectrophotometer at 220 nm. The amino acid sequence of peptides was mainly determined by the dansyl-Edman technique [10]. The amidation states of Glu and Asp were assigned by direct identification by HPLC of the phenylthiohydantoin (PTH) derivatives released during dansyl-Edman sequence analysis. The same procedure was used for the identification of the derivatives of carboxymethylcysteine [11]. The sequence of peptide T6 and T10 was obtained by automated Edman degradation with an Applied Biosystems 410, gas-phase sequencer equipped with an accessory (120A) for on-line detection of PTH derivatives. Sequencing of the N-terminal tryptic peptide T1 was done essentially as described [12].

3. RESULTS AND DISCUSSION

The complete amino acid sequence of ovine Cu, Zn SOD is shown in fig.1. The sequence was reconstructed by isolation and analysis of a complete set of tryptic peptides which were ordered by another complete set of overlapping peptides ob-

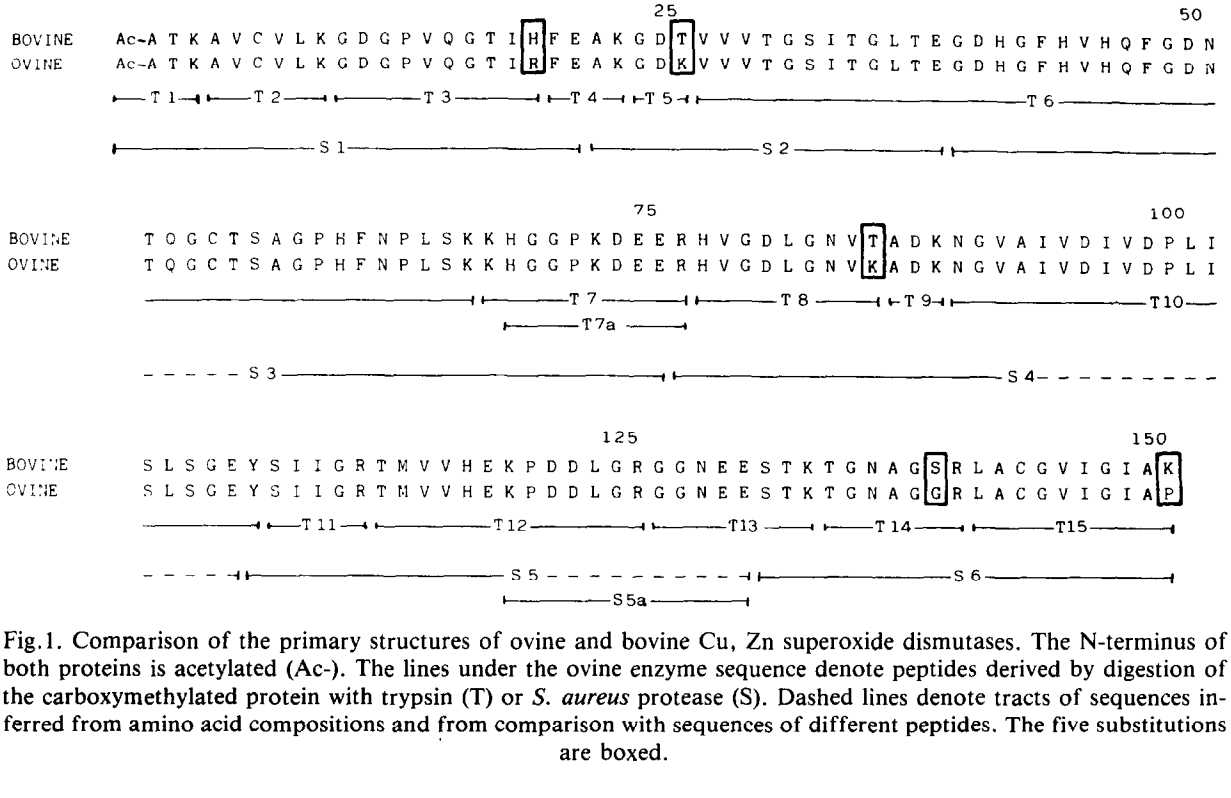

Fig.1. Comparison of the primary structures of ovine and bovine Cu, Zn superoxide dismutases. The N-terminus of both proteins is acetylated (Ac-). The lines under the ovine enzyme sequence denote peptides derived by digestion of the carboxymethylated protein with trypsin (T) or *S. aureus* protease (S). Dashed lines denote tracts of sequences inferred from amino acid compositions and from comparison with sequences of different peptides. The five substitutions are boxed.

tained following digestion of a second sample of carboxymethylated apoprotein with the protease from *S. aureus*, as shown in fig.1. All the peptides were generated by cleavages in accordance with the specificity of the proteolytic enzymes, except for T15 and S6, corresponding to the C-terminal of the protein, and T10, produced by a chymotryptic-like cleavage at a Tyr-Ser bond. For the Glu-specific *S. aureus* protease, the only cleavage at an Asp-X bond was in position 81–82. This bond seems particularly susceptible to *S. aureus* protease, since it was found to be cleaved also in other Cu,Zn SODs [6]. Comparison with the corresponding sequence of the bovine enzyme (fig.1) shows that the two polypeptide chains are identical in length (151 residues), whereas all other known mammalian enzymes of this class are longer (153 residues for horse, human and rat enzymes, 152 for pig; Rotilio [1]). Moreover, the extra residues were always found in the region of residues 17–30, which has been proved to be exceptionally hypervariable with respect to the rest of the protein [13]. In this respect the ovine enzyme is atypical, since, also as far as this region is concerned, it is more similar to the bovine enzyme than any other known SOD (only 2 substitutions while the pig enzyme, the second most similar enzyme, contains 5 changes). One substitution is present in segment 110–150, which forms the active site channel, namely Gly for Ser at residue 140, but it is irrelevant to electrostatic interactions taking place in this region of the protein.

Such a close similarity with the bovine enzyme, which is the most extensively studied enzyme of this class, offers a unique opportunity for considerations on structure-function relationships. In fact, the only major difference between the two enzymes turns out to be the net protein charge. While carboxylate side chains, on a monomer basis, exceed by 6 the positively charged ones (Lys + Arg) in the bovine enzyme, and by 5 in the pig SOD, the excess is 4 in the sheep enzyme. The isoelectric point was measured by electrofocusing to be 8.0 ± 0.2, which is the highest value reported so far for mammalian SODs, and, in any case for SODs for which the primary structure and molecular data are available. While both bovine and ovine enzymes use the same positive charges to guide superoxide toward the active site copper ion, the ovine SOD seems to be not so well suited in that elsewhere on the molecular surface of the enzyme O_2^- is not repelled by negative charges. On this basis the sheep enzyme should be less efficient than the cow SOD. In fact the catalytic constant ($\times 10^9$ $M^{-1} \cdot s^{-1}$) turned out to be 2.27 and 2.03 at pH 9.6, for the cow and sheep enzyme, respectively. While an extensive pulse-radiolytic study at various pH and ionic strength values is in progress to investigate this aspect in more datail, these results confirm that an electrostatic gradient between the active site and the charge on the rest of the protein surface plays a role in determining the enzyme activity [4].

A further point to be mentioned is that, since the sheep SOD is so similar to the bovine enzyme (95% homology) and only differs in the protein charge, its use as pharmacological substitute for the cow enzyme might be worth trying, in view of the expectedly better interactions of cationic proteins with negative charges of natural membranes.

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