Primary structure of porcine Cu,Zn superoxide dismutase

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The complete amino acid sequence of Cu,Zn superoxide dismutase from porcine erythrocytes has been determined. Comparison of the sequence with that of the bovine enzyme shows an overall high degree of homology with conservation of the crucial residues and the presence of two regions prone to variation. In one of these hypervariable regions the insertion of one residue with respect to the bovine enzyme and evidence of structural microheterogeneity has been observed. On the basis of the three-dimensional structure of the bovine enzyme no obvious relationship is apparent between a specific amino acid replacement and the unique pH-dependence pattern of the activity of the porcine enzyme

Superoxide dismutase   Porcine erythrocyte   Amino acid sequence   Microheterogeneity

1. INTRODUCTION

Cu,Zn superoxide dismutases (EC 1.15.1.1) have been shown to be conservative enzymes as far as their structure and function are concerned. X-ray analysis is available for the bovine enzyme [1], but primary structures have been reported for a number of other species [2–8]. These enzymes display a very high catalytic constant for the reaction with O_2^-, approaching diffusion controlled limits [9]. On the grounds of the three-dimensional structure and of chemical modifications of residues of the bovine enzyme, it appears that the catalytic efficiency is determined by the attraction of O_2^- to the copper ion bound at the active site by a set of positively charged amino acid residues, in particular Arg 141 and Lys 134 [10]. Other enzymes of this class studied thus far keep the same catalytic constant between pH 5 and 10 ([9,11], bovine and human enzyme, respectively; our unpublished data, yeast and sheep enzymes) and are reversibly inactivated between pH 10 and 12 ([12] bovine enzyme; our unpublished data, other species). In contrast, the activity of the porcine enzyme, which has an unusually high isoelectric point

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among mammalian superoxide dismutases [13], decreases almost linearly with increasing pH (between pH 7.5 and 12.0) [14]. Elimination of lysine charges by succinylation makes the enzyme activity pH-independent below pH 10. These results prompted us to determine whether the primary structure of the porcine enzyme could explain such peculiar properties.

2. MATERIALS AND METHODS

Superoxide dismutase was prepared from porcine erythrocytes as previously described [14]. Metal-free protein was prepared by dissolving the holoenzyme in 70% formic acid and left for 2 h at 40°C, followed by extensive dialysis against deionized water and lyophilization. Apo-protein was reduced and carboxymethylated with radioactive iodoacetate; two samples (10 mg each) were digested with trypsin or *Staphylococcus aureus* protease, respectively [15]. Isolation of peptides was performed by high-performance liquid chromatography (HPLC) on a reverse-phase column (Brownlee Labs, 10 μm) with gradients of acetonitrile in 0.2% trifluoroacetic acid generated in a Beckman model 420 instrument at a flow rate of 1.2 ml/min. The absorbance of the effluent was

monitored using a Beckman model 165 variable wavelength detector set at 220 and 280 nm. Sequence analysis was carried out using the dansyl-Edman method [16] on the entire peptides or on their endopeptidase subdigestion products after purification. Amide groups were determined and assigned by the following methods: (1) analysis of the amino acids released by action of carboxypeptidases [15], (2) measurements of peptide mobility on electrophoresis at pH 6.5, and (3) in the course of dansyl-Edman degradation, the ethyl acetate extracts that contained the thiazolinone derivatives of Asx or Glx were evaporated to dryness, treated with 1 M HCl at 80°C for 5 min and the resulting phenylthiohydantoin amino acids identified by HPLC [17]. Sequencing of the N-terminal tryptic peptide was done essentially as described in [18]

3 RESULTS AND DISCUSSION

The complete amino acid sequence of Cu,Zn superoxide dismutase from porcine erythrocytes is shown in fig.1. The sequence was deduced by isolation and analysis of a complete set of tryptic peptides which were aligned by another complete set of overlapping peptides obtained following digestion of a second sample of carboxymethylated apoprotein with the protease from *S. aureus*. This work was greatly facilitated, compared with our previous studies on the primary structures of human Cu,Zn [3] and Mn superoxide dismutases [15], by the extensive use of HPLC for peptide purification and assignment of amidation state for Glx and Asx residues
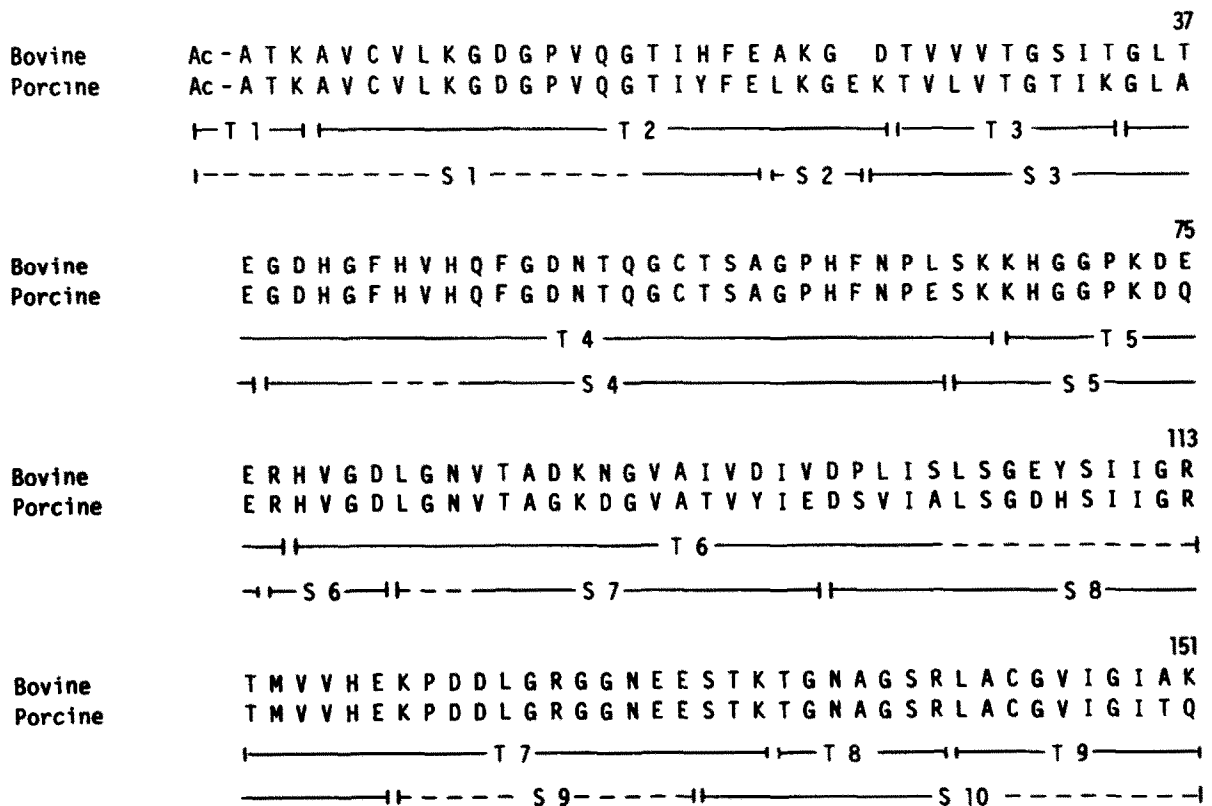
The sequence is composed of 152 amino acids in

Fig 1 Comparison of the primary structures of porcine and bovine Cu,Zn superoxide dismutase The N-terminus of both proteins is acetylated (Ac-) Arrows under the porcine enzyme sequence denote peptides derived by digestion of the carboxymethylated protein with trypsin (T) or *S aureus* protease (S) Dashed arrows denote tracts of sequences inferred from amino acid compositions and from comparison with sequences of different peptides Numbering is that of the bovine enzyme

contrast to the 151 residues of the bovine enzyme. The additional residue is inserted between positions 24 and 25 of the bovine enzyme, in a region, located on the surface of the molecule, previously shown to be hypervariable in the mammalian enzymes [3].

Hypervariability of this region was further confirmed by the finding of a tryptic peptide, T-3a, identical to T-3 but with the conservative substitution of Val for Leu 29, which was easily isolated by HPLC but with a lower yield (4 vs 30%), giving evidence for the presence of structural microheterogeneity. In general, the overall picture of the primary structure of eukaryotic Cu,Zn superoxide dismutases is confirmed, in particular the high degree of homology (85 5% with respect to the bovine enzyme), and the presence of two regions more prone to variation (residues 17–30 and 87–109).

As far as some evolutionary considerations within this class of superoxide dismutases are concerned, it is worth mentioning that sequence homology between porcine and human enzymes (82.3%) is higher than that between the porcine and equine ones (76 5%). This is unexpected on the basis of the phylogenetic distances of these mammals, as confirmed by the sequence differences observed between the cytochrome molecules from the same species [19]. This apparent anomaly could somehow be related to the unique functional behaviour of porcine superoxide dismutase (see section 1 and the following discussion).

In comparison with the bovine enzyme, the porcine superoxide dismutase is even more homologous with respect to the segment including the active site channel. Between residues 110 and 149 the horse enzyme contains 2 substitutions, the human enzyme 4 and the yeast enzyme 12, while the porcine enzyme shows no differences. Therefore, there is no straightforward structural basis that could affect an activity-linked pK in the pH range differentiating the porcine and bovine enzymes, in particular the pK of the lysines located near the active site [10] (Lys 120 and 134 which are 12 and 13 Å from the copper, respectively, and have been conserved in mammalian superoxide dismutases) Work is in progress in our laboratory to modify selectively such lysine side chains in both bovine and porcine superoxide dismutase and thus provide unambiguous evidence for the specific role of these residues in the enzyme activity.

However, it should be mentioned in this context that the sequence data allow comparison of the net protein charge of 3 species having the same numbers of histidine residues, i.e bovine, human and porcine. On a monomer basis, carboxylate side chains exceed by 6 and 7 positively charged residues (Lys plus Arg) in the bovine and human enzyme, respectively, but by 5 in the porcine superoxide dismutase.

The question as to whether the electrostatic potential over a spot of the protein surface [20] is to be considered catalytically as important as specific residues near the active site remains open. In particular, a local change of electrostatic potential may be related to the fact that the absolute value of the catalytic constant of the porcine enzyme at low ionic strength is higher at neutral pH than that of the bovine enzyme [14,21]

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