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Substitution of arginine for lysine 134 alters electrostatic parameters of the active site in shark Cu,Zn superoxide dismutase

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The complete amino acid sequence was determined for the Cu,Zn superoxide dismutase from the shark *Prionace glauca*. The active site region shows the substitution of an Arg for Lys at position 134, which is important for electrostatic facilitation of the diffusion of O_2^- to the catalytically active copper. This change may be related to observed alterations of electrostatic parameters of the enzyme (p*K* of the pH dependence of the enzyme activity, rate of inactivation by H_2O_2), although it preserves a high efficiency of dismutation at neutral pH.

Superoxide dismutase; Amino acid sequence; Electrostatic interaction

1. INTRODUCTION

Primary structures of Cu,Zn superoxide dismutase (SOD) are known from 17 different eukaryotic organisms. Sequence invariance is found for residues that are directly connected with the binding of active site metals and the maintenance of the three dimensional structure, while it seems to be less stringent for cationic residues which in the refined crystallographic model of the bovine enzyme [1] appear to be involved in pre-collision electrostatic guidance of the negatively charged substrate, O_2^-, to the catalytically active copper. This electrostatically facilitated encounter accounts for the high catalytic efficiency of Cu,Zn SOD and reflects a typical pH-dependence curve, which can be fitted by two p*K* values between pH 9 and 11, and the ionic strength dependence of the enzyme activity [2]. In this respect, rather than the conserved Arg 141, which may be considered as part of the pocket around the catalytic Cu ion (5 Å apart), other residues, which are more distantly located on the activity-linked electrostatic channel, play an important role. In the high resolution crystallographic analysis of the bovine enzyme two residues at approx. 12 Å from the copper (Lys 134 and 120) were identified as responsible for the electrostatic facilitation of the enzyme-substrate encounter [1]. Since these residues are somewhat subjected to species variation [3] it is worth studying electrostatic parameters of SOD kinetics in variants lacking either or both residues. While a detailed kinetic study is available for the Lys 120-lacking yeast SOD [2], the present report is the first study on a Lys 134-lacking Cu,Zn SOD, that from the shark *Prionace glauca*.

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2. MATERIALS AND METHODS

Ox and shark SODs were purified as previously described [4,5]. Catalytic constants were evaluated from the dependence of the first-order rate of decay of O_2^- on [SOD] (0.2–1.0 μM) under turnover conditions, $[O_2^-] \gg$ [SOD], by pulse radiolysis [2]. Curve fitting procedures were performed as previously described [2]. Inactivation by hydrogen peroxide was carried

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out as previously reported [6]. Enzyme assays during inactivation processes were carried out by the pyrogallol spectrophotometric method [7].

2.1. *Protein sequence*

Procedures for preparation of the apoprotein and subsequent carboxymethylation were as previously described for the porcine enzyme [8]. To perform an N-terminal sequence analysis on the entire protein, 40 μg of the apoprotein were loaded onto a trifluoroacetic acid-treated glass fiber filter and sequenced with an Applied Biosystems model 470A gas-phase protein sequencer equipped with an Applied Biosystems model 120A PTH analyzer for the on-line detection of PTH amino acids.

Two aliquots of 3 mg of *S*-carboxymethylated apoprotein were suspended in 0.5 ml of 0.1 M ammonium bicarbonate and incubated at 37°C for 3 h with trypsin or *S. aureus* V8 protease, respectively. The enzyme to substrate ratio was 1/40. Purification of the fragments was achieved by high-performance liquid chromatography using a Beckman model 332 instrument, on a macroporus reverse-phase column (Brownlee Labs, Aquapore RP-300; 4.6 × 250 mm, 7 μm) eluted with gradients of 0 to 70% acetonitrile in 0.2% trifluoroacetic acid, at the flow rate of 1 ml/min. Elution of the peptides was monitored on a Beckman 165 spectrophotometer both at 220 and 280 nm.

The amino acid composition of peptides was determined using an LKB 4151 Alpha Plus amino acid analyzer. Sequence analyses were performed after loading aliquots (~1 nmol) of pure peptides onto the gas-phase filter, coated with polybrene and prewashed according to the manufacturer's instructions.

3. RESULTS AND DISCUSSION

The complete amino acid sequence of the shark *Prionace glauca* Cu,Zn SOD is aligned in fig.1 with those of the ox [9] and swordfish [10] enzymes. The sequence was deduced following the analysis of the complete set of tryptic peptides, which were overlapped with the help of the complete set of *S. aureus* V8 protease peptides. This is the first report for an organism representative of the cartilaginous fishes. This sequence displays a 72.1% identity with respect to the bovine se-

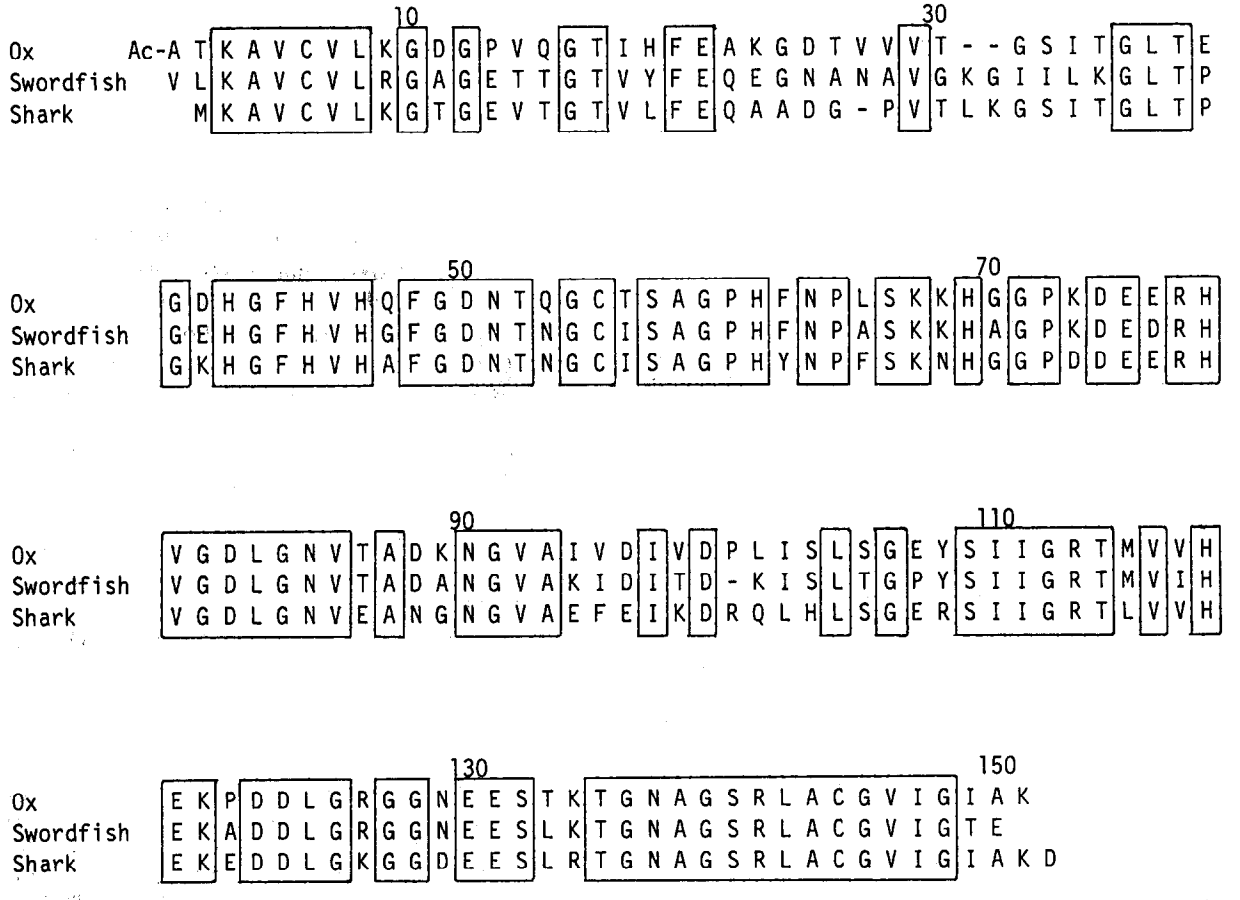

Fig.1. Comparison of the primary structures of shark (*Prionace glauca*), ox and swordfish Cu,Zn superoxide dismutases. Numbering refers to the bovine sequence. Boxes indicate positions at which residues are identical.

quence, taken as representative of the mammalian ones, and 68.2% identity with respect to a teleost SOD, that from swordfish. From the point of view of structure-activity relationships the most interesting feature of the shark Cu,Zn SOD is the absence of Lys 134 which is replaced by an Arg. This residue is a very conserved one and this is the first case of its absence in vertebrates, the other lacking sequences being those from fruit fly and from cabbage [3]. No other charged residues in the electrostatic channel to the active site (Arg 141, Lys 120, Glu 119, Glu 130, Glu 131 in the bovine enzyme) are changed in the shark SOD. Interestingly Glu 131, which is believed to assist Lys 134 in a concerted role for O_2^- guidance to the active site in the bovine enzyme [1] is conserved in the shark enzyme.

The catalytic constant of the shark enzyme was found to be ($k =$) 3.75×10^9 $M^{-1} \cdot s^{-1}$ at neutral pH and $I = 0.02$, a value among the highest found for Cu,Zn SOD [2]. The pH dependence of the activity of the shark SOD in the range pH 6–12 is shown in fig.2 for two salt concentrations together with that of the bovine enzyme as reference. By curve fitting procedures two pK values were calculated for the alkaline activity decline at $I =$ 0.02, at pH 8.6 and 10.6, with a respective contribution of 30% and 70%. These values are lower than those determined for other Cu,Zn SODs [2,11]. The lower pK is strongly affected by ionic strength, suggesting a solvent accessible residue, while the higher pK is nearly insensitive to salt concentration and may be related to Arg 141 as in other SODs [2]. A possible candidate for the residue displaying the lower pK is Lys 120 [11]. It is likely that Arg 134 does not contribute to the pK activity curve, perhaps because of the strong neutralizing effect of the negative charges contributed by Glu 130 and Glu 131. These residues are strategically located at the mouth of the active-site channel with Glu 131 neutralizing Lys 134 in the bovine enzyme three-dimensional model [1]. It is tempting to suggest that in the shark SOD both Glu 131 and Glu 130 carboxyls help electrostatic orientation of the planar guanidinium group of Arg 134. Under these conditions (exposure to solvent, salt bridges) a fairly high pK is expected for this group.

Beside the interaction with O_2^-, also the interaction of Cu,Zn SOD with the inactivating H_2O_2 product [12] is governed by electrostatics. In the presence of H_2O_2 the copper of the bovine enzyme was shown to cycle between the two oxidation states, and the reaction rate was found to be under electrostatic control [13]. This redox process is followed by inactivation of the enzyme and the rate of the process increases with increasing pH.

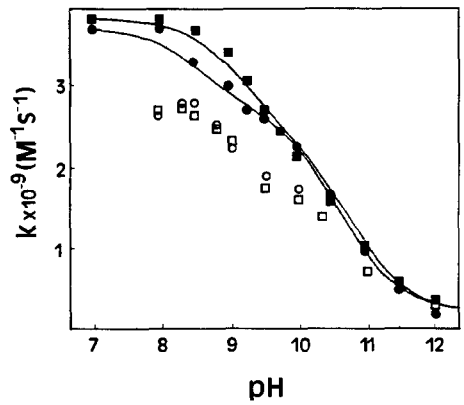

Fig.2. Variation with pH of the catalytic rate constants of ox and shark SODs. Buffers were changed in the different pH ranges as follows: pH 7, Tris/Mes buffer; pH 8–9, Tris/Mops buffer; pH 9 and above, borate buffer. The protein concentration was 0.6 μM. Shark SOD: (●) I 0.02, (○) I 0.1; ox SOD: (■) I 0.02, (□) I 0.1.

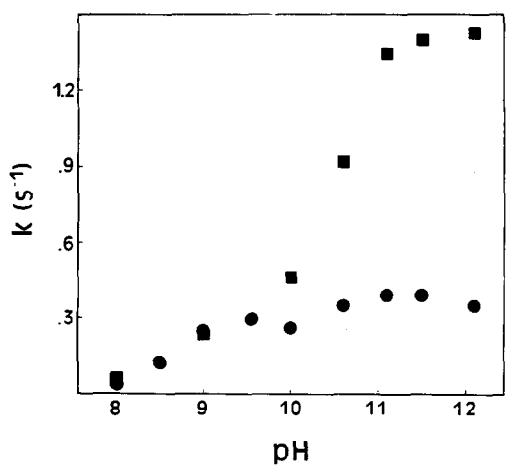

Fig.3. Effect of pH on the rate constant for inactivation of shark (●) and ox (■) SOD by H_2O_2. Enzymes (30 μM) were reacted with 3 mM H_2O_2 in 25 mM PP_i, 25 mM Na_2CO_3 above pH 10 or in 40 mM PP_i, 40 mM $NaHCO_3$, pH 8 and 9. At intervals, aliquots were assayed for residual enzyme activity. The pseudo-first order rate constant values (k) were obtained from semilog plots of residual activity vs time at the indicated pH.

Inactivation of shark Cu,Zn SOD by H_2O_2 was first order with respect to residual activity at all pH values between pH 8 and 12. Fig.3 reports a plot of pseudo-first order rate constants of the inactivation vs pH, for the shark and ox enzymes. These results clearly indicate that the rate of inactivation increases in the same way for the two enzymes between pH 8 and 9.5 approximately. Above this pH the rate further increases in the case of the bovine enzyme, with a pK of about 10.5, in agreement with previous data obtained above 9.6 [6,14], while it is nearly independent of pH in the shark enzyme. The likely explanation for this pH dependence of the H_2O_2 inactivation is that (a) residue(s) with (an) alkaline pK(s) screen(s) the HO_2^- anion (pK = 11.6) somewhat from the copper site where the inactivation occurs [12]. The identical behaviour of ox and shark SODs below pH 9.5 may suggest Lys 120 as a binding site for the peroxide anion. The different combination of charges on the surface of the active site channel of shark SOD may result in the slower rate of the process with respect to the bovine enzyme at higher pH values.

Thus H_2O_2 inactivation appears to be governed by similar electrostatic interactions as the interaction with O_2^-, but with opposite effect, likely because of different size and effective charge on the superoxide and peroxide anions. It can be conclusively suggested that in the case of shark SOD the presence of a cluster formed by Arg 134, Glu 130 and Glu 131 renders the attraction for O_2^- as effective as to result in one of the highest rates of diffusion to the active site, while it apparently inhibits the interaction with H_2O_2 under conditions where Lys 120 is fully deprotonated. This residue may have in the shark protein a pK lower than that observed in mammalian SODs [2] in line with the idea that spatial distribution and resulting electric fields are effective for electrostatic parameters of SODs rather than single residues at specific positions in the linear sequence [15].

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REFERENCES

[1] Getzoff, E.D., Tainer, J.A., Weiner, P.K., Kollman, D.A., Richardson, J.S. and Richardson, A.C. (1983) Nature 306, 287–291.
[2] O'Neill, P., Davies, S., Fielden, E.M., Calabrese, L., Capo, C., Marmocchi, F., Natoli, G. and Rotilio, G. (1988) Biochem. J. 251, 41–46.
[3] Steffens, G.J., Michelson, A.M., Ötting, F., Puget, K., Strassburger, W. and Flohé, L. (1986) Biol. Chem. Hoppe-Seyler 367, 1007–1016.
[4] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[5] Galtieri, A., Natoli, G., Lania, A. and Calabrese, L. (1986) Comp. Biochem. Physiol. 83B, 555–559.
[6] Fuchs, H.J.R. and Borders, C.L., jr (1983) Biochem. Biophys. Res. Commun. 116, 1107–1113.
[7] Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474.
[8] Schininà, M.E., Barra, D., Simmaco, M., Bossa, F. and Rotilio, G. (1985) FEBS Lett. 186, 267–270.
[9] Steinman, H.M., Naik, V.R., Abernethy, J.L. and Hill, R.L. (1974) J. Biol. Chem. 249, 7326–7338.
[10] Rocha, A., Bannister, W.H. and Bannister, J.V. (1984) Eur. J. Biochem. 145, 477–484.
[11] Argese, E., Viglino, P., Rotilio, G., Scarpa, M. and Rigo, A. (1987) Biochemistry 26, 3224–3228.
[12] Bray, R.C., Cockle, S.A., Fielden, E.M., Roberts, P.B., Rotilio, G. and Calabrese, L. (1974) Biochem. J. 139, 43–48.
[13] Viglino, P., Scarpa, M., Rotilio, G. and Rigo, A. (1988) Biochim. Biophys. Acta 952, 77–82.
[14] Hodgson, E.K. and Fridovich, I. (1975) Biochemistry 14, 5294–5298.
[15] Desideri, A., Falconi, M., Parisi, V. and Rotilio, G. (1989) FEBS Lett. 250, 45–48.