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Conservation of local electric fields in the evolution of Cu,Zn superoxide dismutase

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The trend of the electric field and the value of the electric field flux, sensed by the superoxide substrate in the proximity of the active site, were found to be constant in three highly homologous Cu,Zn superoxide dismutases from ox, pig and sheep, which display large differences in net protein charge and distribution of electrically charged surface residues but very similar catalytic rate constants. The spatial relationship of charges on the protein surface apparently has been conserved during the evolution of this enzyme to create electrostatic facilitation of catalysis.

Molecular evolution; Electrostatic catalysis; Superoxide dismutase

1. INTRODUCTION

Cu,Zn superoxide dismutases are a class of dimeric enzymes, composed of equivalent subunits of 16 kDa. The three-dimensional structure has been resolved to a resolution of 2 Å for the bovine erythrocyte enzyme [1] and shows a rigid β-barrel core scaffolding external loops which accommodate the active site, consisting of a cluster of adjacent Cu and Zn ions. These enzymes catalyze the dismutation of superoxide anion into oxygen and hydrogen peroxide at diffusion-limited rates. For this reaction, evidence in favor of an enzyme-substrate recognition process based on electrostatic interactions has been provided by selective chemical modification experiments [2,3] and by investigation of the dependence of the activity of the bovine enzyme on pH and ionic strength [4]. The idea of electrostatic guidance of the negatively charged superoxide substrate to a positively charged active site as an essential feature to explain the selective diffusion to the restricted active-site area, was first proposed by Koppenol [5]. He calculated the coulombic potential, created by charged surface residues of the bovine enzyme, for a fixed value of the water dielectric constant, and found that positive potential was concentrated near the active site, in contrast with the net negative charge of the protein. Recently, we have shown that different Cu,Zn superoxide dismutases that have distinctly more positive net charges of the protein than the bovine enzyme display identical catalytic rate constants [6]. It appeared important to reinvestigate the electrostatic model in relation to variously charged superoxide dismutases, since the hypothesis with respect to the bovine enzyme relied on the existence of an asymmetric distribution of potential between the positively charged active site and the elsewhere negatively charged protein surface. The present results indicate that a specific stereorelationship of charges is preserved in genetic variants of the enzyme rather than global protein charge and charge distribution on the protein surface.

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2. MATERIALS AND METHODS

The calculation of electrostatic parameters was based on

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previously determined primary structures [7–9], which are reported in fig.1. The crystallographic coordinates of the bovine enzyme [1] were used for all of the enzymes, since they show a very high degree of homology (fig.1) and all amino acid substitutions are located on the protein surface [8,9]. Moreover, the profiles for the propensity of flexibility [10], hydropathic character [11] and prediction of secondary structure [12] gave identical patterns for the three enzymes. Therefore, it is reasonable to assume that the substitutions involved do not perturb the overall three-dimensional structure. For the bovine enzyme the same charge assignment as in [5] was assumed in order to give a final net charge on the dimer of $-3e$, where e is the charge on an electron (1.6×10^{-19} C). The porcine enzyme [8] has 21 amino acid substitutions plus one insertion with respect to the bovine enzyme, 12 of which, including the insertion, produce charge variation yielding an overall net charge of $-1e$. The ovine enzyme [9] shows five substitutions for the monomer which result in a net increase of three positively charged residues (overall net charge equal to zero). The charge of the inserted Glu 24 in the pig enzyme was positioned on the C_α of the preceding amino acid residue. Small shifts of this charge did not affect the overall results. The potentials were calculated as in [5].

3. RESULTS AND DISCUSSION

The three proteins chosen here have the highest degree of homology among the Cu,Zn superoxide dismutases sequenced to date, i.e. with respect to the bovine enzyme, 97% for the sheep protein and 86% for the porcine form (fig.1) and thus meet the general criterion that analysis of protein families for which linear sequences and at least one crystallographic structure (that of the bovine enzyme [1]) are available yields more accurate results if the sequences compared show a high degree of homology. On the other hand, they are particularly suitable for studying evolutionary conservation of electrostatic parameters in these enzymes, since they differ markedly from each other as far as the net charge on the protein is concerned [6]. The bovine enzyme is an acidic protein at physiological pH (p*I* 5.2) whereas the porcine enzyme is nearly neutral (p*I* 6.5) and the sheep protein distinctly cationic (p*I* 8.0). Fig.2 shows a contour diagram of the electrostatic potentials of the three proteins considered. The electrostatic difference between the three enzymes is straightforward, as the size of the positively charged regions increases with the total net charge of the protein. However, when the electric field was calculated in the vicinity of the active site (fig.3) this difference vanished. The density of the arrows representing the electric field was identical in the proximity of the active site for all

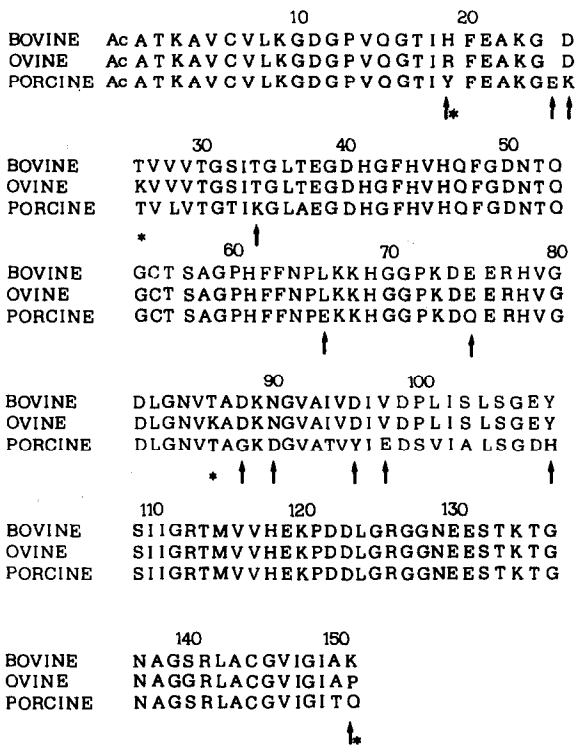

Fig.1. Amino acid sequences of bovine [9], porcine [10] and ovine [11] Cu,Zn superoxide dismutases. The substituted charged residues have been labelled with reference to the sequence of the bovine enzyme: (↑) porcine/bovine change; (*) ovine/bovine change.

three, and all vectors point to the catalytically active copper and not to the adjacent zinc. In particular (fig.4), the arrows focus at a point located 5 Å to the right-hand side of the copper which is in projection the location of Arg 141 [1]. This residue is present in all Cu,Zn superoxide dismutases sequenced to date and has been shown to be essential for enzyme activity [13]. It is also interesting to note that the substantially identical values for the electric field flux correlate fairly well with the respective catalytic constants of the three enzymes (table 1).

A reasonable objection to these results is that in the calculations, the dielectric constant of bulk water ($D = 78.5$) was adopted [5]. A more realistic approach, which solves the Poisson-Boltzmann equation for molecules of arbitrary shapes and incorporates the screening effect of the electrolyte [14], is essential when the influence of the shape of the protein and the shape and absolute magnitude

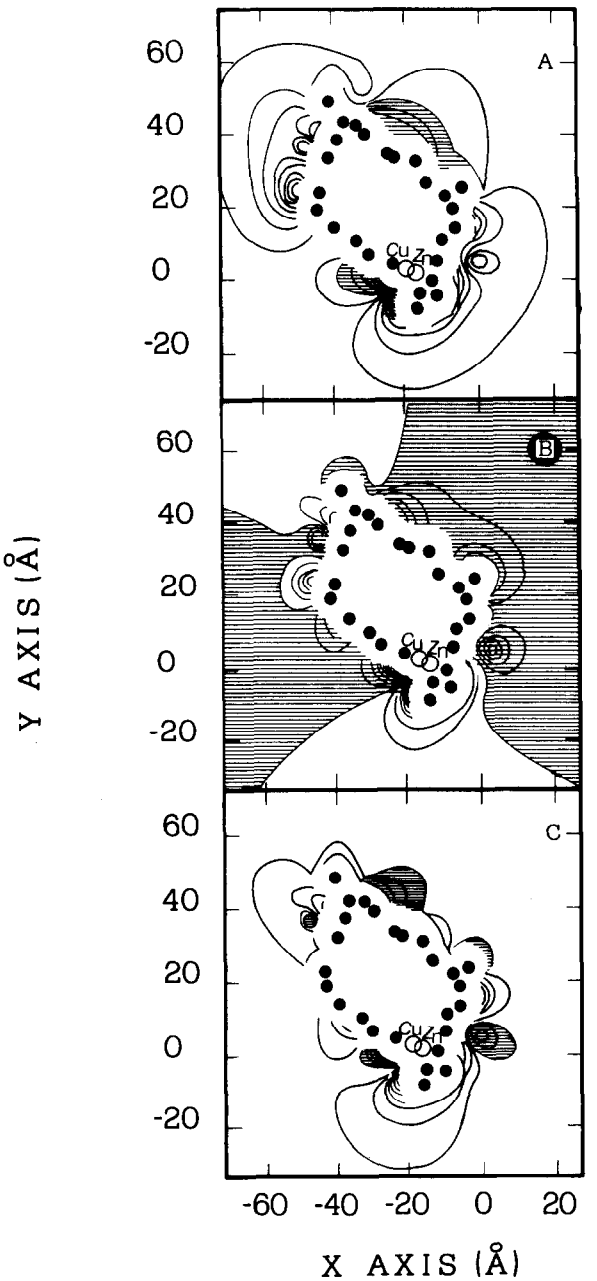

Fig.2. Contour diagrams for the electrostatic potential around (A) bovine, (B) ovine, (C) porcine Cu,Zn superoxide dismutase in a cross-section perpendicular to the crystallographic z-axis for $z = -15$ Å, i.e. the xy plane where the copper of one of the two subunits is located. The electrostatic potential is expressed in units of kT/e where k is Boltzmann's constant and T = 300 K. Positive potential is represented by hatched areas. Copper and zinc are shown as empty circles and are labelled with the appropriate symbol. Full circles indicate the atoms that lie on the edge of the protein surface in the same cross-section. The Zn(II) is not on the same plane of the Cu(II) and is displayed as a projection.

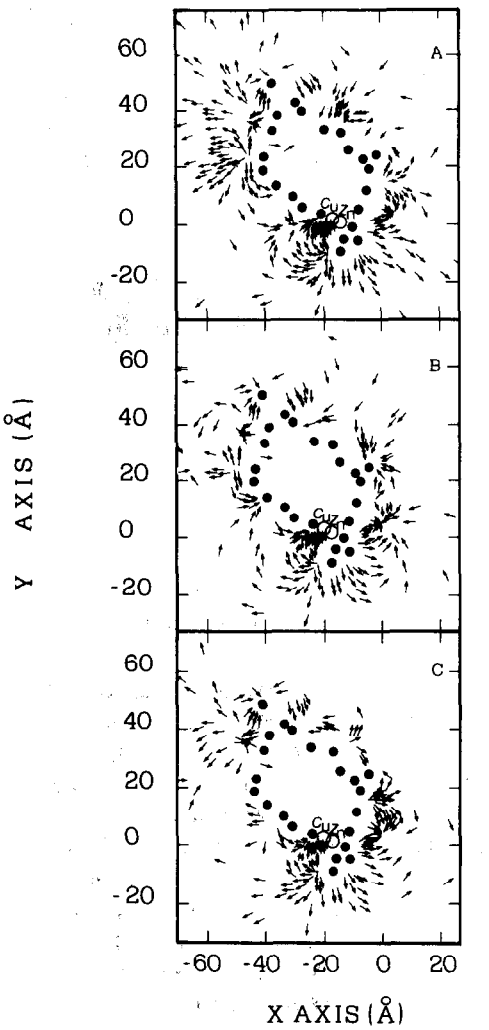

Fig.3. Electric fields in (A) bovine, (B) ovine, (C) porcine Cu,Zn superoxide dismutase displayed in a cross-section perpendicular to the crystallographic z axis at $z = -15$ Å (see fig.1). Arrows represent electric field vectors and their density is proportional to the absolute value of the electric field at that point.

of the field surrounding the protein is to be evaluated. However, the use of such an approach becomes less stringent when molecules of the same spatial structure, i.e. equal dielectric properties, are compared as far as interactions near the protein surfaces are concerned. Using a different value for the constant will change the absolute value of the potential but does not affect the validity of the model, which has been worked out on a comparative basis and is insensitive to perturbations

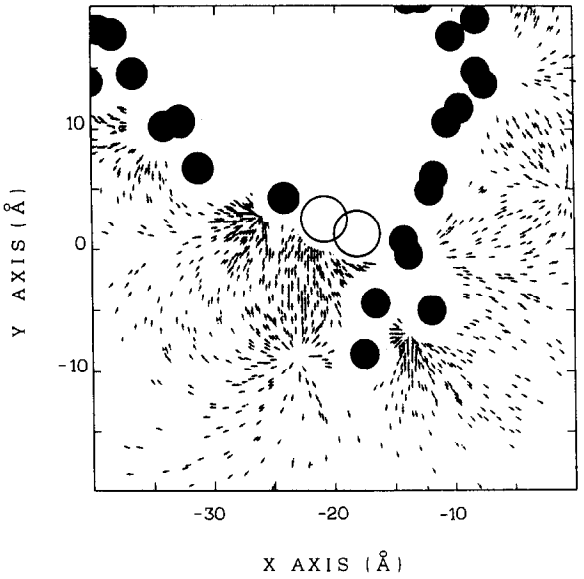

Fig.4. Close-up view of the electric field in the proximity of the active site of bovine Cu,Zn superoxide dismutase.

acting on all three proteins considered. Therefore, only the role of the different charge distributions is to be compared in our analysis. On these bases, it is possible to include the electric field parameter among the features conserved during the evolution of this class of enzymes, beside ligands to the metal ion, conformation-determining residues, and Arg 141. Charged surface residues are apparently more subject to variation. In particular, Lys 120 or Lys 134 (approx. 12 Å distant from the copper) may play essential roles in attracting O_2^- to the active-site copper in the bovine enzyme [15]. However, the absence of Lys 120 and/or Lys 134 in some enzymes from lower eukaryotic species does not affect the value of the catalytic constant [6]. Apparently, co-ordinated mutations have occurred in the evolution of Cu,Zn superoxide dismutase so that changes of the surface charged residues are counterbalanced such that they result in the same three-dimensional array of charges giving rise to a constant electric field between the catalytic active site and the surrounding protein surface. Such an electric field is able to attract negatively charged molecules of small size, like O_2^-, at a diffusion-controlled rate toward the active site. This attraction has apparently been a constant feature of the molecular evolution of this protein, and is very likely to be linked to its distinct biological function.

Table 1

Electric field flux (ϕ) in different Cu,Zn superoxide dismutases

Source of enzyme	$10^{-9} \times k$ ($M^{-1} \cdot s^{-1}$)	ϕ (kTÅ$/e$)
Ox	3.8	23.9
Pig	3.2	23.5
Sheep	3.3	24.2

Values for the bimolecular rate constant are according to [6]. ϕ was calculated as the scalar product between the electric vectors (see fig.3) and a 5 × 5 Å surface perpendicular to the xy plane at an average distance of 15 Å from the active site. The values are expressed in units of kTÅ$/e$

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REFERENCES

[1] Tainer, J.A., Getzoff, E.D., Beem, K.M., Richardson, J.S. and Richardson, D.C. (1982) J. Mol. Biol. 160, 181–217.
[2] Cudd, A. and Fridovich, I. (1982) J. Biol. Chem. 257, 11443–11447.
[3] Cocco, D., Rossi, L., Barra, D., Bossa, F. and Rotilio, G. (1982) FEBS Lett. 150, 303–306.
[4] Argese, E., Viglino, P., Rotilio, G., Scarpa, M. and Rigo, A. (1987) Biochemistry 26, 3224–3228.
[5] Koppenol, W.H. (1981) in: Oxygen and Oxy-radicals in Chemistry and Biology (Rodgers, M.A.J. and Powers, E.L. eds) pp.671–674, Academic Press, New York.
[6] O'Neill, P., Davies, S., Calabrese, L., Capo, C., Marmocchi, F., Natoli, G. and Rotilio, G. (1988) Biochem. J. 251, 41–46.
[7] Steinman, H.M., Naik, V.R., Abernethy, J.L. and Hill, R.L. (1974) J. Biol. Chem. 249, 7326–7338.
[8] Schininà, M.E., Barra, D., Simmaco, M., Bossa, F. and Rotilio, G. (1985) FEBS Lett. 186, 267–270.
[9] Schininà, M.E., Barra, D., Gentilomo, S., Bossa, F., Capo, C., Rotilio, G. and Calabrese, L. (1986) FEBS Lett. 207, 7–10.
[10] Karplus, P.A. and Schultz, G.E. (1985) Naturwissenschaften 72, 212–213.
[11] Fauchere, J.L. and Pliska, W.E. (1983) Eur. J. Med. Chem. 18, 369–375.
[12] Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. 120, 97–120.
[13] Beyer, W.F., Fridovich, I., Mullenbach, G.I. and Hallewell, R. (1987) J. Biol. Chem. 262, 1182–1187.
[14] Klapper, I., Hagstrom, R., Fine, R., Sharp, K. and Honig, B. (1986) Proteins 1, 47–59.
[15] Getzoff, E.D., Tainer, J.A., Weiner, P.K., Kollmann, P.A., Richardson, J.S. and Richardson, D.C. (1983) Nature 306, 287–291.