# COMPETITIVE INHIBITION OF Cu, Zn SUPEROXIDE DISMUTASE BY MONOVALENT ANIONS

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SUMMARY

Polarographic measurements showed that $N_3^-$ and halides inhibit the activity of bovine Cu, Zn superoxide dismutase in a competitive fashion, as previously demonstrated for $CN^-$ and $OH^-$. All anions increase the spin-lattice nuclear magnetic relaxation time ($T_1$) of aqueous solutions of the enzyme as well, but the stability constants measured from $T_1$ data are lower than those calculated from activity data. The results suggest that substrate and anionic inhibitors bind during the catalytic action at the water coordination position of the enzyme copper, and that these inhibitors may have a greater affinity for the cuprous form of the enzyme which is generated in the catalytic cycle.

Copper-zinc superoxide dismutases catalyse the dismutation of superoxide anions by a mechanism in which the copper ion at the active site is alternatively reduced to $Cu^+$ by the first $O_2^-$, giving $O_2$, and reoxidized by the second $O_2^-$, giving $H_2O_2$. The rates of reduction and oxidation are equal and approach the diffusion controlled limit (1).

Inhibition of these enzymes is still poorly characterized at the mechanistic level, mainly because of the low yield of substrate obtained by pulse radiolysis, the only technique available until recently for mechanistic studies of $O_2^-$ dismutation. We have shown that determination of catalytic constants





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in the presence of relatively high concentrations of $O_2^-$ can be obtained with a polarographic method (2) and were thus able to measure $K_m$ and $V_{max}$ values of the copper-zinc superoxide dismutase of bovine red blood cells (3). In this report we have used this method to investigate the mechanism of inhibition of SOD by monovalent anions, which are known to bind to the copper site of the enzyme.

## MATERIALS AND METHODS

Superoxide dismutase was prepared from bovine red blood cells according to the procedure of McCord and Fridovich (4).

All reagents were analytical grade. Twice distilled water was used. High purity mixtures of $O_2$ and $N_2$ were from SIO (Milan). The activity of the enzyme was measured by the polarographic method of catalytic currents (2) with a Model 461 Amel polarographic apparatus (Amel, Milan) at 25°C in 0.02 M borate buffer pH 9.8 saturated with triphenylphosphine oxide and equilibrated with $N_2$ - $O_2$ mixtures with different $O_2$ content. The concentration of superoxide obtained in these conditions was determined as previously reported (4). Longitudinal nuclear magnetic relaxation time ($T_1$) of water protons was measured by a Varian pulsed NMR apparatus working at 16 MHz.

## RESULTS

### Activity measurements

Double reciprocal plots of $1/V_o$ vs $1/[O_2^-]$ at constant inhibitor concentration showed (Fig. 1) that $N_3^-$, $Cl^-$, $Br^-$ and $F^-$ are competitive inhibitors, as already shown for $CN^-$ and $OH^-$ (3) which however are far more effective inhibitors. The inhibition constants calculated from the slopes of such plots, are reported in Table 1, first entry.

Similar values were found when the enzyme activity of the bovine copper superoxide dismutase was measured at constant

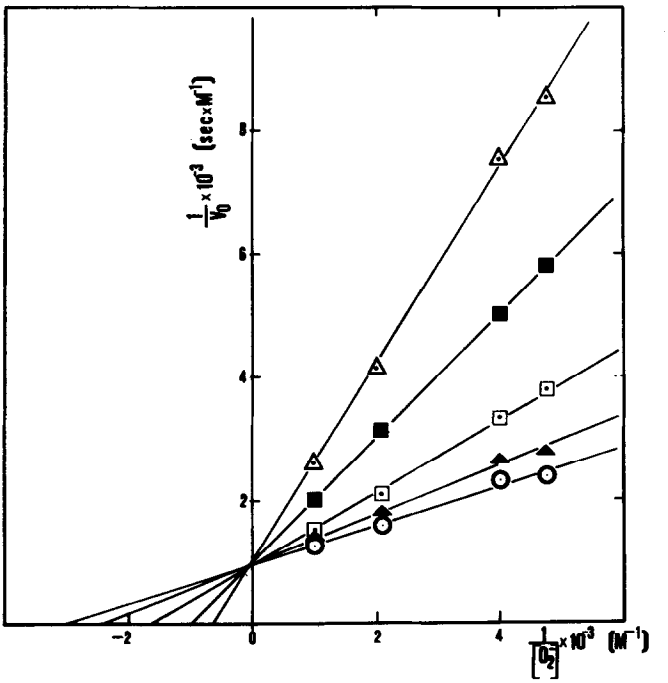


Figure 1 - Double reciprocal plots of initial velocities versus $O_2^-$ concentration. Enzyme concentration : $1 \times 10^{-9}$ M. ⊙ no inhibitor ; ▲ 0.08M $F^-$; ⊡ 0.08M $Cl^-$ ; ■ 0.08M $Br^-$ ; △ 0.05M $N_3^-$ . Ionic strenght:0.1 (by addition of 2M $NaClO_4$)

$[O_2^-]$(air equilibrated solutions) and at five concentrations of the inhibitory anion (Table I, second entry).

Increasing ionic strength decreased the inhibition power of all anions (Table I, third entry). Citrate, $CNS^-$, and $NO_3^-$ had no effect on superoxide dismutase activity.

## $T_1$ measurements

The longitudinal nuclear magnetic relaxation time ($T_1$) of water protons of aqueous solutions of superoxide dismutase was measured at different anion concentrations at pH 9.8. The titration of the paramagnetic contribution to the relaxivity ( $1/T_{1p}$) with $CN^-$, $N_3^-$, $Br^-$, $Cl^-$ and $F^-$ is reported in Figs.2

TABLE I

Stability constants ($M^{-1}$) of superoxide dismutase from activity and $T_1$ measurements: 0.02 M borate buffer, pH 9.8, T= 25°C. Different ionic strengths were obtained by addition of 2M $NaClO_4$.

| Anion | from activity measurements $\mu$=0.1(a) | $\mu$=0.1(b) | $\mu$=0.2(b) | from $T_1$ measurement at $\mu$= 0.12(c) |
|---|---|---|---|---|
| $CN^-$ | $2.9\times10^5$ | $2.4\times10^5$ | $2.3\times10^5$ | $9\times10^4$ |
| $N_3^-$ | 83 | 87 | 68 | 58 |
| $Br^-$ | 28.5 | 29 | 21.5 | 0 |
| $Cl^-$ | 11 | 13.5 | 9.5 | 1.6 |
| $F^-$ | 3.1 | 3.2 | 2.0 | 1 |

a) The constant was measured from Lineweaver-Burk plots such as those of Fig.1
b) The constant was measured from experiments at $[O_2^-] \simeq 2\times10^{-4}M$ and at different anion concentrations.
c) The measurements were made on enzyme solutions containing the same components as the solutions used in polarographic measurement. Ionic strength has no effect on water relaxation of enzyme solutions with or without coordinating ligands.

and 3, while the stability constants measured for the various enzyme-anion complexes by this method are reported in the last entry of Table I. It appears that the different anions affec - ted the relaxivity to different extents, roughly paralleling their effects on activity. However $Br^-$ showed no effect on relaxivity even at concentrations as high as 1M. In the case of $CN^-$, Fig. 3 shows that 2 moles of $CN^-$ ions are bound per mole of protein.

## DISCUSSION

The interaction of the copper binding site of copper, zinc

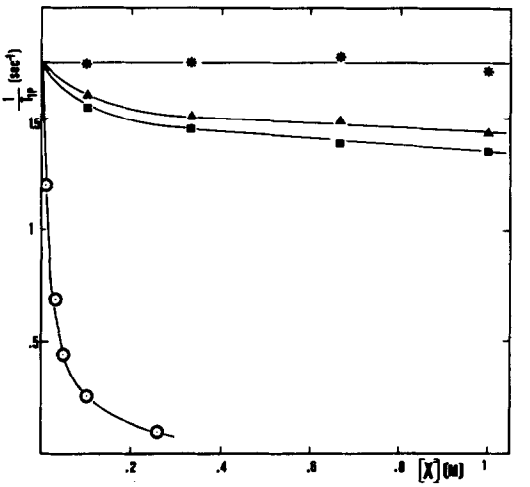


Figure 2 – Paramagnetic contribution to the longitudinal nuclear magnetic relaxation rate of water protons, at 16 MHz of a solution of superoxide dismutase in the presence of different anion concentrations.
✱ $Br^-$; ▲ $F^-$; ■ $Cl^-$; ⊙ $N_3^-$
0.02M borate buffer, pH 9.8. Superoxide dismutase concentration: $2.1 \times 10^{-4}$ M.

superoxide dismutases with monovalent anions has been investigated by magnetic resonance and optical techniques in early reports on this enzyme (5,6,7). $CN^-$, which showed the far highest affinity toward the enzyme-bound copper was suggested to replace a water molecule coordinated to the metal ion. The reaction of all the tested anions ($CN^-$, $N_3^-$, $F^-$, $OH^-$) brought about a conversion of the EPR line shape of the enzyme-bound copper from rhombic to axial (6,7). This pointed to a similar mechanism of reaction in spite of the great difference of stability constants between $CN^-$ and other anions and a conflicting report on the effect of $N_3^-$ on magnetic properties of bovine superoxide dismutase (5). Activity measurements either by pulse radiolysis (1) or polarography (3) showed that $CN^-$ and $N_3^-$ are also inhibitors of the enzyme and that the inhibition constant calculated from kinetic data is roughly comparable to that measured from spectroscopic measurements. The availability of a kinetic method wor-

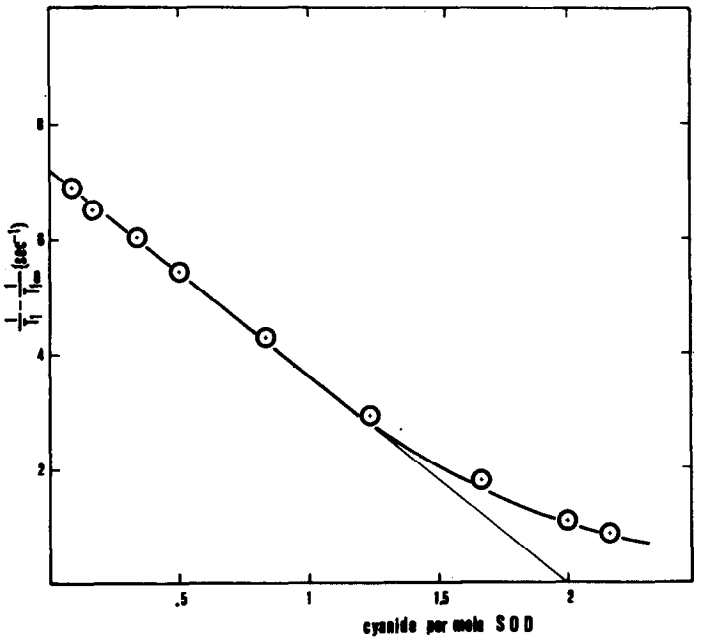


Figure 3 - Relaxivity of water protons at 16 MHz as the function of the molar ratio $[CN^-]$ / [superoxide dismutase]
0.02M borate buffer, pH 9.8. Superoxide dismutase concentration: $9 \times 10^{-4}$ M

king at high $O_2^-$ concentrations led us to study the reaction of the enzyme with monovalent anions in more detail, and, in particular, to compare the activity and equilibrium data in a more precise way. A competitive pattern of inhibition was observed with anions having lower affinity toward enzyme ($N_3^-$ and halides) similarly to that observed with $CN^-$ and $OH^-$ (4) and this confirms that an identical mechanism can be suggested for all anions. In particular, $N_3^-$ behaved as the other anions, at variance with previous suggestions by Fee and Gaber (5) and by Hodgson and Fridovich (8). The former authors proposed another $N_3^-$-binding site with higher affinity than copper because of the lack of $T_1$ effects in the presence of low $N_3^-$ concentrations which produced an altered EPR spectrum. The latter authors suggested that $N_3^-$ binds to copper at a different position of that normal

ly occupied by $H_2O$, as they observed no inhibition of superoxide dismutase activity by 0.01 M $N_3^-$. While the former results were not reproduced in other laboratories (9) the latter data may be imputable to the relatively low $N_3^-$ concentration used (2 ). Therefore, on the basis of our data, including the effect of ionic strength (Table I), it can be proposed that enzyme - -substrate and enzyme-inhibitor complexes are formed in the enzyme reaction and that the binding position of $O_2^-$ and inhibitors is the same, i.e. the water coordination position, as shown by the general effects of anions on the spin-lattice relaxation time of the water protons (Table I, and Figs 2 and 3).

However, the most interesting feature of the data reported in Table I is the differences between stability constants of complexes calculated from polarography and water proton relaxation measurements. Possible explanations are:

a) $O_2^-$ and $H_2O$ bind at different binding sites on the enzyme;
b) conformational changes occur during turnover;
c) the binding constants of some anions for the oxidized ($Cu^{++}$) and reduced ($Cu^{+}$) states of the enzyme copper are different.

As regards point a) the titration of superoxide dismutase with $CN^-$(Fig.3) shows that in the oxidized form of the enzyme, which has two equivalent $Cu^{++}$ ions, one in each of two identical subunits (1), 1 $CN^-$ binds to each $Cu^{++}$ stoichiometrically and there is no $CN^-$ binding sites with stability constant equal to that obtained from activity experiments. Since $CN^-$ competes with $H_2O$ and $O_2^-$ for the copper ions of superoxide dismutase the difference in behaviour of resting and working enzyme appears to be related to the catalytic action of the enzyme.

It is not easy to decide from experiments in turnover conditions whether the observed behavior is due to a change of con - formation during enzyme action or to different values of binding constant of the two oxidation states of the enzyme copper. The latter hypothesis is more plausible on account of the higher stability constants of $CN^-$ and halide ions with the $Cu^+$ (10). $CN^-$

and $Cl^-$ have been demonstrated to bind the cuprous form of the enzyme by $^{35}Cl$ line width measurements (11). On this line it is interesting to notice that, while for the $Cu^+$ free ion the stability constant decreases in the order $I^- \geq Br^- \geq Cl^-$ (10) in the case of superoxide dismutase we found $Br^- \geq Cl^-$ and $I^- \simeq 0$. This result can be interpreted in terms of non accessibility of $I^-$ to the active site pocket of the reduced form of bovine superoxide dismutase. If this assumption is correct the active site should have dimensions between the ionic radius of $Br^-$ and $I^-$ which are 1.87 and 2.12 Å respectively (12).

Lastly it should be pointed out that, in spite of the low value of its stability constant, $Cl^-$ appears to be an effective inhibitor of red cell superoxide dismutase at physiological concentrations.

REFERENCES

1) Fielden,E.M.,Roberts,P.B., Bray,R.C., Mautner,G.N., Rotilio,G. and Calabrese,L. (1974) Biochem.J.,199, 49-60.
2) Rigo,A., Viglino,P. and Rotilio,G. (1975) Anal.Biochem.,68, 1-8.
3) Rigo,A., Viglino,P. and Rotilio,G. (1975)Biochem.Biophys. Res.Commun.,63, 1013-1018.
4) McCord,J.M. and Fridovich,I. (1969) J.Biol.Chem.,244,6049-6055.
5) Fee, J.A. and Gaber,B.P. (1972) J.Biol.Chem.,247, 60-65.
6) Rotilio,G., Finazzi-Agrò A., Calabrese,L., Bossa,F., Guerrieri,P. and Mondovì,B. (1971) Biochemistry 10,616-620.
7) Rotilio,G., Morpurgo,L., Giovagnoli,G., Calabrese,L. and Mondovì,B. (1972) Biochemistry,11, 2182-2187.
8) Hodgson,E.K. and Fridovich,I. (1975) Biochemistry,14, 5214-5299.
9) Rotilio,G., Calabrese,L., Mondovì,B. and Blumberg,W.E. (1974) J.Biol.Chem., 249, 3157-3160.
10) Charlot,G. (1963) "L'analyse qualitative et les reactions en solution", Masson, Paris, 274.
11) Fee,J.A. and Ward,R.L. (1976) Biochem.Biophys.Res.Commun. 71, 427-437.
12) Cotton,F.A. and Wilkinson,C. (1972) "Advanced Inorganic Chemistry" Interscience, New York, 458.