KINETIC STUDY OF O_2^- DISMUTATION BY BOVINE SUPEROXIDE DISMUTASE. EVIDENCE FOR SATURATION OF THE CATALYTIC SITES BY O_2^-

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Summary. Dismutation of O_2^- by bovine copper-zinc superoxide dismutase has been studied at different O_2^- concentrations with a polarographic method. Saturation of the enzyme by the substrate was observed and K_m and Vmax values were calculated. Inhibition by OH^- and CN^- was shown to be of the competitive type. The data support on inner sphere mechanism for the reaction between O_2^- and copper.

A number of metalloproteins present in the aerobic organisms are able to catalyze the dismutation of O_2^- into H_2O_2 and O_2 and are named superoxide dismutases (1). The best known is the enzyme from bovine red cells. Its active site is a cupric ion bound to three histidine residues one of which is also bound to a zinc ion (2-4). Pulse radiolysis measurements have shown that its mechanism of reaction is a ping-pong redox reaction where the rates of oxidation and reduction of copper ion by O_2^- are equal (5).

Recently we have developped a polarographic method which appears to be a powerful analytical procedure to carry out further kinetic studies on the O_2^- superoxide dismutase system (6-7). One advantage of this method is the relatively high concentration of O_2^- which is possible to realize in comparison to pulse radiolysis. In view of this potentiality the aim of this work was to study the behaviour of bovine superoxide dismutase at different O_2^- concentrations in order to control the saturability of the enzy

me with the substrate, which was not observed in previous pulse radiolysis studies (8).

MATERIALS AND METHODS

All reagents were analytical grade. Twice distilled water was used. High purity mixtures of N_2 and O_2 were supplied by SIO (Milan).
Bovine superoxide dismutase was prepared according to the procedure of McCord and Fridovich (9).
The activity measurements were carried out as previously reported (6), at 25°C in .1N borate buffer, pH 9.90, containing 5×10^{-4} M triphenylphosphine oxide and equilibrated with different mixtures of N_2/O_2.
The O_2 concentration in the reaction system was calculated after each experiment from the height of the wave of O_2 reduction to H_2O_2.
An Amel polarographic apparatus mod. 461 and an Amel pH meter mod. 331 (Milan) were used.

RESULTS AND SISCUSSION

O_2^- is generated at the dropping mercury alectrode (DME) in presence of triphenylphosphine oxide (TPO) by reduction of O_2 at -1 V vs a saturated calomel electrode, and the concentration of O_2^- in the reaction layer next to the DME can be considered in first approximation equal to that of the oxygen in the bulk (10). The addition to this system of superoxide dismutase results in the dismutation of O_2^- into H_2O_2 and O_2 which depolarizes the DME and, as a consequence, increases the electrode current. As previously reported (6) the kinetic constant for this process was calculated assuming the rate of O_2^- disappearance given by

$$1) \qquad -\frac{d[O_2^-]}{dt} = k\,[O_2^-]\,[Eo]$$

where Eo is the total enzyme concentration.
However according to the simple kinetic scheme

$$2) \qquad E + O_2^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EO_2^- \xrightarrow{k_3} E + P$$

it results:

$$2) \qquad -\frac{d[O_2^-]}{dt} = \frac{k_3}{K_m + [O_2^-]} [O_2^-] [E]$$

with $K_m = \frac{k_2 + k_3}{k_1}$. This equation is similar to eqn 1) if $K_m \gg [O_2^-]$ or O_2^- concentration can be considered constant during the measure. Since in all activity determinations the superoxide dismutase concentration was such that the reacted fraction of O_2^- was at most .1, $[O_2^-]$ and consequently $\frac{k_3}{K_m + [O_2^-]}$ can be considered as a constant during the activity measurement.

The values of $\frac{k_3}{K_m + [O_2^-]}$ at different O_2^- concentrations are reported in Table 1. The decrease of the $\frac{k_3}{K_m + [O_2^-]}$ with in

TABLE 1

Evidence for saturation of bovine superoxide dismutase by O_2^-

$[O_2^-]$ (a) (mM)	$v_o \times 10^3$ (b) (M/sec)	$\frac{k_3}{Km + [O_2^-]} \times 10^{-9}$
0.044	0.15	3.45
0.078	0.23	2.98
0.18	0.42	2.32
0.35	0.70	1.98
0.88	1.08	1.22

a) The concentration of O_2^- in the reaction layer was calculated as 70% of the oxygen bulk concentration taking in account O_2^- dismutation at this pH (6).

b) $v_o = \frac{k_3}{Km + [O_2^-]} \times [Eo] \times [O_2^-]$ where $[Eo]$, the enzyme concentration, was 1×10^{-9}M.

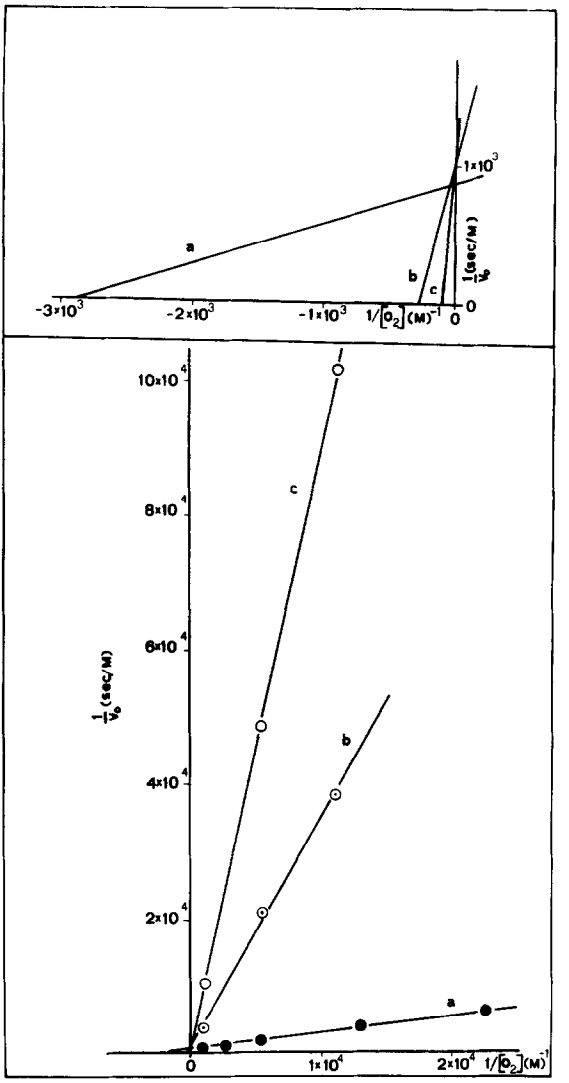

Fig. 1 - Double reciprocal plot of initial velocities (v_o) versus O_2^- concentration with 1 x 10^{-9} M superoxide dismutase. ○ (curve a): at pH 9.9; ⊖ (curve b): at pH 12.0, by addition of concentrated NaOH; ● (curve c): at pH 9.9, in the presence of 1.1 x 10^{-4} NaCN.

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creasing O_2^- values is an evidence for saturation of catalytic sites of the enzyme by O_2^-. In fig. 1, curve a, a series of points from a similar experiment is reported in the form of double reciprocal plot of $\frac{1}{V_o}$ vs $\frac{1}{[O_2^-]}$. A crossing point was clearly detectable on the ordinate axis (see upper part of Fig. 1).

The average values of K_m and k_3 obtained from 4 different series of measures are $(3.55 \pm .08) \times 10^{-4} M^{-1}$ and $(1.01 \pm .21) \times 10^{6}$ sec^{-1} respectively. These values are in good agreement with the previously reported limiting values of K_m (8) and turnover number (5).

Since from the pulse radiolysis measurements (5) no significative difference was observed between the overall kinetic constant $\frac{k_3}{K_m}$ and the kinetic constants k_1 relative to the process of reduction or oxidation of the enzyme catalytic sites, see eqn. 2), k_2 must be negligible respect to k_3. As a consequence it results $K_m = \frac{k_3}{k_1}$ which means that K_m is a steady state constant.

The observed saturation of the enzyme by O_2^- led us to re-examine some previously described inhibition phenomena in this light.

The curves b) and c) of Fig. 1 correspond respectively to the inhibition of superoxide dismutase by OH^- and CN^-. It appears that both OH^- and CN^- display a competitive type of inhibition.

In discussing these results, it should be recalled that a large set of spectroscopic data was interpreted in terms of copper coordination by a water molecule with a pK about pH 11 (11,12) which was apparently displaced on reaction with cyanide (2,13). Since activity measurements in the pH range 10-12 (14) and in the presence of cyanide (8) showed reversible inhibition by OH^- and CN^-, some relevance of this water molecule to the catalytic mechanism appeared likely. However no simple mechanistic explanation was possible since no evidence for saturation kinetics was available. On the other hand the results presented above give a direct kinetic evidence that O_2^- binds to the enzyme copper at the water coordination site and that this site is where inhibition by cyanide takes place. Furthermore, they support an inner sphere mechanism for the electron transfer between O_2^- and the copper in copper-zinc superoxide dismutases.

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