SATURATION BEHAVIOR OF SUPEROXIDE DISMUTATION CATALYZED BY THE

IRON CONTAINING SUPEROXIDE DISMUTASE OF <u>E. Coli</u> B[*]

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SUMMARY. The iron containing superoxide dismutase from <u>E. coli</u> B is shown to
catalyze superoxide dismutation by a mechanism which exhibits saturation
kinetics. This behavior is quite different from that observed previously
with bovine Zn/Cu- and iron-containing superoxide dismutase from <u>P. leiognathi</u>.
Two parameters of catalysis were measured in the pH range 7.2 to 10.4: k_{cat}
was found to be independent of pH and Km varied with the function Km = Km(low pH)
$[1 + \exp(pH - 8.8)]$. These results implicate a group in the catalytic mechanism
which ionizes with pKa = 8.8.

INTRODUCTION

Superoxide dismutases are metalloproteins which catalyze Reaction (1).

$$2 \ O_2^- + 2 \ H^+ \xrightarrow{k_s} H_2O_2 + O_2 \qquad (1)$$
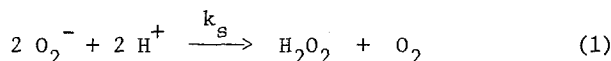
Iron containing superoxide dismutases have been isolated from a variety of

microorganisms [1-8] and from one plant system [9]. Generally, these proteins

are composed of two identical subunits having a molecular weight of ∿19,000

with each apparently binding a single Fe^{3+} [3,10], although there is one re-

port of an iron containing dismutase having four identical subunits [4].

Villafranca [11,12] has demonstrated, with nuclear magnetic relaxation

techniques, the presence of at least one water molecule associated with the Fe^{3+} of the protein from E. coli B. Slykhouse and Fee [10] studied the spectral and anion binding properties of this protein, noting an unusual discrepancy between the affinity of the Fe^{3+} for F^- and N_3^- and the ability of these anions to inhibit the catalysis of superoxide dismutation. Spectral measurements indicated that these anions bind to the Fe^{3+} with dissociation constants (K_d) of 1-2 mM whereas activity measurements revealed inhibition constants of ∿10 mM for N_3^- [13] and ∿40 mM for F^- [10,13]. Examination of the pH dependence of N_3^- binding showed that K_d was independent of pH in the range 4.5 to 7.5 but increased by a factor of 10 per unit pH in the range 9-10 [14]. Therefore, K_d is influenced by an ionizing group on or near the Fe^{3+} having a pKa ∿8.7. Spectral studies of the Fe^{3+} also indicated an ionizing group in this pH range, and it was tentatively assigned to the hydrolysis of a water molecule bound to Fe^{3+} [14]. Examination of N_3^- binding to the Fe^{3+} by temperature-jump relaxation revealed that the limiting rate is reached at high concentrations of N_3^- suggesting that direct coordination of the anion to Fe^{3+} is preceded by association with a non-chromophoric site on the protein which was termed the "anion binding pocket" [14].

We report here that catalysis of superoxide dismutation by the iron-containing superoxide dismutase from E. coli shows saturation kinetics which can be interpreted in terms of the Michaelis-Menten formalism. This behavior was observed by two independent experimental procedures for examining O_2^- in aqueous solution: a stopped-flow spectrophotometric method [15] and pulsed radiolysis [16]. It is noteworthy that the iron-containing superoxide dismutase of P. leiognathi [17] does not show saturation behavior.

MATERIALS AND METHODS

Potassium superoxide was obtained from Alfa Products (Danvers, Massachusetts) and was stored in a dessicator prior to use. 18-Crown-6 ether was purchased from PCR, Inc. (Gainesville, Florida) and used without further purification. Spectral grade dimethylsulfoxide (DMSO) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and stored over $CaCl_2$ prior to use. Solutions of superoxide in DMSO were prepared by a slight modification of the method of Valentine and Curtis [18]; the final concentration of superoxide was close to 25 mM.
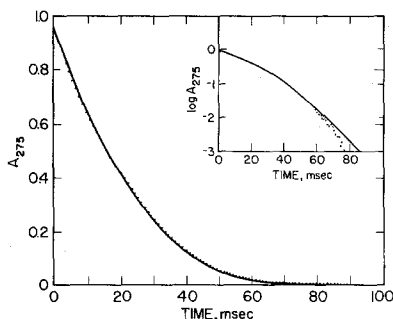
Figure 1. Example of superoxide dismutation catalyzed by the iron containing superoxide dismutase from E. coli B. The initial concentration of O_2^- was 0.5 mM as obtained in a stopped-flow experiment (see text); [Fe] as dismutase, 400 nM; 25 mM pyrophosphate buffer, pH 8.5; and [EDTA], 0.1 mM; T = 22°C. The dots are experimental points. The solid line was obtained by a solution to the rate expression (2, in text) using k_s = 1.7 x 10^4 $M^{-1}s^{-1}$, k_{cat} = 3.3 x $10^4 s^{-1}$, and Km = 1.25 x 10^{-4}M.

Iron containing superoxide dismutase was purified from E. coli B cell paste (Grain Products, Muscatine, Iowa), according to the procedure of Slykhouse and Fee [10].[1] The protein so purified contains 1.8 ± 0.2 g-atom Fe per 38,700 g protein. Concentrations of superoxide dismutase are expressed as $[Fe^{3+}]$ and were measured using E_{350} = 1850 $[Fe]^{-1}cm^{-1}$ [10]. All other materials were of the highest commercial quality available and glass distilled water was used throughout.

Stopped-flow spectrophotometric measurements of superoxide dismutation were carried out using the mixing apparatus described previously [15]. Briefly, this device mixes one part of a 25 mM solution of superoxide in DMSO with 25 parts of an aqueous solution. The superoxide is observed at 275 nm and E_{275} = 1000 $M^{-1}cm^{-1}$ [19]. The dead-time of the flow system is slightly less than 10 msec, limiting our observations of superoxide decay to pH values above ∿7.2 [20]. Each kinetic trace was stored as an array of 12 bit binary words in a Data General Nova 2/10 computer (On Line Instrument Systems, Athens, Georgia). Kinetic traces were simulated by a numerical solution of the rate equation using the fourth-order Runge-Kutta method [21]. Since only two par-ameters were unknown best fits to the observed data were visually determined.

The pulsed radiolysis experiments were performed using the system and the general procedures described previously [16]. The O_2^- was observed at 250 nm and its concentration determined using E_{250} = 2000 $M^{-1}cm^{-1}$ [19].

RESULTS

Figure 1 illustrates a typical decay curve for the dismutation of super-oxide in the presence of iron-containing superoxide dismutase. The initial

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[1]The reliability of the preparation is better if the sonication supernatant is brought to 0.1 M KCl and allowed to stand overnight at 4°C prior to the heat treatment [10].

concentration of O_2^- in this experiment was ∿0.5 mM. The inset log plot shows

that the decay is neither first-order (linear) nor second-order (concave

upward). The curves are understood in terms of the rate expression

$$-\frac{d[O_2^-]}{dt} = 2k_s[O_2^-]^2 + \frac{k_{cat}[Fe][O_2^-]}{Km + [O_2^-]} \qquad (2)$$

where the first term describes the spontaneous dismutation, Reaction (1),

and the second term can be derived from a scheme of reactions which involves

an equilibrium association of superoxide with the active site of the protein.

The parameters k_{cat} and Km will be defined in the context of the chosen

mechanism. Here we prefer to treat them as composite constants, particular

values of which, along with a knowledge of k_1, allow quite accurate simu-

lations of the kinetic curves. Thus, the data of Fig. 1 are fitted with

$k_s = 1.7 \times 10^4 M^{-1}s^{-1}$,[2] $k_{cat} = 3.3 \times 10^4 s^{-1}$, and Km = 1.25×10^{-4}M. In

this context, Km should be the concentration of O_2^- which produces half-

maximal velocity. Since the stopped-flow apparatus is not well suited to test

this statement, pulsed-radiolysis experiments were employed to provide an

independent measure of saturation.

The results presented in Fig. 2 confirm that the iron containing super-

oxide dismutase exhibits saturation kinetics. This is evident from the

linearity of the photomultiplier output (mV) vs. time and from the Lineweaver-

Burk plot (inset of Fig. 2) from which $k_{cat} = 2.1 \times 10^4 s^{-1}$ and Km = 7.8×10^{-5}M

were obtained. These values are in excellent agreement with those obtained

from the stopped-flow experiment. At low concentrations of O_2^-, < 30 μM, the

turnover rate constant was found to be $3 \pm 1 \times 10^8 M^{-1}s^{-1}$ which also agrees

with the ratio of k_{cat}/Km obtained from both sets of experiments. Additional

studies, which will be described elsewhere, have revealed that the parameters

are somewhat sensitive to temperature, buffers, and protein concentration.

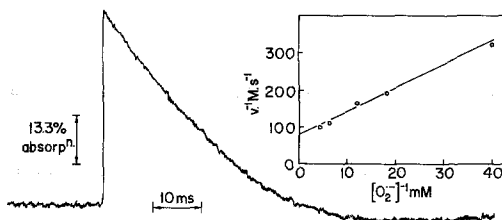[2]The value of k_1 was measured independently for each buffer system and
pH used.

Figure 2. Example of superoxide dismutation catalyzed by the iron containing superoxide dismutase from E. coli B. in the pulse radiolysis experiment. The initial concentration of O_2^- was 0.22 mM produced on irradiation of a 0.48 μM Fe as superoxide dismutase, O_2 saturated, 10 mM pyrophosphate, pH 8.4 solution, 0.1 mM EDTA, and 0.1 M ethanol. Inset. Double reciprocal plot of initial velocity against the initial O_2^- concentration which was changed by varying the intensity of radiation in the pulse.

With these problems in mind we have carried out a preliminary study of the pH dependence of Km and k_{cat}, and the results are presented in Fig. 3. Under these conditions the value of k_{cat} = 3.5 ± 0.6 x $10^4 s^{-1}$ over the pH range 7.2 to 10.4. By contrast, Km is nearly independent of pH below 8.5 but increases rapidly above pH 9.5. This dependence of Km can be rationalized if an ionization on the protein occurs (Reaction (3)) such that only the low pH form can bind O_2^-.

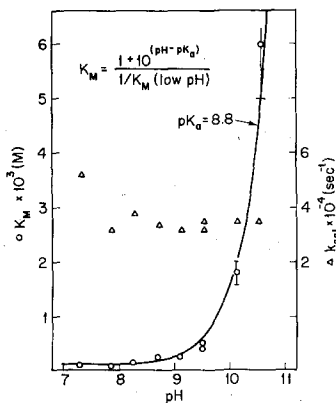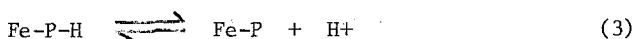$$Fe-P-H \rightleftharpoons Fe-P + H+ \qquad\qquad (3)$$

Figure 3. The pH dependence of the kinetic parameters, k_{cat} and Km, for the catalysis of superoxide dismutation by the iron containing superoxide dismutase from E. coli B.
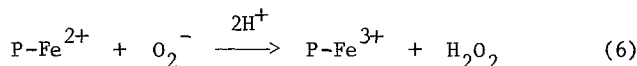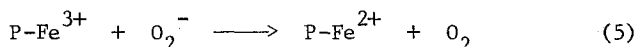
from which

$$Km = Km(\text{low pH})(1 + \exp(pH - pK_a)) \qquad (4)$$

can be derived. When this expression is fitted to the data by a non-linear

regression procedure, a value for pK_a of 8.8 ± 0.1 is found.

DISCUSSION

In a detailed study of the mechanism of the iron containing superoxide dis-

mutase from P. leiognathi, Lavelle et al [17] found the active iron in the

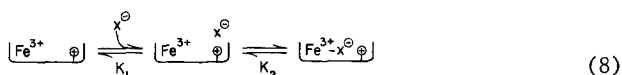protein to function by an oxidation-reduction cycle (Reactions [5] and [6])

$$P\text{-}Fe^{3+} + O_2^- \longrightarrow P\text{-}Fe^{2+} + O_2 \qquad (5)$$

$$P\text{-}Fe^{2+} + O_2^- \xrightarrow{2H^+} P\text{-}Fe^{3+} + H_2O_2 \qquad (6)$$

with a turnover constant of $5.5 \times 10^8 M^{-1} s^{-1}$ (based on protein concentration)

which was independent of pH in the range $6.2 - 9$, moreover, there was no in-

dication of saturation at concentrations of O_2^- as high as 300 μM. This be-

havior is very similar to that found earlier for the bovine Zn/Cu protein [16].

By contrast, the data presented above show that catalysis by the E. coli iron

protein exhibits saturation which can be presumed to arise from a reversible

association of the protein with O_2^- (Reaction (7))

$$O_2^- + P\text{-}Fe \rightleftharpoons O_2^-\text{-}P\text{-}Fe \qquad (7)$$
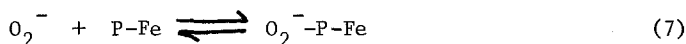
Since preliminary results (O'Neill et al, Unpublished) have indicated that the

E. coli protein also utilizes an oxidation-reduction cycle the dismutases

from the two bacterial sources are evidently not mechanistically identical

with respect to superoxide dismutation.

Because both azide binding to $P\text{-}Fe^{3+}$ and catalysis of superoxide dis-

mutation show saturative kinetic behavior it is instructive to compare these

processes. The binding of azide to $P\text{-}Fe^{3+}$ has been shown to occur by the

following minimal mechanism (Reaction (8)),

$$[Fe^{3+}\ \varphi] \underset{K_1}{\overset{X^{\ominus}}{\rightleftharpoons}} [Fe^{3+}\ \overset{X^{\ominus}}{\varphi}] \underset{K_2}{\rightleftharpoons} [Fe^{3+}\text{-}X^{\ominus}\varphi] \qquad (8)$$

where K_1 = 10 mM, k_2 = 4 x $10^5 s^{-1}$, and k_{-2} = 5 x $10^3 s^{-1}$ at pH 7.4 [14].

Thus, the forward rate constant for azide binding is k_f = k_2/K_1 = 4 x $10^8 M^{-1} s^{-1}$.

This value is similar to the independently measured, second-order turnover

rate constant (3 ± 1 x $10^8 M^{-1} s^{-1}$, pH 7.4) for catalyzed superoxide dis-

mutation and this value is approximately equal to k_{cat}/Km (2.5 x $10^8 M^{-1} s^{-1}$,

pH 7.4). The similarity of these values, obtained in different laboratories

with different preparations of protein, suggests some mechanistic analogy

between azide binding to $P\text{-}Fe^{3+}$ and catalysis of O_2^- dismutation.[3] However,

Km does not directly correspond with K_1 of azide binding as the latter is

independent of pH, and k_{cat} cannot directly correspond to k_2 since the latter

decreases markedly as pH increases while k_{cat} does not. Since k_{cat} and Km

are composite constants, their derivation from a detailed reaction scheme

must await completion of a detailed study of the transient features of

catalysis.

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[3] Preliminary studies (O'Neill et al, Unpublished) have shown the oxidation of $P\text{-}Fe^{2+}$ by O_2^- to be extremely rapid (k > 2 x $10^9 M^{-1} s^{-1}$) in the pH range of interest, suggesting the Fe^{3+} form of the protein may be involved in the rate limiting step.

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