SATURATION BEHAVIOR OF SUPEROXIDE DISMUTATION CATALYZED BY THE
IRON CONTAINING SUPEROXIDE DISMUTASE OF E. COLI B*

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SUMMARY. The iron containing superoxide dismutase from E. coli 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 P. leiognathi.
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 $K_m$ varied with the function $K_m = K_m(\text{low pH})
[1 + \exp(pH - 8.8)]$. These results implicate a group in the catalytic mechanism
which ionizes with $pK_a = 8.8$.

INTRODUCTION

Superoxide dismutases are metalloproteins which catalyze Reaction (1).

$$2 \text{O}_2^- + 2 \text{H}^+ \xrightleftharpoons[k_{\text{cat}}]{k_s} \text{H}_2\text{O}_2 + \text{O}_2$$

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 $\text{Fe}^{3+}$ [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

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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 $\sim$10 mM for N$_3^-$ [13] and $\sim$40 mM for F$^-$ [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 $\approx$8.7. Spectral studies of the Fe$^{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.
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]. 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] 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 parameters 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 superoxide 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 $\sim 0.5$ 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

$$\frac{d[O_2^-]}{dt} = 2k_s [O_2^-]^2 + \frac{k_{cat}[Fe][O_2^-]}{K_m + [O_2^-]}$$

(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 $K_m$ 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 simulations of the kinetic curves. Thus, the data of Fig. 1 are fitted with $k_s = 1.7 \times 10^4 M^{-1}s^{-1}$, $k_{cat} = 3.3 \times 10^4 s^{-1}$, and $K_m = 1.25 \times 10^{-4} M$. In this context, $K_m$ 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 superoxide 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 $K_m = 7.8 \times 10^{-5} M$ were obtained. These values are in excellent agreement with those obtained from the stopped-flow experiment. At low concentrations of $O_2^-$, $< 30 \mu M$, 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 $k_{cat}/K_m$ 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 $K_m$ and $k_{cat}$, and the results are presented in Fig. 3. Under these conditions the value of $k_{cat} = 3.5 \pm 0.6 \times 10^4 \text{s}^{-1}$ over the pH range 7.2 to 10.4. By contrast, $K_m$ is nearly independent of pH below 8.5 but increases rapidly above pH 9.5. This dependence of $K_m$ can be rationalized if an ionization on the protein occurs (Reaction (3)) such that only the low pH form can bind $O_2^-$.

\[
\text{Fe-P-H} \rightleftharpoons \text{Fe-P} + \text{H}^+ \quad (3)
\]

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

\[ \text{Km} = \text{Km}(\text{low pH})(1 + \exp(p\text{H} - pK_a)) \]  

(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 dismutase 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])

\[
P^{-}Fe^{3+} + O_2^- \longrightarrow P^{-}Fe^{2+} + O_2 \quad (5) \]

\[
P^{-}Fe^{2+} + O_2^- + 2H^+ \longrightarrow P^{-}Fe^{3+} + H_2O_2 \quad (6) \]

with a turnover constant of \( 5.5 \times 10^8 \text{M}^{-1}\text{s}^{-1} \) (based on protein concentration) which was independent of pH in the range 6.2 – 9, moreover, there was no indication of saturation at concentrations of \( O_2^- \) as high as 300 \( \mu \text{M} \). This behavior 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))

\[
O_2^- + P^{-}Fe \rightleftharpoons O_2^-P^{-}Fe \quad (7) \]

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 \( P^{-}Fe^{3+} \) and catalysis of superoxide dismutation show saturative kinetic behavior it is instructive to compare these processes. The binding of azide to \( P^{-}Fe^{3+} \) has been shown to occur by the following minimal mechanism (Reaction (8)),

\[
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\]
\[ \begin{align*}
\text{Fe}^{3+} + 3\text{NO}_2^- & \rightleftharpoons \text{Fe}^{3+} + 3\text{NO}_2^- \\
\text{Fe}^{3+} + 3\text{NO}_2^- & \rightleftharpoons \text{Fe}^{3+} + 3\text{NO}_2^- \\
\end{align*} \] (8)

where \( K_1 = 10\, \text{mM} \), \( k_2 = 4 \times 10^5\, \text{s}^{-1} \), and \( k_{-2} = 5 \times 10^3\, \text{s}^{-1} \) at pH 7.4 \([14]\).

Thus, the forward rate constant for azide binding is \( k_f = k_2/K_1 = 4 \times 10^8\, \text{M}^{-1}\text{s}^{-1} \).

This value is similar to the independently measured, second-order turnover rate constant \( (3 \pm 1 \times 10^8\, \text{M}^{-1}\text{s}^{-1}, \text{pH 7.4}) \) for catalyzed superoxide dismutation and this value is approximately equal to \( k_{\text{cat}}/K_m \) \( (2.5 \times 10^8\, \text{M}^{-1}\text{s}^{-1}, \text{pH 7.4}) \). The similarity of these values, obtained in different laboratories with different preparations of protein, suggests some mechanistic analogy between azide binding to P-Fe \(^{3+} \) and catalysis of \( \text{O}_2^- \) dismutation.\(^3\) However, \( K_m \) does not directly correspond with \( K_1 \) of azide binding as the latter is independent of pH, and \( k_{\text{cat}} \) cannot directly correspond to \( k_2 \) since the latter decreases markedly as pH increases while \( k_{\text{cat}} \) does not. Since \( k_{\text{cat}} \) and \( K_m \) are composite constants, their derivation from a detailed reaction scheme must await completion of a detailed study of the transient features of catalysis.

REFERENCES


\(^3\) Preliminary studies (O'Neill et al, Unpublished) have shown the oxidation of P-Fe\(^{2+} \) by \( \text{O}_2^- \) to be extremely rapid \((k > 2 \times 10^8\, \text{M}^{-1}\text{s}^{-1}\) in the pH range of interest, suggesting the Fe\(^{3+} \) form of the protein may be involved in the rate limiting step.