Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 114-120

SATURATION BEHAVIOR OF THE MANGANESE-CONTAINING SUPEROXIDE DISMUTASE FROM *PARACOCCUS DENITRIFICANS*

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Received March 23, 1983

ABSTRACT : A pulse radiolysis study of the Mn-superoxide dismutase from *Paracoccus denitrificans* has shown that, at concentration of $O_2^{\cdot-}$ below 0.8×10^{-4}M, the catalyzed dismutation of $O_2^{\cdot-}$ is a first order reaction with regard to $O_2^{\cdot-}$. At concentration of $O_2^{\cdot-}$ above 0.8×10^{-4}M, the Mn-superoxide dismutase is shown to catalyze superoxide dismutation with a mechanism which exhibits saturation kinetics. This behavior was previously found in the bovine Cu/Zn-superoxide dismutase and in the Fe-superoxide dismutase from *Photobacterium leiognathi*. Two parameters of catalysis were determined from pH 5 to pH 11 : the rate constant k was pH independent at basic pH. The variation of Km with pH indicated that the enzyme possessed an ionizable group with pK 9.8 which participates to the substrate binding.

Superoxide dismutases (superoxide : superoxide oxidoreductase, E.C. 1.15.1.1) (S.O.D.) are enzymes which catalyze the reaction : $O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{2H^+} O_2 + H_2O_2$. They are classified into three groups according to the metal of their active sites : Cu/Zn, Fe and Mn. At low concentrations of $O_2^{\cdot-}$, the catalyzed reaction has been proved to be first order with regard to $O_2^{\cdot-}$ (1-4), as the spontaneous decay is second order with regard to $O_2^{\cdot-}$ (5). The behavior of S.O.D. in regard to larger concentrations of $O_2^{\cdot-}$ has been previously studied. This led to different conclusions. First studies on the bovine erythrocyte Cu/Zn-S.O.D. have shown that the enzyme was not saturated by its substrate until 2.1×10^{-4}M $O_2^{\cdot-}$ (1, 6). However, this S.O.D. was later proved to be saturated by its substrate in studies where concentrations of $O_2^{\cdot-}$ could reach 8.8×10^{-4}M (7). No saturation behavior was observed for the Fe-S.O.D. from *Photobacterium leiognathi* (2). But, more recently, the Fe-S.O.D. from *Escherichia coli* was proved to be saturated by $O_2^{\cdot-}$ (4) and to be in agreement with the Michaelis theory. We present here a pulse radiolysis study of the kinetics of a Mn-S.O.D. isolated from *Paracoccus denitrificans* (8). This Mn-S.O.D. is here shown to be saturated by its substrate. It is a new example of S.O.D. which is

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saturated by $O_2^{\cdot-}$. A study of the influence of pH on the catalytic properties of this S.O.D. is also presented.

MATERIALS AND METHODS

The Mn-S.O.D. from *P. denitrificans* was purified as described previously (8). Samples used were about 4500 units/mg protein. For definition of units and protein assay, see (8). The samples were dialyzed against 0.002M appropriate buffer before pulse radiolysis experiments. Buffers used were Na acetate buffer for pH < 6, K phosphate buffer for pH between 6 and 8 and Na borate buffer for pH > 8. The enzyme was irradiated in 0.16M Na formate, 0.002M buffer in tridistilled water. Irradiations were performed using a double-beam Febetron 708 which generates electron pulses of 800 KeV (4 ns at half-height). Fast spectrophotometric analysis was carried out with a standard device including a xenon lamp, a monochromator, and a photomultiplier. The solutions were irradiated in a fused-silica microcuvette (10 mm optical path-length x 3mm x 20mm). Doses per pulse were in the range of 5 to 40 krad. Experimental curves were delivered as transmitted intensity at 245 nm (ε = 2000 $mole^{-1}.1.cm^{-1}$) plotted against time. These curves were digitalized and concentration of $O_2^{\cdot-}$ calculated for every point on computer. These points were fitted with a calculated curve corresponding to the equation :

$-\frac{d[O_2^{\cdot-}]}{dt} = k_{cat}[O_2^{\cdot-}] + ks_{app}[O_2^{\cdot-}]^2$ which gives after integration the analytic form $y = \frac{a.e^{-bx}}{1-ce^{-bx}}$ (see below).

RESULTS

Kinetics at low concentration of $O_2^{\cdot-}$ (<0.8x10^{-4}M)

The decay of $O_2^{\cdot-}$ observed in the presence of S.O.D. is the result of both spontaneous decay and catalyzed decay. The catalyzed decay has been proved to be first order with regard to $O_2^{\cdot-}$ in various studies (1-3). If the decay catalyzed by the Mn-S.O.D. from *P. denitrificans* is also first order with regard to $O_2^{\cdot-}$, the equation of catalyzed decay is :

$-\frac{d[O_2^{\cdot-}]}{dt} = k_{cat}[O_2^{\cdot-}]$. The spontaneous decay is due to the reaction (5) :

$O_2^{\cdot-} + HO_2^{\cdot} \xrightarrow{H^+} O_2 + H_2O_2$, with $HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot-}$ (pK = 4.8). The corresponding equation is :

$-\frac{d[O_2^{\cdot-}]}{dt} = ks\,[O_2^{\cdot-}][HO_2^{\cdot}] = ks_{app}\frac{[H^+]}{K_4}[O_2^{\cdot-}]^2$ with $ks_{app} = ks.10^{pK_A-pH}$. The equation for the observed decay of $O_2^{\cdot-}$ is subsequently :

$-\frac{d[O_2^{\cdot-}]}{dt} = k_{cat}[O_2^{\cdot-}] + ks_{app}[O_2^{\cdot-}]^2$. The integration of this equation gives the expression : $[O_2^{\cdot-}] = \frac{a.e^{-bt}}{1-c.e^{-bt}}$ with $a = c \times \frac{k_{cat}}{ks_{app}}$, $b = k_{cat}$, $c = \frac{[O_2^{\cdot}]_o}{[O_2^{\cdot-}]_o + k_{cat}/ks_{app}}$ and $[O_2^{\cdot-}]$ = initial concentration of $O_2^{\cdot-}$. The best fit of this expression with experimental curves of decay of $O_2^{\cdot-}$ was calculated from pH 5 to pH 11, zone where the enzyme is stable (it retains whole activity after 15 min incubation at every used pH). Figure 1 presents an example of fitting of an experimental curve with a calculated curve. Such

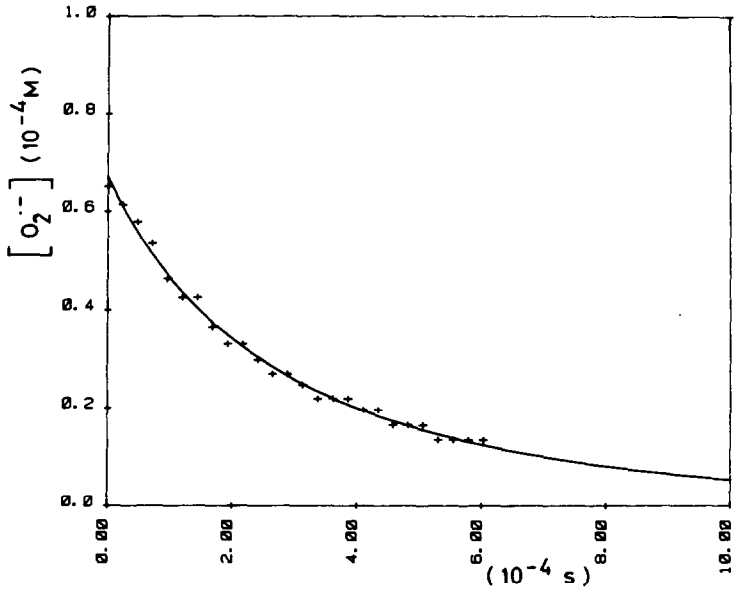


Figure 1. Best fit of a curve with analytical form $y = \frac{a.e^{-bx}}{1-c.e^{-bx}}$ with experimental data of decay of $O_2^{\cdot-}$ at pH 5, in presence of 4 µM Mn-S.O.D. from *P. denitrificans* at initial concentration of $O_2^{\cdot-}$ $0.6x10^{-4}$M. Crosses are points obtained after digitalization of the experimental curve of decay, the solid line is the calculated curve.

results were obtained at every pH. We could then conclude that the decay of $O_2^{\cdot-}$ catalyzed by the Mn-S.O.D. from *P. denitrificans* is first order with regard to $O_2^{\cdot-}$ at concentrations of $O_2^{\cdot-}$ below $0.8x10^{-4}$M. The first order apparent rate constant k_{cat} determined was found to be proportional to the S.O.D. concentration. k_{cat} was divided by the enzyme concentration to give a second order constant $k_2 = 3.33x10^8$ $1.mole^{-1}.s^{-1}$ at pH 7.9.

Kinetics at higher concentrations of $O_2^{\cdot-}$ (>$0.8x10^{-4}$M)

When the initial concentration of $O_2^{\cdot-}$ was increased, the initial velocity of catalyzed decay was observed to deviate from a straight line (Figure 2). This indicated a saturation behavior for the dismutation of $O_2^{\cdot-}$ catalyzed by the Mn-S.O.D. from *P. denitrificans*. In assuming that the enzyme followed Michaelis laws, we have drawn a Lineweaver-Burk plot for two concentrations of S.O.D. (Figure 3) in order to determine the Michaelis constants of the enzyme. The straight lines were calculated by linear regression. The value of the Michaelis constant Km determined by the plot was $2 \pm 0.4x10^{-4}$M at pH 7.9. The values of maximal velocities were also obtained from this plot. They were divided by the S.O.D. concentrations to give the value of the enzyme rate constant k which was $7.76 \times 10^{-4} s^{-1}$ at pH 7.9. The values of initial velocities calculated with the Michaelis equation and these values of constants fitted with experimental points

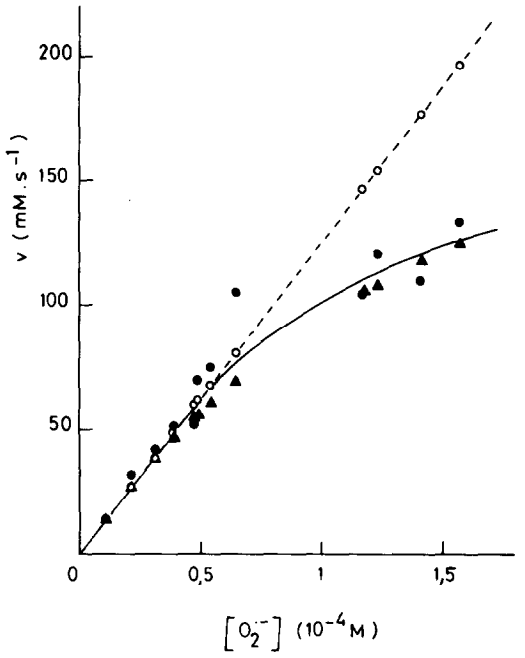


Figure 2. Initial velocity of decay of $O_2^{\cdot-}$ catalyzed by 3.68 µM Mn-S.O.D. from *P. denitrificans* at pH 7.9, as a function of initial concentration of $O_2^{\cdot-}$. (•) experimental points ; (o) calculated values of velocities according to first order kinetics : $v = k_{cat}[O_2^{\cdot-}]$, $k_{cat} = 1260 s^{-1}$; (▲) calculated values of velocities according to Michaelis equation :

$$v = k[SOD] \frac{[O_2^{\cdot-}]}{Km + [O_2^{\cdot-}]}, \quad k = 7.76 \times 10^{-4} s^{-1}, \quad Km = 2.10^{-4} M.$$

Solid line is experimental curve, dotted line is the straight line corresponding to first order kinetics.

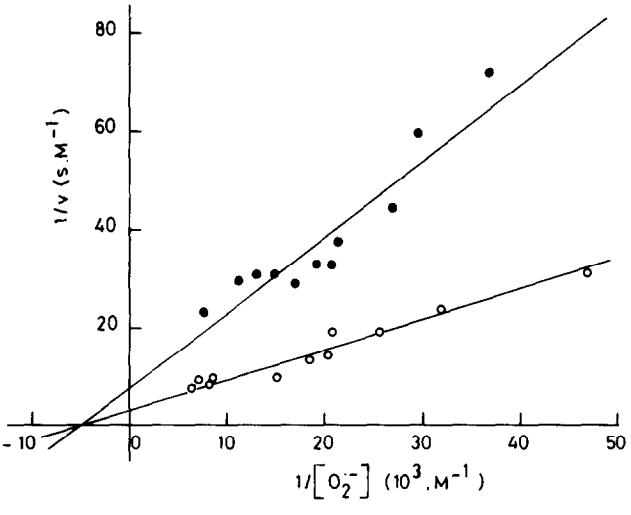


Figure 3. Lineweaver-Burk plot for the Mn-S.O.D. from *P. denitrificans* at pH 7.9. (•) [SOD] = 3.68 µM, (o) [SOD] = 1.84 µM. Lines were obtained by linear regression.

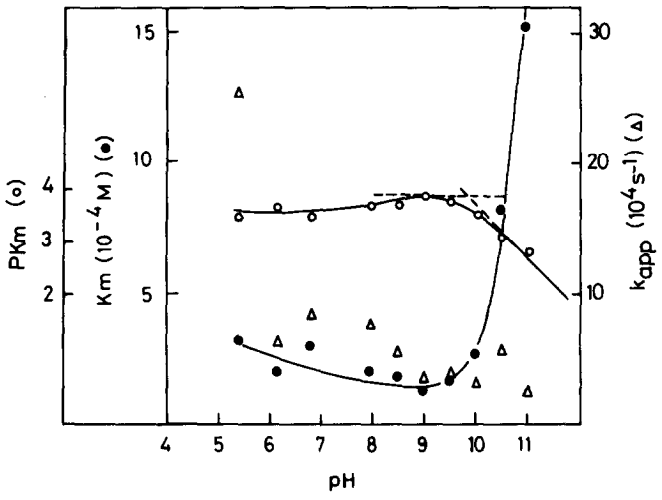


Figure 4. Rate constant of the Mn-S.O.D. from *P. denitrificans*, Michaelis constant Km and pKm (= -log Km) as a function of pH.

(Figure 2). So the Mn-S.O.D. from *P. denitrificans* was concluded to be a Michaelian enzyme which establishes an enzyme-substrate complex. The values of parameters determined by Lineweaver-Burk plots are consistent with the value of k_2 found for low concentrations of $O_2^{\cdot-}$. When $O_2^{\cdot-}$ is low, the expression $-\frac{d[O_2^{\cdot-}]}{dt} = \frac{k[SOD][O_2^{\cdot-}]}{Km + [O_2^{\cdot-}]}$ becomes : $-\frac{d[O_2^{\cdot-}]}{dt} \simeq \frac{k}{Km}[SOD][O_2^{\cdot-}] = k_2[SOD][O_2^{\cdot-}]$. The value of $\frac{k}{Km}$ obtained at pH 7.9 is 3.88×10^8 $1.mole^{-1}.s^{-1}$, which is fairly near the value of k_2 : 3.33×10^8 $1.mole^{-1}.s^{-1}$ obtained above.

pH dependence of the rate constant k of the superoxide dismutase

The rate constant k of the Mn-S.O.D. was determined from Lineweaver-Burk plots in a wide range of pH (Figure 4). It is nearly constant at basic pH (> 7). It seems to increase at acidic pH.

pH dependence of the Michaelis constant Km

The Michaelis constant Km has also been calculated from Lineweaver-burk plots at different pH (Figure 4). It increased at high pH. On a plot of pKm = -log Km against pH (Figure 4), the graph presented a sharp increase. The lines tangent to the curve crossed at pH 9.8. Such an angle in this graph allows to reveal the presence of an ionizable group which participates directly to the enzyme-substrate bond. The crossing point gives the value of the pK_A of this group, which is 9.8. As the only pK_A of the substrate is 4.8, we can attribute surely the ionizable group to the protein. So the Mn-S.O.D. from *P. denitrificans* possesses an ionizable group with a pK of 9.8 which participates to the substrate binding.

DISCUSSION

The reaction of catalyzed dismutation has been shown to be first order with regard to $O_2^{\cdot-}$ at low concentrations of $O_2^{\cdot-}$, as in previous kinetic studies of the three classes of S.O.D. (1-3). It means that the reaction occurs in steps involving one $O_2^{\cdot-}$ anion only, as proposed in these different studies. Besides, we have observed that the Mn-S.O.D. from *P. denitrificans* has also been shown to be saturated by its substrate with kinetics in good agreement with Michaelis laws. This means that an enzyme-substrate complex is formed during catalysis. It is the first example of a Mn-S.O.D. having a Michaelian behavior. The bovine erythrocyte Cu/Zn-S.O.D. (7) and the Fe-S.O.D. from *E. coli* (4) have also been found to be Michaelian enzymes.

In view of these results, assuming a ping-pong mechanism as proposed by McAdam et al. (3) for another S.O.D., the following catalytic scheme is proposed :

$$[Mn^{3+}] + O_2^{\cdot-} \rightleftharpoons [Mn....O_2]^{\cdot 2+} \longrightarrow [Mn^{2+}] + O_2$$
$$[Mn^{2+}] + O_2^{\cdot-} \rightleftharpoons [Mn....O_2]^{\cdot +} \xrightarrow{2H^+} [Mn^{3+}] + H_2O_2$$

Scheme I

However, an increase of the rate constant k of the enzyme at acidic pH would not be accounted for by such a mechanism. This increase at acidic pH would strongly suggest that the protonated form $HO_2^{\cdot}$ reacts in place of $O_2^{\cdot-}$, introducing a factor pH-dependent in the kinetic equation, at acidic pH ($[HO_2^{\cdot}] = [O_2^{\cdot-}]\frac{[H^+]}{K_A}$). It is not very probable that $HO_2^{\cdot}$ could bind to the enzyme in another enzyme-substrate complex. A more probable scheme is presented :

$$[Mn^{3+}] + O_2^{\cdot-} \rightleftharpoons [Mn....O_2]^{\cdot 2+} \longrightarrow [Mn^{2+}] + O_2$$
$$[Mn^{2+}] + O_2^{\cdot-} \text{ (or } HO_2^{\cdot}) \xrightarrow[(\text{or } H^+)]{2H^+} [Mn^{3+}] + H_2O_2$$

Scheme II

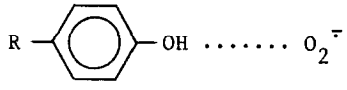
We can also suppose, as Weinstein and Bielski (9) for a complex Cu(II)-Histidine, that the complex $[Mn....O_2]^{\cdot 2+}$ itself reacts on the second $O_2^{\cdot-}$:

$$[Mn^{3+}] + O_2^{\cdot-} \rightleftharpoons [Mn....O_2]^{\cdot 2+}$$
$$[Mn....O_2]^{\cdot 2+} + O_2^{\cdot-} \xrightarrow{2H^+} [Mn^{3+}] + O_2 + H_2O_2$$

Scheme III

The only difference between schemes II and III is that in scheme III, the complex reacts with $O_2^{\cdot-}$ before giving the $[Mn^{2+}]$ form. The data available don't allow to decide what scheme is correct. Further kinetic studies at acidic pH are also necessary to know if $HO_2^{\cdot}$ is really implied in this reaction.

The pH-dependence of the Michaelis constant revealed that the enzyme has an ionizable group which participates to the substrate binding. Its pK_A is 9.8. The Fe-S.O.D. from *E. coli* (4) was also found to possess an ionizable group, but with a different pK_A (8.8). One must remark that this pK_A is very near the pK_A of the side-chain of tyrosine (10.07). It is then possible that one tyrosine residue may be implied in the binding of the substrate. The binding could be realized by electrostatic interaction illustrated as follows, between the proton of phenolic group and $O_2^{\cdot-}$ anion :



At pH 9.8, the proton is released, and the $O_2^{\cdot-}$ would not be more retained by the anionic phenol. A precise study of the influence of chemical modifications of tyrosines would be very useful to verify this hypothesis.

ACKNOWLEDGEMENTS

We wish to thank J. Chevrel for expert technical assistance in pulse radiolysis experiments. We are very grateful to Dr. P.M. Vignais for valuable advice, and to Dr. P. Térech for calculations on computer.

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