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ONE-ELECTRON TRANSFER REACTIONS IN BIOCHEMICAL SYSTEMS

VIII. KINETIC STUDY OF SUPEROXIDE DISMUTASE

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SUMMARY

The overall kinetic study of the inhibition by superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) of the oxygen-mediated reduction of cytochrome *c* in the xanthine oxidase reaction showed that the catalytic reaction of superoxide dismutase was first order with respect to enzyme and to substrate. The present value for k_d/k_c^2 was found to be much different from the results obtained in the experiments of pulse radiolysis while the ratio of k_{sod}/k_c seemed to be similar regardless of experimental conditions, where k_d, k_c and k_{sod} were the rate constants of the noncatalytic dismutation of superoxide radicals and of reactions of the radical with cytochrome *c* and superoxide dismutase, respectively.

Superoxide dismutase inhibited the formation of oxyperoxidase in the peroxidase-oxidase reaction. It was found that the superoxide radical reacted with ferric peroxidase and superoxide dismutase at similar rates.

INTRODUCTION

Superoxide radicals were suggested to participate in the reduction of cytochrome *c* by xanthine oxidase[1] and in the peroxidase-oxidase reactions[2]. The generation of the radicals has been shown by direct demonstration with the use of ESR spectroscopy in the reactions of oxidase[3] and peroxidase[4]. The finding of an enzyme, called superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1), which catalytically scavenges these radicals[5], has developed a method suitable for dealing with the kinetics of the radical reactions[6-8]. Rate constants of reactions of superoxide radicals with cytochrome *c* and superoxide dismutase have been measured. In these experiments the radicals were formed by autoxidation of reduced flavin[6] and pulse radiolysis[7,8] and the concentration of the radical reached the level measurable by optical or ESR spectroscopy.

Using superoxide dismutase several reactions have been found to involve the superoxide radical as an active intermediate. It seems likely that the steady-state concentration of the radical is very low in most of these reactions. Although the absolute values for the rate constants may not be given it would be of importance to study the kinetics of the overall reactions of superoxide radicals under the physiological conditions in which the radical is continuously supplied in small amounts.

MATERIALS AND METHODS

Milk xanthine oxidase was prepared by the method of Hart and Bray[9]. The concentration of the enzyme was calculated using the value of $E_{450\ \mathrm{nm}} = 37.8$ $\mathrm{mM^{-1} \cdot cm^{-1}}$ per molecule of enzyme-bound FAD. Superoxide dismutase was prepared from dry seeds of green pea (*Pisum sativum*) and the concentration of the enzyme was calculated from $E_{680\ \mathrm{nm}} = 0.29\ \mathrm{mM^{-1} \cdot cm^{-1}}$ (ref. 10). Peroxidase was prepared from horseradish roots according to Shannon *et al.*[11] and the RZ value ($E_{403\ \mathrm{nm}}$ to $E_{280\ \mathrm{nm}}$) of the enzyme was 3.2. The enzyme preparation used was a mixture of isoenzymes B and C according to Paul[12] and the concentration was calculated from $E_{403\ \mathrm{nm}} = 100\ \mathrm{mM^{-1} \cdot cm^{-1}}$. Cytochrome *c* was obtained from Boehringer. The rate of cytochrome *c* reduction was calculated assuming that $\Delta E_{550\ \mathrm{nm}}$ between the oxidized and reduced states is $19.6\ \mathrm{mM^{-1} \cdot cm^{-1}}$.

Absorbance measurements were carried out with a Hitachi recording spectrophotometer, Model 124, equipped with a thermostatically controlled cell compartment. The reaction temperature was 25 °C.

RESULTS AND DISCUSSION

Reaction of superoxide with cytochrome c

It has been confirmed that xanthine oxidase reduces O_2 and *p*-benzoquinone by a mixed mechanism of one-electron and two-electron transfers[13,14,15]. When O_2 is reduced by the enzyme system in solutions equilibrated with air, the reactions would be:

$$\text{xanthine} + O_2 + H_2O \rightarrow \text{uric acid} + H_2O_2 \qquad (1)$$

$$\text{xanthine} + 2\ O_2 + H_2O \rightarrow \text{uric acid} + 2\ O_2^- + 2\ H^+ \qquad (2)$$

According to Fridovich[13] Reactions 1 and 2 occur at a ratio depending on the pH and the concentrations of xanthine and O_2. The superoxide anions thus generated, dismutate spontaneously as follows,

$$2\ O_2^- + (2\ H^+) \xrightarrow{k_d} O_2 + H_2O_2 \qquad (3)$$

or alternately act to reduce cytochrome *c*,

$$O_2^- + \text{cytochrome } c^{3+} \xrightarrow{k_c} O_2 + \text{cytochrome } c^{2+} \qquad (4)$$

Fig. 1 shows the relationship between the initial rate of reduction of cytochrome *c* and its concentration. Then the following equation is obtained at the steady state,

$$V = V_c + V_d \qquad (5)$$

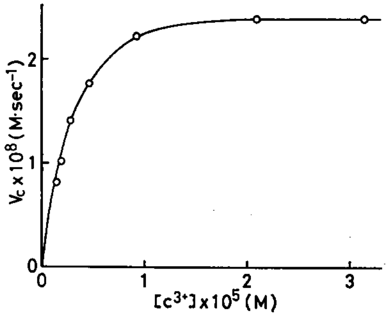

Fig. 1. Dependence of the reduction rate of ferricytochrome *c* on its concentration. The initial rate of formation of ferrocytochrome *c* was observed at 550 nm. Cuvettes contained 40 μM xanthine, 9 nM xanthine oxidase and various amounts of ferricytochrome *c* in 0.05 M potassium phosphate buffer at pH 7.8 with 0.1 mM EDTA. The reaction was started by the addition of xanthine oxidase.

where V is the rate of superoxide generation by xanthine oxidase and V_c and V_d can be formulated as follows,

$$V_c = k_c[c^{3+}][O_2^-] \tag{6}$$

$$V_d = k_d[O_2^-]^2 \tag{7}$$

V is equal to the initial rate of reduction of cytochrome *c* (V_c) at the saturating level of its concentration (Fig. 1). From Eqns 5, 6 and 7, the following equation is obtained,

$$\frac{V}{V_c} = 1 + \frac{k_d}{k_c^2} \frac{V_c}{[c^{3+}]^2} \tag{8}$$

The relationship between V/V_c and $V_c/[c^{3+}]^2$ is shown in Fig. 2. From the slope of the straight line the ratio k_d/k_c^2 was measured to be $5.0 \cdot 10^{-4}$ M·s.

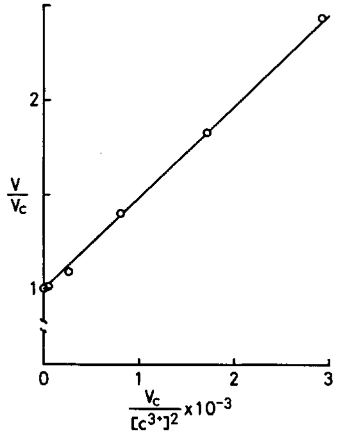

Fig. 2. Proportionality of V/V_c and $V_c/[c^{3+}]^2$. The data of Fig. 1 were plotted according to Eqn 8. Abbreviations: $[c^{3+}]$, the concentration of ferricytochrome *c*; V_c, reduction rate of ferricytochrome *c*; V, saturating level of V_c.

At a saturating level of cytochrome *c*, V_d becomes negligible compared with V_c. Under such a condition the effect of superoxide dismutase was investigated. The rate of reduction of cytochrome *c* decreased when superoxide dismutase increased as shown in Fig. 3.

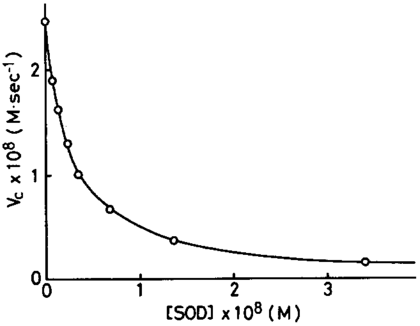

Fig. 3. Inhibitive effect of superoxide dismutase on the reduction rate of ferricytochrome *c*. The reaction mixture contained 17 μM ferricytochrome *c*, 9 nM xanthine oxidase, 50 μM xanthine and various amounts of superoxide dismutase in 0.05 M potassium phosphate buffer at pH 7.8 with 0.1 mM EDTA.

In this case the generated superoxide anions decay mostly through Reaction 4 and the enzyme-catalyzed dismutation reaction.

$$2\ O_2^- + (2\ H^+) \xrightarrow[(SOD)]{k_{sod}} O_2 + H_2O_2 \tag{9}$$

Therefore, the following equation is made at the steady state,

$$V = V_c + V_{sod} \tag{10}$$

where V_{sod} represents the rate of enzymic decay of superoxide anions. If V_{sod} is assumed to be represented as

$$V_{sod} = k_{sod}[SOD]\ [O_2^-] \tag{11}$$

the following relation can be derived from Eqns 6, 10 and 11,

$$\frac{V}{V_c} = 1 + \frac{k_{sod}}{k_c[c^{3+}]}[SOD] \tag{12}$$

The plot shown in Fig. 4 indicates that the above postulates are reasonable. The assumption that the enzyme catalyzes the decay by a second-order process for the superoxide was graphically proved to be not applicable to this case. The angular coefficient gave the value of $7.1 \cdot 10^3$ for the ratio k_{sod}/k_c.

Rotilio *et al.*[8], studying the dependence of V_{sod} on the concentration of substrate, superoxide, have concluded that the overall rate is governed by a second-order rate constant for reaction between enzyme and superoxide. A similar result was also reported by Klug *et al.*[7]. The concentration of superoxide, generated by pulse radiolysis was around 10^{-5} M in both experiments.

Although it was almost impossible to directly measure the superoxide concentration in the present experiment, V_{sod} could be plotted against the superoxide

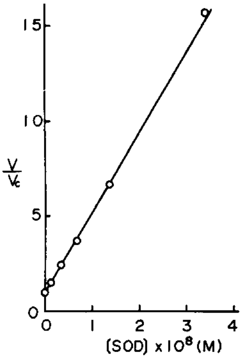

Fig. 4. Relationship between V/V_c and [SOD]. The data of Fig. 3 were plotted according to Eqn 12. Abbreviations are as described in Fig. 2.

concentration calculated as follows. The concentration of superoxide could be calculated from V_c with use of the known value of k_c. At a given V in Eqn 10, V_{sod} was obtained by subtracting V_c from V. Fig. 5 shows a plot of V_{sod} against the concentration (below μM) of superoxide. The reaction of superoxide dismutase was thus found to be first order with respect to superoxide under the physiological conditions where the substrate was generated gradually in small amounts.

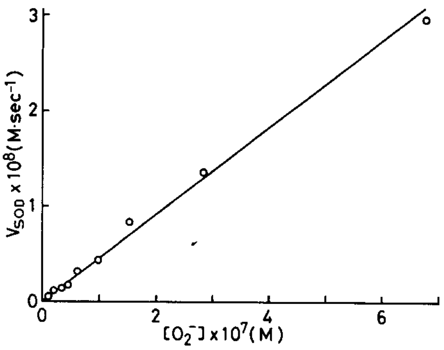

Fig. 5. Relationship between the reaction rate of superoxide dismutase (V_{sod}) and the concentration of superoxide anion. The initial rate of formation of ferrocytochrome c was measured at every level of xanthine oxidase concentration. V_{sod} and the concentration of superoxide anion were calculated as described in the text. Reactions were carried out in 0.05 M phosphate buffer at pH 7.8, containing 16 μM ferricytochrome c, 0.5 nM superoxide dismutase, 50 μM xanthine and 0.1 mM EDTA. The concentration of xanthine oxidase ranged from 1 to 37 nM.

The values of k_d/k_c^2 and k_{sod}/k_c were measured at various pH values and the results are listed in Table I. These values are similar to those calculated from the replots of the results obtained in the xanthine oxidase system by Fridovich and Handler[1] and Fridovich[13] but are quite different from those calculated from individual rate constants obtained directly in the reaction system in which superoxide anions are generated by pulse radiolysis. The discrepancy cannot yet be explained. Our values of k_c and k_{sod} should be tentative since these are calculated with use of the k_d values obtained in the reaction system of pulse radiolysis.

TABLE I

RATE CONSTANTS OF REACTIONS OF SUPEROXIDE AND THEIR RATIOS

pH	k_d/k_c^2 $(M \cdot s)$	k_d* $(M^{-1} \cdot s^{-1})$	k_c $(M^{-1} \cdot s^{-1})$	*Method of O_2^- generation*	*Ref.*
5.0	$2.8 \cdot 10^{-3}$	$1.9 \cdot 10^{7}$	$8.2 \cdot 10^{4}$	Xanthine oxidase	This paper
5.5	$1.1 \cdot 10^{-3}$	$1.1 \cdot 10^{7}$	$9.8 \cdot 10^{4}$		
7.0	$7.1 \cdot 10^{-4}$	$4.5 \cdot 10^{5}$	$2.5 \cdot 10^{4}$		
7.8	$5.0 \cdot 10^{-4}$	$7.1 \cdot 10^{4}$	$1.2 \cdot 10^{4}$		
8.5	$3.5 \cdot 10^{-5}$	$1.5 \cdot 10^{4}$	$2.1 \cdot 10^{4}$		
7.8	$1.5 \cdot 10^{-4}$**			Xanthine oxidase	1
8.5	$1.2 \cdot 10^{-6}$***		$1.1 \cdot 10^{5}$	Pulse radiolysis	17
8.4			$1.6 \cdot 10^{5}$	Autoxidation of reduced flavin	6

pH	k_{sod}/k_c	k_{sod} $(M^{-1} \cdot s^{-1})$	*Method of O_2^- generation*	*Ref.*
7.0	$4.1 \cdot 10^{3}$	$1.0 \cdot 10^{8}$	Xanthine oxidase	This paper
8.5	$5.6 \cdot 10^{3}$	$1.2 \cdot 10^{8}$		
7.0	$3 \cdot 10^{3}$†		Xanthine oxidase	13
8.5	$1.6 \cdot 10^{4}$††	$1.8 \cdot 10^{9}$	Pulse radiolysis	7, 17
5.3 ~ 9.5		$(1.6 \pm 0.3) \cdot 10^{9}$	Pulse radiolysis	8

* Direct measurements. Superoxide was generated by pulse radiolysis[16].

** Calculated from the replot of the data of Fridovich and Handler[1].

*** Obtained from the values of k_d (ref. 16) and k_c (ref. 17).

† Calculated from the replot of Fridovich's data[13].

†† Obtained from the values of k_{sod} (ref. 7) and k_c (ref. 17).

Reaction of superoxide with horseradish peroxidase

Oxyperoxidase was found to be formed when a small amount of H_2O_2 was added to an aerobic solution of dihydroxyfumarate[2] or NADH[18] which is the substrate of the peroxidase–oxidase reaction. The stoichiometry of the reaction has led to following equations[2].

$$2\ YH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} 2\ YH\cdot + 2\ H_2O \tag{13}$$

$$YH\cdot + O_2 \longrightarrow Y + O_2^- + H^+ \tag{14}$$

$$\text{peroxidase} + O_2^- \xrightarrow{k_1} \text{oxyperoxidase} \tag{15}$$

As shown in Fig. 6, when superoxide dismutase was added after oxyperoxidase had been formed it decomposed into the ferric enzyme with a half decay time of about 3 min in agreement with the result previously reported for the autodecomposition of oxyperoxidase[19,20].

Fig. 7 shows the time course of the absorbance change at 418 nm when superoxide dismutase was added before the reaction started. Transient absorption which was not affected by superoxide dismutase would be ascribed to peroxidase Compound II, an obligative intermediate of the enzyme in the peroxidase catalysis. The difference between the time course in the presence of a saturating

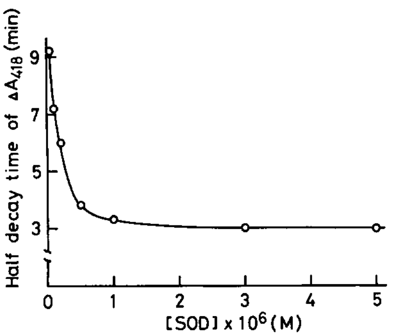

Fig. 6. Effect of superoxide dismutase on the apparent decay time of oxyperoxidase. Oxyperoxidase was formed by the addition of 1.5 μM H_2O_2 into a reaction mixture composed of 8 μM horseradish peroxidase, 0.1 mM NADH and 0.1 M acetate buffer at pH 5.0. Superoxide dismutase was added 1 min after H_2O_2 was added. The decrease of oxyperoxidase was measured as a decrease in absorbance at 418 nm. In the absence of superoxide dismutase the gradual increase of oxyperoxidase was observed as shown in Fig. 7.

amount of superoxide dismutase and others would roughly show reaction curves of oxyperoxidase formation due to the reaction of ferric peroxidase and superoxide. The amount of oxyperoxidase reached its maximal value about 20 s after H_2O_2 was added. Judging from the visible spectra shown in Fig. 8 it could be concluded that the enzyme consisted of ferric and oxy forms and no appreciable amount of Compound II was present after that time. In the absence of superoxide dismutase a slow increase of oxyperoxidase could be seen after the rapid reaction as reported previously[18].

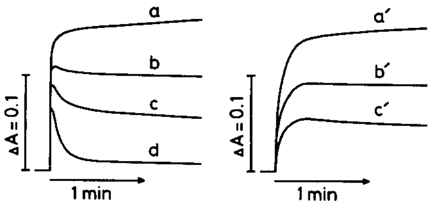

Fig. 7. Effect of superoxide dismutase on the formation of oxyperoxidase. The left figure shows increases in absorbance at 418 nm when 1.5 μM H_2O_2 was added to a solution containing 8.4 μM horseradish peroxidase, 0.1 mM NADH and various amounts of superoxide dismutase in 0.1 M acetate buffer at pH 5.0. The concentrations of superoxide dismutase were 0(a), 0.64(b), 1.9(c) and 13(d) μM. The right figure shows the approximate time courses of oxyperoxidase formation, calculated by assuming that the transient formation of Compound II of horseradish peroxidase was shown by the typical curve of d and was similarly involved in other reaction curves. Consequently, curves a′, b′ and c′ were obtained as a–d, b–d and c–d, respectivlely.

If the concentration of H_2O_2 added is low enough and the concentration of ferric peroxidase is assumed to remain constant the following relation can be deduced,

$$\frac{[\text{total superoxide generated}] - [\text{formed oxyperoxidase}]}{[\text{formed oxyperoxidase}]} = \frac{\int k_{\text{sod}}\,[\text{SOD}]\,[\text{superoxide}]\,\mathrm{d}t}{\int k_{\text{i}}[\text{ferric peroxidase}][\text{superoxide}]\,\mathrm{d}t} = \frac{k_{\text{sod}}\,[\text{SOD}]}{k_{\text{i}}[\text{ferric peroxidase}]} \qquad (16)$$

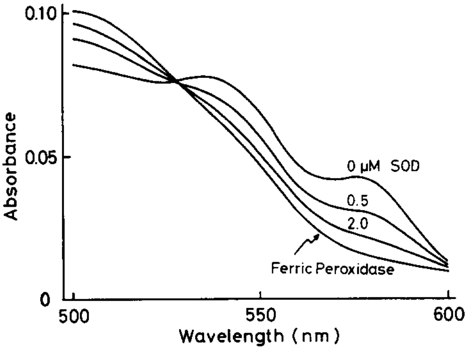

Fig. 8. Identification of the intermediates of Fig. 7 as oxyperoxidase. Spectra were scanned from 600 nm in the time range between 20 and 55 s after 1.5 μM H_2O_2 was added into solutions containing 8.5 μM horseradish peroxidase, 0.1 mM NADH and indicated amounts of superoxide dismutase in 0.1 M acetate buffer at pH 5.0. No appreciable peroxidase Compound II was observed in these spectra.

According to the mechanism shown in Reactions 13 and 14 the total molar amount of superoxide generated should be twice the added H_2O_2 and would equal the amount of oxyperoxidase formed in the absence of superoxide dismutase. From the results of Fig. 7 the ratio k_{sod}/k_i was calculated to be about 4. The results are summarized in Table II. Though the value of k_i is not confirmative it can be said that the reactions of the superoxide anion with ferric peroxidase and with superoxide dismutase occur at similar rates. If H_2O_2 is formed in the reaction between superoxide and superoxide dismutase, peroxidase should remain in the form of Compound II in the presence a sufficient amount of NADH. Thus, the results of Figs 7 and 8 might suggest that the reaction of superoxide and superoxide dismutase in a stoichiometric amount does not produce H_2O_2 but forms an intermediate product of the enzyme. The details of the reaction are now under investigation.

TABLE II

REACTION OF SUPEROXIDE ANION WITH PEROXIDASE

pH	*SOD* (*μM*)	*Oxyperoxidase formed* (*μM*)	k_{sod}/k_i*	k_i ($M^{-1}\cdot s^{-1}, \times 10^7$)	
5.0	0	2.3			
	0.64	1.6	4.6	3.5**	35***
	1.9	0.9	4.7	3.4**	34***
5.5	0	2.2			
	0.64	1.2	6.8	3.1**	24***
	1.9	0.7	6.4	3.3**	25***

* The ratio was calculated according to Equation 16. The concentration of ferric peroxidase was tentatively calculated as 1/2 ([total peroxidase] + [final ferric peroxidase]).

** Calculated with use of the k_{sod} values of $1.6 \cdot 10^8$ $M^{-1}\cdot s^{-1}$ (pH 5.0) and of $2.1 \cdot 10^8$ $M^{-1}\cdot s^{-1}$ (pH 5.5).

*** Calculated with use of the k_{sod} values of $1.6 \cdot 10^9$ $M^{-1}\cdot s^{-1}$ (ref. 8).

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