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A KINETIC STUDY OF REDUCTION OF BOVINE ERYTHROCYTE SUPEROXIDE DISMUTASE WITH POTASSIUM HEXACYANOFERRATE(II)

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When the two copper ions at the active sites of bovine erythrocyte superoxide dismutase were reduced by $Fe(CN)_6^{4-}$, a single stage kinetic process was observed. This result indicates that the two copper sites in bovine erythrocyte superoxide dismutase are equivalent for reduction by $Fe(CN)_6^{4-}$

Bovine erythrocyte superoxide dismutase(Cu_2Zn_2SOD) is a dimeric metalloenzyme containing zinc(II) and copper(II) ions in each subunit.[1] Copper ions are essential for the enzymic activity in the catalytic cycle.[2] The results of detailed kinetic investigation of bovine erythrocyte superoxide dismutase has led to the conclusion that only half of the copper sites of the enzyme is functional in catalysis.[3] Lowrence et al.[4] also showed that the addition of $K_3[Fe(CN)_6]$ to the enzyme resulted in a nearly stoichiometric loss of titratable copper in the oxidized enzyme up to 0.5 equivalent of copper by coulometry. Recently, ^{19}F NMR experiments indicated that two copper sites of the enzyme in steady turnover involved the catalytic function.[5] Therefore, we are interested in studing whether the two copper sites of the enzyme would be equivalent or not for the reduction with reducing agents. In this paper, the reduction rates of copper ions in bovine erythrocyte superoxide dismutase with $K_4[Fe(CN)_6]$ were measured.

Bovine erythrocyte superoxide dismutase was prepared by the method of McCord and Fridvich.[6] The copper and zinc contents were measured by a Shimadzu AA-630-12 atomic absorption spectrophotometer. The concentration of bovine erythrocyte superoxide dismutase was determined from the copper content by atomic absorption spectrophotometry. The ratio of copper/zinc was almost 1.0. Its purity was 85%. The purity of the enzyme was also checked by high performance liquid chromatography[7] and was 92%. The enzyme activity was measured by the method of Sugiura et al.[8] which was a modified method of McCord and Fridvich.[6] Its specific activity was 3600-4500 unit/mg. Time course of the reduction of bovine erythrocyte superoxide dismutase with $K_4[Fe(CN)_6]$ was monitored by the absorbance at 420 nm (Hitachi model 557 spectrophotometer) where $Fe(CN)_6^{3-}$ has high molar absorbance(ε=990 $M^{-1}cm^{-1}$)† but $Fe(CN)_6^{4-}$ is transparent. Equilibrium dialysis was performed by equilibrium dialysis cells (capacity 1ml) and its procedure was described in the previous paper.[9]

Fee et al.[10] have determined the Cu(II)/Cu(I) redox potential of bovine

† 1 M = 1 mol dm^{-3} in this paper.

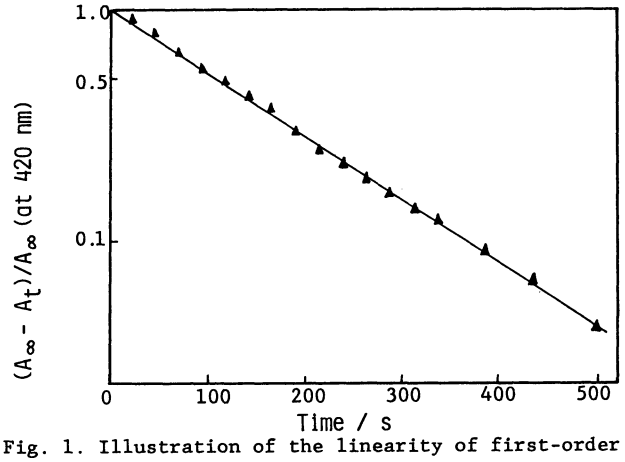


Fig. 1. Illustration of the linearity of first-order plots for a run with bovine erythrocyte superoxide dismutase(5.5×10^{-5}M), $K_4Fe(CN)_6$(2×10^{-3}M), pH 6.0, 0.1 M phosphate buffer, temperature 10°C, at 420nm

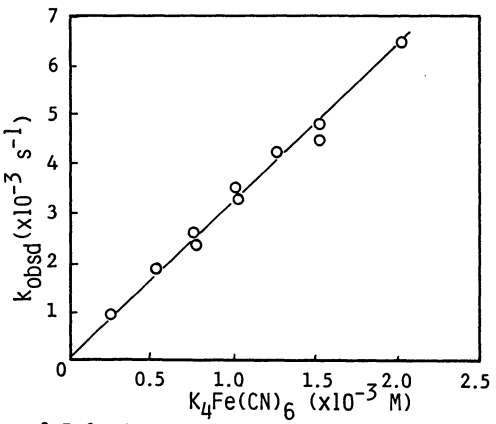


Fig. 2. Relationship between pseudo-first-order rate constant and concentration of $K_4Fe(CN)_6$, pH 6.0, 0.1 M phosphate buffer, temperature 10 °C.

erythrocyte superoxide dismutase with $Fe(CN)_6^{4-}$

$$ECu(II) + Fe(CN)_6^{4-} \rightleftharpoons ECu(I) + Fe(CN)_6^{3-} \quad (1)$$

The redox potential of ECu(II) is pH dependent and decreases with increasing pH, so that kinetic experiments in this paper were performed in 0.1 M phosphate buffer (pH 6.0) and in the presence of large excess of $Fe(CN)_6^{4-}$ against bovine erythrocyte superoxide dismutase. Slow change of the absorbance at 420 nm were observed after $Fe(CN)_6^{4-}$ and bovine erythrocyte superoxide dismutase were mixed quickly. Figure 1 shows the time dependence of the absorbance at 420 nm. Plots of $\log[(A_\infty-A_t)/A_\infty]$ vs. time were linear for over 90% of the course of the reaction. The pseudo-first-order rate constant(k_{obsd}) was derived from the slope of the line. The fraction which was reduced to ECu(I) from ECu(II) by $Fe(CN)_6^{4-}$ was calculated to be 80-90% from the copper content and A_∞. The reduced content is not dependent on the concentration of $Fe(CN)_6^{4-}$. Figure 2 shows a linear relationship between the pseudo-first-order rate constant(k_{obsd}) and the concentration of $Fe(CN)_6^{4-}$. This result indicates that the reaction is first-order with respect to the concentration of $Fe(CN)_6^{4-}$. The effect of the concentration of bovine erythrocyte superoxide dismutase on the reaction rate was tested with 1.0×10^{-3} M $Fe(CN)_6^{4-}$. The pseudo-first-order rate constant(k_{obsd}) of the reaction was found to be constant in the concentration range of the enzyme (0.23×10^{-4} - 1.1×10^{-4} M). These results indicate that the rate of the reaction from ECu(II) to ECu(I) with $Fe(CN)_6^{4-}$ is an overall second-order reaction and may be defined as:

$$d[ECu(I)]/dt=k_{obsd}[ECu(II)]=k_{app}[Fe(CN)_6^{4-}][ECu(II)] \quad (2)$$

where k_{obsd} is pseudo-first-order rate constant and k_{app} is apparent second-order rate constant. The k_{app} was obtained from the slope of the line in Fig. 2 and was 3.2 M^{-1} s^{-1} at 10 °C.

In order to determine the charge at the reaction site of the enzyme for $Fe(CN)_6^{4-}$, the dependence of the reaction rate constant on ionic strength was determined. In the reaction of plastocyanine with ascorbate, [11] the apparent charge of the reaction sites was determined from the following relationship between log k_{app} and $\mu^{1/2}$

(μ: ionic strength).

$$\log k_{app} = \log k_0 + 1.04 \cdot Z_A Z_B \sqrt{\mu} \qquad (3)$$

where Z_A and Z_B are the charges of species. This equation was used to determine the charge of the reaction site in the enzyme for $Fe(CN)_6^{4-}$. As shown in Fig. 3, the reduction rate of bovine erythrocyte superoxide dismutase by $Fe(CN)_6^{4-}$ is dependent on the ionic strength. When NaCl was used as an electrolyte, the relationship between log k_{app} and $\mu^{1/2}$ is linear as shown in Fig. 3, so that Eq. 3 set up. From the slope of the line in Fig. 3, the charge of the reaction site of the enzyme for $Fe(CN)_6^{4-}$ was calculated to be +0.97(NaCl).

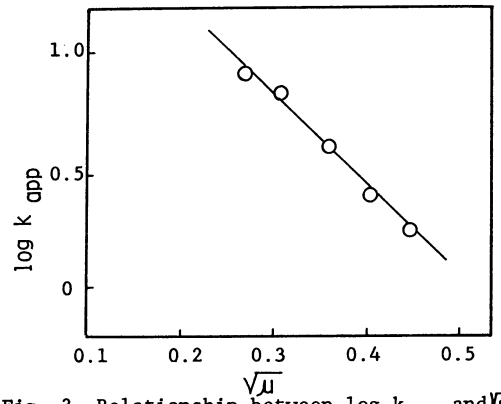


Fig. 3. Relationship between log k_{app} and $\sqrt{\mu}$ 0.05M phosphate buffer(pH 6.0) and various concentration of NaCl, temperature 10°C.

To make sure the independence of the species of electrolytes, the same experiment was performed by using KNO_3 as electrolyte. In this experiment, the charge of the reaction site of the enzyme for $Fe(CN)_6^{4-}$ in bovine erythrocyte superoxide dismutase was +0.91. This value is very close to that obatined by using NaCl as an electrolyte.

The copper ion of a subunit in bovine erythrocyte superoxide dismutase was reduced by $Fe(CN)_6^{4-}$ at pH 6.0 (0.1 M phosphate buffer). The reduced content of copper ions in the enzyme was 80-90% and the reduction was observed to be incomplete. The incomplete reduction of bovine erythrocyte superoxide dismutase was found in the reduction by H_2O_2[12] and an excess amount of hydrate electrons also failed to reduce 13-21% of the copper ion in the enzyme.[3] A more recent paper[13] has indicated that the enzyme may be contaminated with nonfunctional copper ion that has similar EPR properties.

The over 90% of the copper ions in bovine erythrocyte superoxide dismutase was reduced with pseudo-first-order form by $Fe(CN)_6^{4-}$. This behavior indicates that over 90% of the copper ions in the enzyme is homogeneous. Lowrence's experiment[4] indicated that only the half of the copper ions in the enzyme was reduced in the presence of $K_3[Fe(CN)_6]$. They interpreted this result due to the binding of $Fe(CN)_6^{3-}$ to the copper ions in a subunit. But, in our experiment, $Fe(CN)_6^{4-}$ is oxidized by bovine erythrocyte superoxide dismutase to $Fe(CN)_6^{3-}$, which does not interfere with the reduction process of the enzyme. Therefore, the equilibrium dialysis experiments have been carried out to examine whether $Fe(CN)_6^{3-}$ would bind to the enzyme or not. The equilibrium dialysis of bovine erythrocyte superoxide dismutase (1.3×10^{-4} M) in the presence of $K_3[Fe(CN)_6]$ (1.0×10^{-4} M) indicated that $Fe(CN)_6^{3-}$ did not bind to the native enzyme.

One of the acceptable interpretation of the single stage kinetic process which is observed in Fig. 1 is statistical kinetics in which the two copper sites of bovine erythrocyte superoxide dismutase are equivalent and k_1 is equal to $2k_2$.

$$ECu(II)Cu(II) \xrightarrow{k_{1obsd}} ECu(II)Cu(I) \xrightarrow{k_{2obsd}} ECu(I)Cu(I) \qquad (4)$$

Substitution of these relationship into a general equation for biphasic kinetics yields

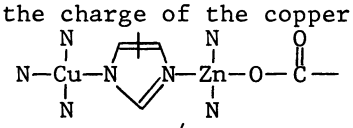
$$A_t = 2C_0\varepsilon_0 + \frac{C_0\varepsilon_0}{k_{1obsd} - k_{2obsd}}\left[2k_{2obsd}e^{-k_{1obsd}\cdot t} - k_{1obsd}e^{-k_{1obsd}\cdot t} - k_{1obsd}e^{-k_{2obsd}\cdot t}\right] \qquad (5)$$

where C_0 is the initial concentration of the enzyme and ε_0 is the molar absorbance of $Fe(CN)_6^{3-}$ at 420 nm.[14] k_{1obsd} is equal to $2k_{2obsd}$ and $2C_0\varepsilon_0$ is equal to A_∞, so that the absorbance change can be expressed as:

$$(A_\infty - A_t)/A_\infty = e^{-k_{2obsd} \cdot t} \quad (6)$$

Accordingly, the rate constant(k_{obsd} in Eq. 2) for overall reaction obtained from the single stage kinetics corresponds to true rate constant(k_{2obsd}) for the monofunctinal reactant ECu(II)Cu(I). And k_{1obsd} was obtained from $2k_{2obsd}$. The second-order rate constants(k_1 and k_2) are obtained by dividing the pseudo-first-order values k_{1obsd} and k_{2obsd} with the concentration of $Fe(CN)_6^{4-}$. Therefore, k_2 is equal to k_{app}. k_1 and k_2 were 6.4 M^{-1} s^{-1} and 3.2 M^{-1} s^{-1}, respectively.

The charge of the reaction site of the enzyme for $Fe(CN)_6^{4-}$ was calculated to be +0.9 — +1.0 from Eq. 3. The negative charges of carboxylate and imidazole residue, coordinating to the copper and zinc ions, neutralize a part of charges of the metal ions. Hence the charge of the copper ion in the active sites would be almost +1.0.



Therefore, $Fe(CN)_6^{4-}$ would react directly with the copper ion at the active site.

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