

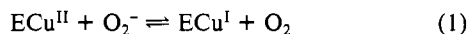
Electron-Transfer Reaction between $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ and Copper(II)/Copper(I) Ions in Bovine Erythrocyte Superoxide Dismutase: pH Dependence and Inhibition by Various Kinds of Anions[†]

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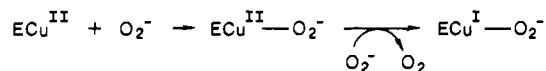
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The pH dependence of the redox reaction between $\text{Cu}(\text{II})/\text{Cu}(\text{I})$ and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ in superoxide dismutase has been studied by kinetic methods. The rates of reduction of copper(II) ions in superoxide dismutase by $\text{Fe}(\text{CN})_6^{4-}$ were almost constant between pH 5.0 and 8.2. But the rates of oxidation of copper(I) by $\text{Fe}(\text{CN})_6^{3-}$ were constant below pH 6.0 and increased with increasing pH from 6.0 to 8.2 (10-fold increase for one unit of pH). This behavior indicates that the protonated species of copper(I) ion in superoxide dismutase is the main reaction species with $\text{Fe}(\text{CN})_6^{3-}$ below pH 6.0 but the deprotonated species of copper(I) ion in superoxide dismutase is the main reaction species with $\text{Fe}(\text{CN})_6^{3-}$ from pH 6.0 to 8.2. This is the reason why the apparent equilibrium constant for the redox reaction between $\text{Cu}(\text{II})/\text{Cu}(\text{I})$ in superoxide dismutase and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ is dependent on pH. The reduction rates of copper(II) ion in superoxide dismutase by $\text{Fe}(\text{CN})_6^{4-}$ in the presence of various kinds of anions have been studied by kinetic methods. Cyanide and N_3^- adducts of copper(II) ion in superoxide dismutase, which form a square-planar coordination geometry around copper(II) ion in the enzyme, cannot accept an electron from $\text{Fe}(\text{CN})_6^{4-}$, but OCN^- and SCN^- adducts of copper(II) ion in superoxide dismutase, which form a five-coordination geometry around copper(II) ions, can accept an electron. Therefore, the ability of electron acceptance from $\text{Fe}(\text{CN})_6^{4-}$ to copper(II) ion in superoxide dismutase is governed by the coordination geometry around copper(II) ions in the anion adduct of the superoxide dismutase. The redox equilibrium data between $\text{Cu}(\text{II})/\text{Cu}(\text{I})$ in superoxide dismutase and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ in the presence or absence of anions indicate that the coordination of N_3^- and CN^- to copper(II) ions in superoxide dismutase caused the large shift in redox potential because of a very low affinity of these anions for copper(I) ion in superoxide dismutase. On the other hand, the OCN^- and SCN^- adducts of superoxide dismutase did not cause a large shift in redox potential of copper ion in the enzyme, because these anions may also coordinate to copper(I) ion in the enzyme.

Bovine superoxide dismutase (SOD) is a dimeric metalloenzyme containing zinc(II) and copper(II) ions in each subunit.^{1,2} The enzyme is found to be an extremely efficient catalyst for the dismutation of superoxide ions. The reaction mechanism of this enzyme is described by^{2,3}



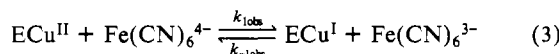
Although there is no direct evidence, it is believed that ECu^{II} is reduced in the inner-sphere redox reaction by O_2^- , because various anions bind to the copper ions in the enzyme and inhibit enzyme activity with a competitive type of pattern.⁴ However, recently, the reduction mechanism of ECu^{II} , which proceeds through an outer-sphere reaction, was proposed by Rosi et al.⁵ and Osman et al.⁶



Therefore, the electron-transfer reaction is very important for understanding the reaction mechanism in this enzyme.

Various kinds of anions inhibit enzyme activity. Many researchers^{3,7-9} have reported the mode of anion binding to SOD. Bertini et al.⁸ proposed two modes of anion binding to SOD: first, anions (N_3^- , CN^-) replace the water molecule that coordinates to the copper ion in SOD and cause rearrangement of the protein in such a way to end up in a basal position, and second, anions (i.e. SCN^- , OCN^-) replace one of the basal histidines. Recently, it was shown that histidine 44 in SOD is the one that undergoes detachment upon anion binding.^{10,11}

In a previous paper¹² and the work of Pladziewicz et al.,¹³ the electron-transfer reaction rate from $\text{Fe}(\text{CN})_6^{4-}$ to the copper(II) ion in SOD is an overall second-order reaction.



We are interested in the pH dependence of the electron transfer from $\text{Fe}(\text{CN})_6^{4-}$ to the copper(II) ion in SOD and the electron

transfer from $\text{Fe}(\text{CN})_6^{4-}$ to anion adducts of the copper(II) ion in SOD that have various coordination geometries, because the electron transfer from substrate (superoxide ion) to copper(II) ion in the enzyme is inhibited by anions and is a very important reaction.

Materials and Methods

Reagents and Enzyme. Bovine erythrocyte superoxide dismutase (SOD) was purified from bovine erythrocyte by the method of McCord and Fridovich.¹⁴ The enzyme was dialyzed against a 0.02 M buffer. Protein concentration was estimated by the method of Lowry, and the copper and zinc contents were measured with a Shimadzu AA-630-12 flame atomic absorption spectrophotometer. All the other reagents were of analytical grade.

Kinetic Measurements. Kinetic studies were monitored at 420 nm by a Hitachi 557 spectrophotometer, which was thermostated at 25 ± 0.5 °C with a NESLAB EX-200 instrument. Potassium ferrocyanide was dissolved in 0.02 M buffer (pH 5.0-7.0, MES buffer; pH 7.2-8.2, HEPPS buffer). In order to protect the ferrocyanide from oxidation by oxygen, the reaction was carried out under nitrogen atmosphere. Ionic

[†] Abbreviations: SOD, bovine erythrocytes superoxide dismutase; MES, 2-morpholinoethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-propanesulfonic acid; 6-HDA, 6-hydroxydopamine.

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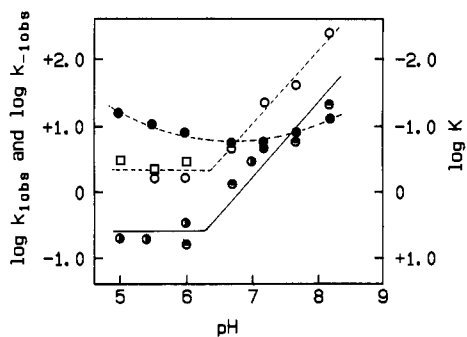


Figure 1. pH dependence of $k_{1\text{obs}}$, $k_{-1\text{obs}}$, and K : (●) $k_{1\text{obs}}$; (○) $k_{-1\text{obs}}$ directly obtained by kinetics; (□) $k_{-1\text{obs}}$ indirectly obtained by $k_{1\text{obs}}/K$; (●) apparent equilibrium constant (K), which was obtained by the equilibrium method by Fee et al.¹⁸ (●) apparent equilibrium constant (K), which was obtained by the kinetic method ($K = k_{1\text{obs}}/k_{-1\text{obs}}$).

strength was adjusted to 0.15 by NaClO_4 in all experiments.

Redox Equilibrium of Copper Ion in SOD in the Presence of Anion.

Copper ion of SOD and potassium ferrocyanide solutions of which concentrations were equivalent were mixed with a 1:1 volume ratio in 0.02 M MES buffer (pH 6.0, $I = 0.15$). The reduced copper content per total copper ion in SOD ($\text{ECu}^{\text{I}}/(\text{ECu}^{\text{I}} + \text{ECu}^{\text{II}})$) was about 75% in the absence of anions. After the addition of anions, the absorbance at 420 nm was changed with binding of the anion to copper ion in SOD, and it was measured by the Hitachi 557 spectrophotometer. The concentration of ferricyanide produced by reduction of copper(II) ion in SOD by ferrocyanide ion could be calculated by the absorbance at 420 nm. The content of reduced copper ion of SOD in the presence of azide ion was measured at 660 nm (d-d* transition of copper(II) ion in SOD), because the SOD-azide complex has an absorption band around 400 nm.

EPR and T_1 Measurements. X-band EPR spectra were recorded on a JEOL JES-FE2XG instrument at 23 ± 0.5 °C. The spin-lattice relaxation times of the water proton, T_1 , were measured on a JEOL FX-100 instrument with the inversion recovery method at 25 °C. Dissociation constants of some anions were calculated through nonlinear least-squares fitting for both the EPR spectra and the water proton relaxation data obtained at various anion concentrations. The dissociation constant of SCN^- could not be measured by the previous methods because no EPR and T_1 change was observed. Therefore, it was determined by the difference spectra method described by Dooley and McGuirl.¹⁵

Enzyme Activity Measurement. Enzyme activity in the presence of 0.13 M anion was measured at pH 6.0 by using the 6-hydroxydopamine (6-HDA) assay method of Heikkilä and Cabbat.¹⁶ Because the enzyme activity in the presence of OCN^- could not be measured by using the 6-HDA assay, its activity was measured by the xanthine-xanthine oxidase method.¹⁴ The enzyme activity was measured by Shimadzu UV-200 thermostated at 25 ± 0.5 °C.

Results

pH Dependence of the Cu(II)/Cu(I) Redox Reaction of Bovine Superoxide Dismutase with $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$. The Cu(II)/Cu(I) redox reaction of bovine superoxide dismutase with $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ is shown by eq 3. The redox potential of ECu^{II} in eq 3 is pH dependent and decreases with increasing pH, so that eq 3 proceeds completely to the right in the presence of a large excess of $\text{Fe}(\text{CN})_6^{4-}$ at pH 5.0–7.2.

A slow change in the absorbance at 420 nm was followed after quick mixing of the excess $\text{Fe}(\text{CN})_6^{4-}$ with bovine superoxide dismutase. Plots of $\log [(A_\infty - A_t)/A_\infty]$ vs time were linear for 90% of the reaction. The pseudo-first-order rate constant (k_{obs}) was derived from the slope of the line. The relationship between k_{obs} and the concentration of $\text{Fe}(\text{CN})_6^{4-}$ was linear, and k_{obs} was constant in the concentration range of the enzyme (0.23×10^{-4} to 1.1×10^{-4} M). These results indicated that the reaction from ECu^{II} to ECu^{I} with $\text{Fe}(\text{CN})_6^{4-}$ is an overall second-order reaction and that its rate is described by:

$$d[\text{ECu}^{\text{I}}]/dt = k_{\text{obs}}[\text{ECu}^{\text{II}}] = k_{1\text{obs}}[\text{Fe}(\text{CN})_6^{4-}][\text{ECu}^{\text{II}}] \quad (4)$$

where k_{obs} is the pseudo-first-order rate constant and $k_{1\text{obs}}$ is the

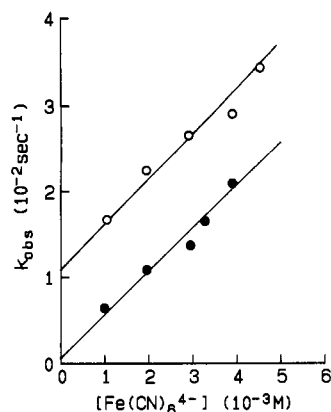


Figure 2. Relationship between k_{obs} and concentration of $\text{Fe}(\text{CN})_6^{4-}$ in the presence and absence of a constant concentration of excess $\text{Fe}(\text{CN})_6^{3-}$ at pH 7.2: (●) $[\text{Fe}(\text{CN})_6^{3-}] = 0$ M; (○) $[\text{Fe}(\text{CN})_6^{3-}] = 2 \times 10^{-4}$ M. The enzyme concentration was 3.0×10^{-5} M; 0.02 M MES buffer at 25 °C was used.

second-order rate constant for the reduction of ECu^{II} by $\text{Fe}(\text{CN})_6^{4-}$. A plot of $k_{1\text{obs}}$ values obtained by eq 4 at pH 5.0–7.2 is shown in Figure 1. In order to measure the oxidation reaction ($k_{-1\text{obs}}$ in eq 3) of ECu^{I} by $\text{Fe}(\text{CN})_6^{3-}$, the reaction described by eq 3 was allowed to reach equilibrium by the addition of an appropriate constant excess of $\text{Fe}(\text{CN})_6^{3-}$ in the presence of an excess of $\text{Fe}(\text{CN})_6^{4-}$. The approach to the equilibrium of the reaction in eq 3 is first order, and the derived apparent first-order rate constant (k_{obs}) is shown as¹⁷

$$\ln \left(\frac{A_e - A_0}{A_e - A_t} \right) = k_{\text{obs}} t = (k_{1\text{obs}}[\text{Fe}(\text{CN})_6^{4-}] + k_{-1\text{obs}}[\text{Fe}(\text{CN})_6^{3-}])t \quad (5)$$

where $k_{-1\text{obs}}$ is the second-order rate constant for oxidation of ECu^{I} by $\text{Fe}(\text{CN})_6^{3-}$ and A_e is the absorbance of attainment of the equilibrium state. At a constant concentration of excess $\text{Fe}(\text{CN})_6^{3-}$, a plot of k_{obs} for approach to equilibrium vs $[\text{Fe}(\text{CN})_6^{4-}]$ is a linear line with an intercept. The slope is $k_{1\text{obs}}$ and the intercept point value is $k_{-1\text{obs}}[\text{Fe}(\text{CN})_6^{3-}]$. The slope of the straight line in the presence and absence of excess $\text{Fe}(\text{CN})_6^{3-}$ should be the same, because the slope of the line is $k_{1\text{obs}}$ in both eq 4 and 5. In Figure 2, the relationship between k_{obs} and $[\text{Fe}(\text{CN})_6^{4-}]$ in the presence of 2×10^{-4} M $[\text{Fe}(\text{CN})_6^{3-}]$ was linear and was parallel to the line that was obtained by the condition when $\text{Fe}(\text{CN})_6^{3-}$ was absent. This behavior indicated the validity of eq 5. The $k_{-1\text{obs}}$ values obtained from eq 5 between pH 5.5 and pH 7.2 are shown in Figure 1. The apparent equilibrium constants ($K = k_{1\text{obs}}/k_{-1\text{obs}}$) of eq 3 were determined by the method of Fee and DiCorleto,¹⁸ so that it was possible to calculate $k_{-1\text{obs}}$ by using $k_{1\text{obs}}/K$. This $k_{-1\text{obs}}$ value was also plotted in Figure 1. At pH 5.5 and 6.0, $k_{-1\text{obs}}$ was indirectly calculated from the apparent equilibrium constant (K), and it was almost the same as those obtained directly from eq 5.

At high pH (pH > 7.7), the apparent equilibrium constant (K) from eq 3 is very small, because the redox potential of the copper ion in SOD is pH dependent and decreases with increasing pH. Then, eq 3 does not completely proceed to the right even in the presence of a large excess of $\text{Fe}(\text{CN})_6^{4-}$. $k_{1\text{obs}}$ and $k_{-1\text{obs}}$ in eq 3 are given by the following equations:¹⁹

$$\ln \left(\frac{A_0^2 - A_e A_t}{(A_t - A_e) A_0} \right) = k_{1\text{obs}}[\text{Fe}(\text{CN})_6^{4-}] \frac{A_0 + A_e}{A_0 - A_e} t \quad (6)$$

$$k_{-1\text{obs}} = k_{1\text{obs}}[\text{Fe}(\text{CN})_6^{4-}] \frac{A_e}{(A_0 - A_e)^2} \quad (7)$$

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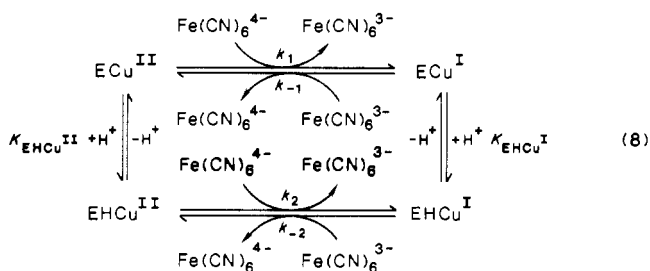
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where A_0 is the absorbance at zero time, A_t is the absorbance at time t , and A_e is absorbance of the attainment of the equilibrium state. Plots of data at pH 7.7 from eq 6 are shown in Figure 3. In Figure 3, there is good linearity between $\ln [(A_0^2 - A_e A_t) / ((A_t - A_e) A_0)]$ and time. The slope of the line is $k_{1\text{obs}}[\text{Fe}(\text{CN})_6^{4-}]((A_0 + A_e) / (A_0 - A_e))$. $((A_0 + A_e) / (A_0 - A_e))$ is determined by the absorbance at zero time and at an equilibrium state. The $[\text{Fe}(\text{CN})_6^{4-}]$ used in excess is almost constant throughout the entire reaction, so that $k_{1\text{obs}}$ can be obtained from eq 6. Also, $k_{-1\text{obs}}$ can be obtained by eq 7. The values $k_{1\text{obs}}$ and $k_{-1\text{obs}}$ obtained from the data at pH 7.7 in Figure 3 were $7.2 \pm 0.2 \text{ M}^{-1} \text{ s}^{-1}$ and $39 \pm 9 \text{ M}^{-1} \text{ s}^{-1}$, respectively. In very high concentrations of $\text{Fe}(\text{CN})_6^{4-}$ ($> 2 \times 10^{-3} \text{ M}$) at pH 7.7, eq 4 is also relevant because eq 3 tends to the right in the presence of a very high concentration of $\text{Fe}(\text{CN})_6^{4-}$ even at pH 7.7. The value of $k_{1\text{obs}}$ obtained from eq 4 was $8.0 \pm 1.0 \text{ M}^{-1} \text{ s}^{-1}$ and was almost consistent with that obtained from eq 6. This agreement between $k_{1\text{obs}}$ obtained by eq 4 and that obtained by eq 6 at pH 7.7 shows the validity of eq 6 used to calculate $k_{1\text{obs}}$.

The values $k_{1\text{obs}}$ and $k_{-1\text{obs}}$ obtained at various pHs are shown in Figure 1. $k_{1\text{obs}}$ is almost independent of pH while $k_{-1\text{obs}}$ is very dependent on pH. At a low pH from 5.0 to 6.0, $k_{-1\text{obs}}$ was almost independent of pH, but $k_{-1\text{obs}}$ increased with increasing pH from 6.0 to 8.2 ($k_{-1\text{obs}}$ increased 10 times with only a pH change of one). Apparent equilibrium constants (K) of eq 3 at various pHs can be calculated by the ratio $k_{1\text{obs}}/k_{-1\text{obs}}$ and are shown in Figure 1. The apparent equilibrium constants (K) at pH 5.0, 5.5, 6.0, and 7.0 were also measured by the methods of Fee and DiCorleto¹⁸ and are shown in Figure 1. The logarithm of the apparent equilibrium constant (K) was independent on pH below 6.0 but increased linearly to a high pH, having a slope of 1.0/pH. The pH dependence of the apparent equilibrium constants was consistent with that reported by Fee and DiCorleto.¹⁸

The pH dependence of the apparent equilibrium constant (K) in eq 3 obtained by the kinetic method was consistent with that reported by Fee and DiCorleto.¹⁸ But, surprisingly, $k_{1\text{obs}}$ was almost constant from pH 5.0 to 8.2 while $k_{-1\text{obs}}$ had a constant value below pH 6.0 but increased linearly to pH 8.2 with a slope of 1.0/pH unit above pH 6.0. This is why the apparent equilibrium constant (K) in eq 3 was dependent on pH. To interpret this behavior, the following equation is proposed:



$$K_{\text{EHCu}^{\text{II}}} = \frac{[\text{ECu}^{\text{II}}][\text{H}^+]}{[\text{EHCu}^{\text{II}}]} \quad (9)$$

$$K_{\text{EHCu}^{\text{I}}} = \frac{[\text{ECu}^{\text{I}}][\text{H}^+]}{[\text{EHCu}^{\text{I}}]} \quad (10)$$

The apparent equilibrium constant (K) in eq 3 is represented by the following kinetic equation:

$$K = \frac{([\text{ECu}^{\text{I}}] + [\text{EHCu}^{\text{I}}])[\text{Fe}(\text{CN})_6^{3-}]}{([\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}]} = \frac{k_{1\text{obs}}}{k_{-1\text{obs}}} = \frac{(k_1 K_{\text{EHCu}^{\text{II}}}/[\text{H}^+] + k_2) / (1 + K_{\text{EHCu}^{\text{II}}}/[\text{H}^+])}{(k_{-1} K_{\text{EHCu}^{\text{I}}}/[\text{H}^+] + k_{-2}) / (1 + K_{\text{EHCu}^{\text{I}}}/[\text{H}^+])} \quad (11)$$

In eq 11, $K_{\text{EHCu}^{\text{I}}}$ is much smaller than 10^{-9} M ,²⁰ so that, at a pH

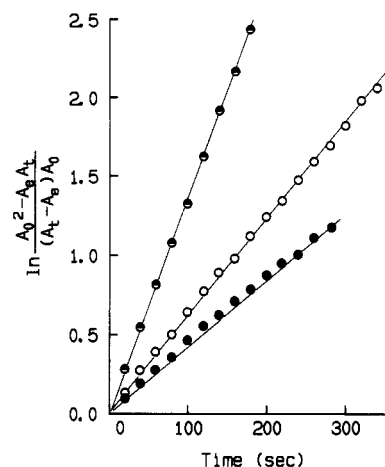


Figure 3. Relationship between $\ln ((A_0^2 - A_e A_t) / ((A_t - A_e) A_0))$ and time at pH 7.7. The enzyme concentration was $4.0 \times 10^{-5} \text{ M}$; 0.02 M HEPES buffer ($I = 0.15$) at 25°C was used. $[\text{Fe}(\text{CN})_6^{4-}]$: (●) $2.5 \times 10^{-4} \text{ M}$; (○) $5.0 \times 10^{-4} \text{ M}$; (◐) $1.5 \times 10^{-3} \text{ M}$.

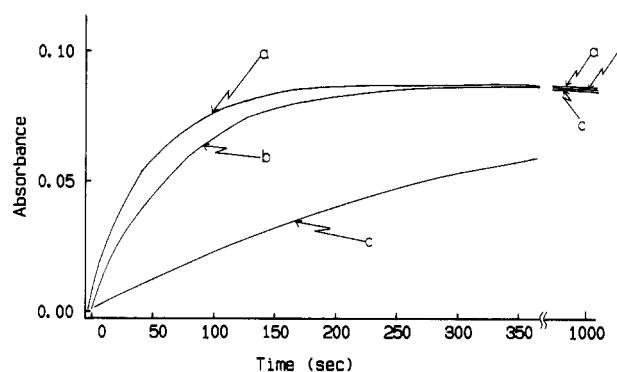


Figure 4. Time course of the $\text{Fe}(\text{CN})_6^{4-}$ reaction with copper(II) ion in SOD in the absence and presence of CN^- at pH 6.0 in 0.02 M MES buffer ($I = 0.15$, NaClO_4): (a) 0 M CN^- ; (b) $1.0 \times 10^{-2} \text{ M}$ CN^- ; (c) $1.0 \times 10^{-1} \text{ M}$ CN^- . The concentration of SOD is $4.7 \times 10^{-5} \text{ M}$ and that of $\text{Fe}(\text{CN})_6^{4-}$ is $2 \times 10^{-3} \text{ M}$.

lower than 8.2, the term $(1 + K_{\text{EHCu}^{\text{I}}}/[\text{H}^+])$ in eq 11 is almost equal to 1. In Figure 1, $k_{1\text{obs}}$ was almost constant, so that it implies two possibilities: one is when $k_1 = k_2$; the other is when $K_{\text{EHCu}^{\text{II}}} \gg 10^{-5} \text{ M}$ and $k_1 K_{\text{EHCu}^{\text{II}}}/[\text{H}^+] > k_2$. Under these conditions, eq 11 can be simplified to

$$\frac{k_{1\text{obs}}}{k_{-1\text{obs}}} = \frac{([\text{ECu}^{\text{I}}] + [\text{EHCu}^{\text{I}}])[\text{Fe}(\text{CN})_6^{3-}]}{([\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}]} = \frac{k_1}{k_1 K_{\text{EHCu}^{\text{I}}}/[\text{H}^+] + k_2} \quad (12)$$

At a lower pH than 6.0, $k_{-1\text{obs}}$ is almost consistent with k_{-2} , because $k_{-1} K_{\text{EHCu}^{\text{I}}}/[\text{H}^+]$ is much smaller than k_{-2} . But, at a pH higher than 6.0, $k_{-1\text{obs}}$ is governed by $k_{-1} K_{\text{EHCu}^{\text{I}}}/[\text{H}^+]$, so that it has a slope of 1.0/pH in Figure 1. A false $\text{p}K_a$ of the apparent equilibrium constant (K) in Figure 1 results when $k_{-1} K_{\text{EHCu}^{\text{I}}}/[\text{H}^+] = k_{-2}$. k_1 and k_{-2} are obtained from the data in Figure 1. k_1 is $\sim 10 \text{ M}^{-1} \text{ s}^{-1}$ and k_{-2} is $\sim 2 \text{ M}^{-1} \text{ s}^{-1}$. Unfortunately, k_{-1} cannot be determined, because $K_{\text{EHCu}^{\text{I}}}$ is unknown. However, it is predicted that $K_{\text{EHCu}^{\text{I}}}$ is much smaller than 10^{-9} M .²⁰ If $K_{\text{EHCu}^{\text{I}}}$ is smaller than 10^{-9} M , k_{-1} calculated by eq 12 is much larger than $1600 \text{ M}^{-1} \text{ s}^{-1}$. Therefore, ECu^{I} in eq 8 donates the electron to $\text{Fe}(\text{CN})_6^{3-}$ much easier than EHCu^{I} ($\gg 800$ times). Therefore, the deprotonation of the EHCu^{I} species is very important for electron transfer from EHCu^{I} to $\text{Fe}(\text{CN})_6^{3-}$. In eq 8, $K_{\text{EHCu}^{\text{I}}}$ is smaller than 10^{-9} M and $K_{\text{EHCu}^{\text{II}}}$ is larger than 10^{-5} M , so that EHCu^{I} and ECu^{II} are main species in the pH range from 5.0 to 8.2.

Inhibition of Electron Transfer from $\text{Fe}(\text{CN})_6^{4-}$ to the Copper(II) Ion in SOD by Various Types of Anions. A typical time regime

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Table I. Dissociation Constants of Various Anions with SOD by Various Methods and Inhibition of SOD Activity by Anions^a

anions	dissoc const, M ⁻¹			% inhibition content ^b	
	EPR	T ₁ ⁻¹	kinetic method	xanthine-xanthine oxidase ^c	6-HDA ^c
CN ⁻	1.1 (±0.2) × 10 ⁻²	1.5 (±0.4) × 10 ⁻²	9.9 (±2) × 10 ⁻³		74
N ₃ ⁻	1.7 (±0.2) × 10 ⁻²	1.7 (±0.3) × 10 ⁻²	2.2 (±0.3) × 10 ⁻²		96
OCN ⁻	3.9 (±1.4) × 10 ⁻²			38 ^d	
SCN ⁻	(5 (±3) × 10 ⁻²) ^f				35 (20) ^e

^a All experiments were run in 0.02 M MES buffer at pH 6.0 (*I* = 0.15 (NaClO₄); 25 °C). ^b The inhibition content was obtained by the ratio (initial rate in the presence of SOD and anion (0.13 M))/(that in the presence of only anion (0.13 M)). The concentration of the anion was 130 mM. ^c 6-HDA and xanthine oxidase methods depend on ref 16 and 14, respectively. ^d The inhibition of the enzyme activity by OCN⁻ can not be measured by the 6-HDA method. ^e Reference 22. ^f The dissociation constant of SCN⁻ was determined by the difference spectrophotometric method.

for the reduction of SOD with Fe(CN)₆⁴⁻ (2 × 10⁻³ M; 25 °C, *I* = 0.15, in 0.02 M MES buffer at pH 6.0) in the presence of various cyanide concentrations is shown in Figure 4.

In Figure 4, the reduction rate was decreased with increasing cyanide concentration but the limiting absorbance changes (*A*_∞) were almost consistent with each other. Plots of log ([*A*_∞ - *A*]_{*t*})/[*A*_∞] vs time in the presence and absence of cyanide ions have a linear correlation for over 90% of the course of the reaction. The pseudo-first-order rate constant (*k*_{obs}) was derived from the slope of the line. The values of *k*_{obs(anion)} and *k*_{obs(control)} represent apparent reduction rate constants of copper(II) ion to copper(I) ion in SOD by Fe(CN)₆⁴⁻ in the presence and absence of anions, respectively. The relationship between the *k*_{obs(anion)}/*k*_{obs(control)} ratio and anion concentrations is shown in Figure 5. The values of *k*_{obs(anion)} and *k*_{obs(control)} are the pseudo-first-order reduction rate constants in the presence and absence of anion, respectively. The changes in EPR and T₁⁻¹ in the presence of various concentration of anions are also shown in Figure 5. The T₁⁻¹ values and the change in the shape of the EPR spectra with increasing concentrations of the anion were almost the same as those reported by Bertini et al.⁸

In Figure 5A, the *k*_{obs(anion)}/*k*_{obs(control)} ratio decreased with increasing concentration of cyanide or azide ion. There was also a similar change of the EPR spectra and T₁⁻¹ (water proton relaxation rate). The dissociation constants of CN⁻ and N₃⁻ obtained by electron-transfer kinetics, EPR, and water proton relaxation methods, are shown in Table I and are almost consistent with each other in the range of standard deviation for the different methods. At a constant concentration of CN⁻ and N₃⁻ (0.026 M), the values of *k*_{obs(anion)}/*k*_{obs(control)} did not change by increasing the concentration of Fe(CN)₆⁴⁻ from 1.5 × 10⁻³ to 6.0 × 10⁻³ M.

In Figure 4, the *A*_∞ value was almost consistent with that of the control in spite of the presence of the highest concentration of CN⁻ (6.5 × 10⁻² M). The same behavior was observed with N₃⁻. It is known that CN⁻ and N₃⁻ replace a water that can coordinate to the copper(II) ion in SOD and form a square-planar geometry around the copper(II) ion in the active site.⁹ The electron transfer kinetics, EPR, and T₁⁻¹ in the presence of N₃⁻ and CN⁻ indicate that the direct electron transfer from Fe(CN)₆⁴⁻ to the copper(II) ions bound by CN⁻ or N₃⁻ ions in SOD is inhibited by CN⁻ or N₃⁻. The dissociation constants of N₃⁻ and CN⁻ obtained by electron-transfer kinetics are almost consistent in the range of standard deviation with those obtained by the EPR and T₁⁻¹ methods. Therefore, in the presence of CN⁻ and N₃⁻, the electron transfer will occur through the native form in which anions are released from copper(II) ion of the enzyme.

Electron-transfer reaction kinetics have also been studied in the presence of SCN⁻ and OCN⁻, which form a five-coordinate structure around the copper ions in the active site without replacing the water molecule. These results are shown in Figure 5B. EPR spectra, but not the electron-transfer rate, were significantly changed by increasing of the concentration of OCN⁻. The dissociation constant of OCN⁻ obtained by EPR spectra was 3.9 × 10⁻² M (Table I). The EPR spectra of the copper ion in SOD in the presence of 0.13 M OCN⁻ showed that OCN⁻ is binding to 80% of the copper(II) ions in SOD, but *k*_{obs(OCN⁻)}/*k*_{obs(control)} remained 80%. Therefore, the electron transfer from Fe(CN)₆⁴⁻ to the copper(II) ions in SOD is only slightly affected by the

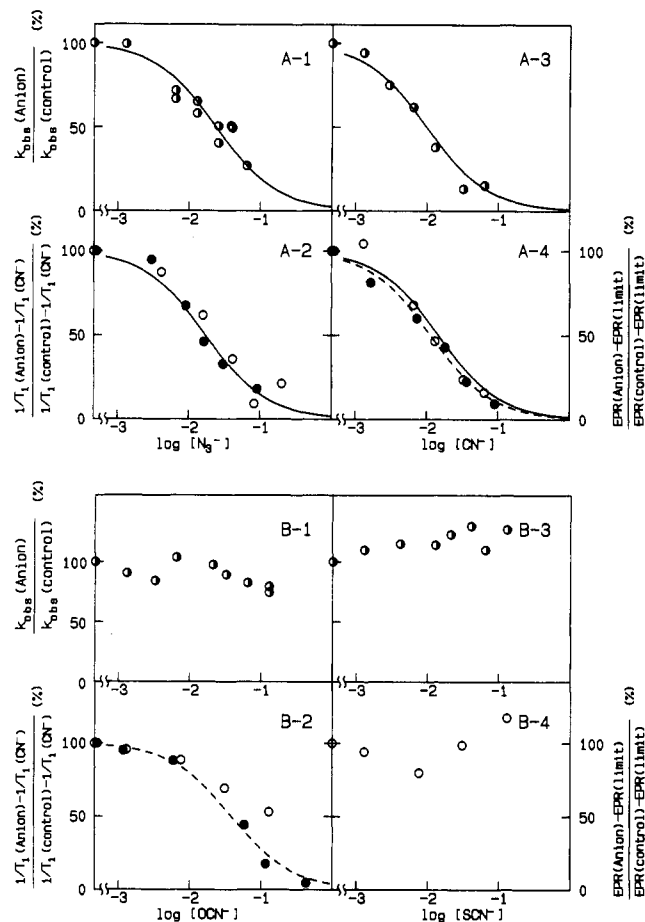


Figure 5. Influence of anions on the electron-transfer rate from Fe(CN)₆⁴⁻ to the copper ion, the EPR spectrum of the copper ion in SOD, and T₁⁻¹ of the water proton (0.02 M MES buffer, pH 6.0, *I* = 0.15 (NaClO₄)). The value of *k*_{obs(anion)}/*k*_{obs(control)} was the ratio of rate constants in the presence and absence of anions. (1/T_{1(anion)} - 1/T_{1(CN⁻)})/(1/T_{1(control)} - 1/T_{1(CN⁻)}) was obtained by subtracting the value of 1/T₁ obtained in the presence of CN⁻ (2 × 10⁻¹ M) from 1/T₁ obtained in the presence and absence of anions. (EPR_{anion} - EPR_{limit})/(EPR_{control} - EPR_{limit}) was obtained by subtracting the limit value of EPR intensity (the EPR intensity for an anion completely bound to the enzyme was calculated by a microcomputer) from the EPR intensity in the presence and absence of an anion. Influence of azide ions: (A-1) electron-transfer rate (25 °C, ●, 2 × 10⁻³ M Fe(CN)₆⁴⁻); (A-2) EPR spectrum (●) and T₁⁻¹ (○) (room temperature, 23 °C; 2.6 × 10⁻⁴ M SOD). Influence of CN⁻ ions: (A-3) electron-transfer rate (25 °C; ○, 2 × 10⁻³ M Fe(CN)₆⁴⁻); (A-4) EPR spectrum (●, --) and T₁⁻¹ (○, —) (room temperature, 23 °C; 2.6 × 10⁻⁴ M SOD). Influence of OCN⁻ and SCN⁻ ions: (B-1 and B-3) electron-transfer rate (25 °C; ●, 2 × 10⁻³ M Fe(CN)₆⁴⁻); (B-2 and B-4) EPR spectrum (●, --) and T₁⁻¹ (○) (room temperature 23 °C; 5.0 × 10⁻⁴ M SOD).}}

addition of OCN⁻. On the other hand, the binding of SCN⁻ increased the rate of electron transfer up to 120%. The dissociation constant obtained by the difference spectra method was 5.0 × 10⁻² M. In the presence of 0.13 M SCN⁻, 72% of the copper(II) ions in SOD are bound by SCN⁻. However, SCN⁻ did not inhibit the

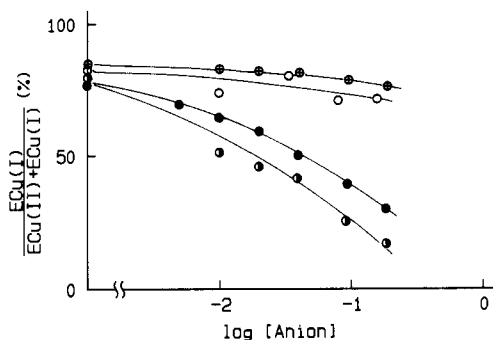


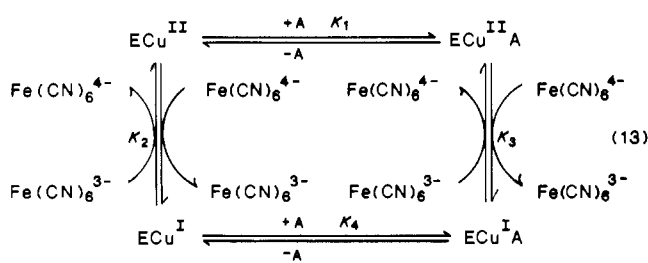
Figure 6. Shift in the redox equilibrium of copper ions in SOD in the presence of anions at pH 6.0: (●) SCN^- ; (○) OCN^- ; (●) N_3^- ; (○) CN^- . A 0.02 M MES buffer at pH 6.0 ($I = 0.15$; 25°C) was used; 1 mL each of a SOD (4×10^{-5} M) solution and a $\text{Fe}(\text{CN})_6^{4-}$ (8×10^{-5} M) solution containing various concentrations of anions were mixed.

electron transfer from $\text{Fe}(\text{CN})_6^{4-}$ to the copper(II) ion in SOD (Figure 5B).

These results indicate that OCN^- and SCN^- , which have five-coordinate geometry around the copper ions in the enzyme active site without replacing the coordinated water molecule,^{8,9} do not affect the electron-transfer rate from $\text{Fe}(\text{CN})_6^{4-}$ to the copper(II) ion in the active site. Therefore, the direct electron transfer from $\text{Fe}(\text{CN})_6^{4-}$ to the copper(II) ions bound to OCN^- or SCN^- occurs very easily.

Why does the cyanide or azide adduct of copper(II) ions not accept an electron from $\text{Fe}(\text{CN})_6^{4-}$ and why does the thiocyanate or cyanate adduct accept it? Lawrence and Sawyer²¹ pointed out that copper(II) in SOD bound by CN^- was not reduced by potentiometric titration and proposed that the cyanide adduct of copper(II) ion in SOD had low redox potential. Therefore, redox equilibrium in eq 3 was measured in the presence of various kinds of anions.

Redox Equilibrium between Cu(II)/Cu(I) in SOD and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ in the Presence or Absence of Various Anions. Redox equilibria in eq 3 were measured in the presence of various concentration of anions. When $\text{Fe}(\text{CN})_6^{4-}$ was added to SOD in a 1:1 ratio of $[\text{Fe}(\text{CN})_6^{4-}]$ to $[\text{ECu}^{\text{II}}]$ at pH 6.0, about 75% of the copper ion in SOD was reduced, but in the presence of CN^- or N_3^- , the lower content of copper ion in SOD was reduced. Therefore, the redox equilibrium in eq 3 was shifted by the anions. The relationships between reduced copper content ($[\text{ECu}^{\text{I}}]/([\text{ECu}^{\text{I}} + \text{ECu}^{\text{II}}])$) and concentration of an anion at pH 6.0 are shown in Figure 6. The contents of $[\text{ECu}^{\text{I}}]/([\text{ECu}^{\text{I}} + \text{ECu}^{\text{II}}])$ were decreased by increasing the concentration of N_3^- and CN^- but were almost constant in the presence of SCN^- and OCN^- . The shift of the equilibrium in eq 3 in the presence of anion is given by



$$\begin{aligned}
 K_1 &= \frac{[\text{ECu}^{\text{II}}][\text{A}]}{[\text{ECu}^{\text{II}}\text{A}]} & K_2 &= \frac{[\text{ECu}^{\text{I}}][\text{Fe}(\text{CN})_6^{3-}]}{[\text{ECu}^{\text{II}}][\text{Fe}(\text{CN})_6^{4-}]} \\
 K_3 &= \frac{[\text{ECu}^{\text{I}}\text{A}][\text{Fe}(\text{CN})_6^{3-}]}{[\text{ECu}^{\text{II}}\text{A}][\text{Fe}(\text{CN})_6^{4-}]} & K_4 &= \frac{[\text{ECu}^{\text{I}}][\text{A}]}{[\text{ECu}^{\text{I}}\text{A}]} \\
 K_1 K_2 &= K_3 K_4
 \end{aligned} \quad (14)$$

Table II. Redox and Binding Equilibria of Anions for Copper Ions of SOD in eq 8^a

	K_1	K_2	K_3	K_4
CN^-	3.8×10^{-3}	4.2	$\ll 2 \times 10^{-2}$	$\gg 1$
N_3^-	8.8×10^{-3}	6.1	$\ll 5 \times 10^{-2}$	$\gg 1$

^a $K_1 = ([\text{ECu}^{\text{II}}][\text{A}])/[\text{ECu}^{\text{II}}\text{A}]$, $K_2 = ([\text{ECu}^{\text{I}}][\text{Fe}(\text{CN})_6^{3-}])/([\text{ECu}^{\text{II}}][\text{Fe}(\text{CN})_6^{4-}])$, $K_3 = ([\text{ECu}^{\text{I}}\text{A}][\text{Fe}(\text{CN})_6^{3-}])/([\text{ECu}^{\text{II}}\text{A}][\text{Fe}(\text{CN})_6^{4-}])$, $K_4 = ([\text{ECu}^{\text{I}}][\text{A}])/[\text{ECu}^{\text{I}}\text{A}]$.

where A is an anion. The apparent reduced copper content in the presence of anion is shown as

$$\frac{\text{reduced copper ion}}{\text{total copper ion}} = \frac{[\text{ECu}^{\text{I}}] + [\text{ECu}^{\text{I}}\text{A}]}{[\text{ECu}^{\text{I}}] + [\text{ECu}^{\text{II}}] + [\text{ECu}^{\text{I}}\text{A}] + [\text{ECu}^{\text{II}}\text{A}]} = \frac{1}{1 + \frac{1 + [\text{A}]/K_1}{K_2(1 + [\text{A}]/K_4)}} \quad (15)$$

The fitting of the N_3^- and CN^- data in Figure 6 to eq 15 has been done by a nonlinear least-square fit using a microcomputer. K_1 , K_2 , and K_4 were determined by using CN^- and N_3^- data. K_3 was calculated by eq 14. These values are shown in Table II. In Table II, K_4 was larger than 1.0. This result indicates that the binding of N_3^- and CN^- for the reduced form of SOD is very weak and that the processes of the reaction occurred through K_1 and K_2 in eq 13.

Inhibition of SOD Activity by Various Kinds of Anions. It is generally known that N_3^- and CN^- strongly inhibit enzyme activity while SCN^- does not inhibit it.^{4,22} But, it is not known whether OCN^- can inhibit enzyme activity. Therefore, the enzyme activity was measured in the presence of OCN^- and shown in Table I with the inhibition data of other anions. OCN^- (0.13 M) gave weak inhibition of the enzyme activity (38%) at the concentration where the EPR spectrum completely changed. This behavior was similar to that of SCN^- . On the other hand, N_3^- and CN^- strongly inhibited the enzyme activity in the same buffer condition.

In this study, it was shown that the electron transfer from $\text{Fe}(\text{CN})_6^{4-}$ to copper(II) ion in SOD is inhibited by the binding of N_3^- and CN^- but not SCN^- and OCN^- . Also N_3^- and CN^- but not OCN^- and SCN^- strongly inhibit enzyme activity. Therefore, the fact that the N_3^- and CN^- adducts of the copper(II) ion of SOD do not have the ability to accept an electron may interfere with enzyme activity and be the reason for this inhibition.

Discussion

The pH dependence of the apparent equilibrium constant (K in eq 3) of the redox reaction between Cu(II)/Cu(I) in SOD and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ was determined by the kinetic method and was consistent with that obtained by Fee et al.^{18,20} The pH independence of the apparent equilibrium constant (K) below pH 6.0 predicted that a transition of some type is observed at low pH.²⁰ The electron-transfer kinetic data indicated that the pH dependence of K below pH 6.0 was dependent on the direct electron transfer from a protonated copper(I) species in SOD (EHCu^{I}) to $\text{Fe}(\text{CN})_6^{3-}$. Kinetic data do not indicate the presence of a low-pH form of copper(II) ion in SOD (EHCu^{II}), because the electron transfer from $\text{Fe}(\text{CN})_6^{4-}$ to copper(II) ion in SOD showed pH independence in the range pH 5.0–8.2. ECu^{I} in eq 8 donates the electron to $\text{Fe}(\text{CN})_6^{3-}$ much easier than EHCu^{I} ($\gg 800$ times). The implication of this behavior suggests that the presence of the proton on the imidazole does not facilitate the oxidation reaction but rather deprotonation facilitates the reaction by making available the imidazole ligand to bind to the oxidized enzyme. Superoxide ions that coordinate to Cu(I) ion in SOD may easily

accept a proton from the imidazole ring in EHCu^{I} and the electron transfer will subsequently occur from $\text{Cu}(\text{I})$ ion in SOD to the superoxide ion.

CN^- and N_3^- adducts of copper(II) ion in SOD, which form the square-planar coordination geometry by replacing the water molecule,^{8,9,23} could not accept an electron from $\text{Fe}(\text{CN})_6^{4-}$, but OCN^- and SCN^- adducts of copper(II) ion in SOD, which form the five-coordinate structure without replacing the water molecule,^{8,9,23} could accept an electron from $\text{Fe}(\text{CN})_6^{4-}$. Therefore, the ability of copper(II) ion in SOD to accept an electron from $\text{Fe}(\text{CN})_6^{4-}$ may be governed by the coordination geometry of the anion adduct around copper(II) ion in SOD. The redox equilibrium data between $\text{Cu}(\text{II})/\text{Cu}(\text{I})$ and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ in the presence or absence of anions indicated that the coordination of N_3^- and CN^- to copper(II) ion in SOD caused the large shift in the redox potential because of a very low affinity of these ions for copper(I) ion in SOD. The low affinity for copper(I) ion was recently proposed by Fee et al.²⁴ for enzyme activity kinetics with O_2^- . On the other hand, the adduct of OCN^- and SCN^- of SOD did not cause a large shift in the redox potential of copper ion in SOD, because these anions may also coordinate to the copper(I) ion in SOD. It was reported by Strothkamp et al.²² that the SCN^- adduct of SOD has enzyme activity. Our results also supported his results and showed that the OCN^- adduct of SOD may also have enzyme activity. Therefore, the five-coordinate geometry around copper(II) ion in SOD may be very important for enzyme activity.

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Appendix

In the pH-dependence experiment between $\text{Cu}(\text{II})/\text{Cu}(\text{I})$ in SOD and $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ (eq 8), the reduced velocity of copper(II) ion in SOD by $\text{Fe}(\text{CN})_6^{4-}$ is shown by

$$\bar{V} = k_{1\text{obs}}([\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}] \quad (\text{A1})$$

The rate is also the sum of the contributions of ECu^{II} and EHCu^{II} .

$$\bar{V} = (k_1[\text{ECu}^{\text{II}}] + k_2[\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}] \quad (\text{A2})$$

Equations A1 and A2 are combined:

$$k_{1\text{obs}} = \frac{k_1[\text{ECu}^{\text{II}}] + k_2[\text{EHCu}^{\text{II}}]}{[\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}]} = \frac{\frac{k_1 K_{\text{EHCu}^{\text{II}}}}{[\text{H}^+]} + k_2}{\frac{K_{\text{EHCu}^{\text{II}}}}{[\text{H}^+]} + 1} \quad (\text{A3})$$

where $K_{\text{EHCu}^{\text{II}}}$ is the equilibrium constant of protonation of ECu^{II} in eq 8. In the same way, $k_{-1\text{obs}}$ is obtained:

$$k_{-1\text{obs}} = \frac{k_{-1}[\text{ECu}^{\text{I}}] + k_{-2}[\text{EHCu}^{\text{I}}]}{[\text{ECu}^{\text{I}}] + [\text{EHCu}^{\text{I}}]} = \frac{\frac{k_{-1} K_{\text{EHCu}^{\text{I}}}}{[\text{H}^+]} + k_{-2}}{\frac{K_{\text{EHCu}^{\text{I}}}}{[\text{H}^+]} + 1} \quad (\text{A4})$$

where $K_{\text{EHCu}^{\text{I}}}$ is the equilibrium constant of protonation of ECu^{I} . At the equilibrium condition of eq 8, the reduced rate of copper(II) ion (\bar{V}) and oxidized rate of copper(I) ion (\bar{V}) in SOD by $\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$ are equal.

$$k_{1\text{obs}}([\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}] = k_{-1\text{obs}}([\text{ECu}^{\text{I}}] + [\text{EHCu}^{\text{I}}])[\text{Fe}(\text{CN})_6^{3-}] \quad (\text{A5})$$

The rearrangement of eq A5 and the insertion by eq A3 and A4 in to eq A5 produce

$$\frac{([\text{ECu}^{\text{I}}] + [\text{EHCu}^{\text{I}}])[\text{Fe}(\text{CN})_6^{3-}]}{([\text{ECu}^{\text{II}}] + [\text{EHCu}^{\text{II}}])[\text{Fe}(\text{CN})_6^{4-}]} = \frac{k_{1\text{obs}}}{k_{-1\text{obs}}} = \frac{\left(\frac{k_1 K_{\text{EHCu}^{\text{II}}}/[\text{H}^+] + k_2}{K_{\text{EHCu}^{\text{II}}}/[\text{H}^+] + 1} \right)}{\left(\frac{k_{-1} K_{\text{EHCu}^{\text{I}}}/[\text{H}^+] + k_{-2}}{K_{\text{EHCu}^{\text{I}}}/[\text{H}^+] + 1} \right)}$$

This equation corresponds to eq 11.

Registry No. SOD, 9054-89-1; Cu, 7440-50-8; N_3^- , 14343-69-2; CN^- , 57-12-5; SCN^- , 302-04-5; OCN^- , 22400-26-6; $\text{Fe}(\text{CN})_6^{4-}$, 13408-63-4; $\text{Fe}(\text{CN})_6^{3-}$, 13408-62-3.

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