# Electron Transfer between Copper and Zinc Superoxide Dismutase and Hexacyanoferrate(II)

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The electron-transfer rate between oxidized copper, zinc superoxide dismutase and $Fe(CN)_6^{4-}$ has been measured for several mutants of human SOD and compared to the rate obtained with the native enzyme. Among the results, the following are meaningful: (i) substitution of the positive Arg at position 143 with a neutral group decreases the electron transfer rate, (ii) mutation of Thr-137 has little influence, and (iii) substitution of Glu-133 with the neutral Gln increases the rate. Such pattern is similar to that of activity towards superoxide dismutation and to that of affinity for the anion $N_3^-$. It is proposed that $Fe(CN)_6^{4-}$ approaches the copper ion at the entrance of the cavity. $^{13}C$ NMR measurements on $Co(CN)_6^{3-}$ allow an estimate of the distance between the terminal N and copper in wild type SOD of 3.9 Å. Such distance is consistent with direct nitrogen to copper electron transfer. No reduction occurs in the case of zinc-depleted derivatives.

## Introduction

Copper, zinc superoxide dismutase (SOD hereafter) catalyzes the dismutation of superoxide anion with very high efficiency.[1-4] The reaction mechanism has been proposed to occur through the following steps[5]

$$Cu^{II}Zn^{II}SOD + O_2^- \rightarrow Cu^{I}Zn^{II}SOD + O_2$$

$$Cu^{I}Zn^{II}SOD + O_2^- + 2H^+ \rightarrow Cu^{II}Zn^{II}SOD + H_2O_2$$

It is possible that the oxidation of $O_2^-$ occurs through direct $O_2^-$–$Cu^{2+}$ interaction.[6,7] Recently the electron transfer reaction from $Fe(CN)_6^{4-}$ to copper(II) ion in SOD has been investigated under a variety of conditions through kinetic methods.[8-10] The electron-transfer reaction with $Fe(CN)_6^{4-}$ is a second-order process

$$Cu^{II}Zn^{II}SOD + Fe(CN)_6^{4-} \underset{k_{-1obs}}{\overset{k_{1obs}}{\rightleftharpoons}} Cu^{I}Zn^{II}SOD + Fe(CN)_6^{3-}$$

and the reduction rate constant at excess of $Fe(CN)_6^{4-}$ over $Cu^{II}Zn^{II}SOD$, can be obtained by the equation

$$d[Cu^{I}Zn^{II}SOD]/dt = k_{obs}[Cu^{II}Zn^{II}SOD] = k_{1obs}[Fe(CN)_6^{4-}][Cu^{II}Zn^{II}SOD]$$

where $k_{obs}$ is the pseudo-first-order rate constant and $k_{1obs}$ is the second-order rate constant for the reduction of ECu(II) by $Fe(CN)_6^{4-}$.[10]

Our goal is to determine the way in which electrons are supplied to the copper(II) ion by hexacyanoferrate(II), and, in particular, whether the electron transfer occurs in the active cavity. In this case a direct electron jump from nitrogen to copper or a direct anion–metal interaction are reasonable mechanisms. We have determined the reduction rate constants for some mutants of human SOD, in which catalytically relevant residues have been removed or introduced, and compared such rates with the activity towards superoxide dismutation and the affinity for azide. Furthermore, the nature of the interaction between copper and the anion has been investigated by $^{13}C$ NMR relaxation rate measurements carried out on $^{13}C$-enriched $Co(CN)_6^{3-}$ anion, a structural analog of $Fe(CN)_6^{4-}$, in the presence of the enzyme.

The mutants investigated in the present study are Arg143–Ile, Thr137–Ser, Glu133–Gln, Lys136–Gln, and Asp124–Asn. Arg-143 is located at the entrance of the cavity, close to the copper ion and probably assists the substrate in entering the cavity.[6,11-17] The Ile-143 mutant has only 11% the activity of the wild type.[15] Opposite to Arg-143 there is Thr-137, involved in a hydrogen-bonding network, together with Glu-133.[18] Substitution of Thr-137 with Ser causes an increase of the hydrophilicity of the cavity, even if it does not affect the enzymatic activity.[19,20] Removal of a negative charge at position 133 provides a substantially more active enzyme.[18] Lys-136 has been supposed to be the responsible of the pH-dependent properties of the enzyme at high pH,[21] but its role is still a matter of debate.[22] Asp-124, on the other hand, forms a long-range bridge between the zinc and the copper sites, connecting, through hydrogen bonds, the Zn-bound His-71, to the Cu-bound His-46.[11] Removal of this

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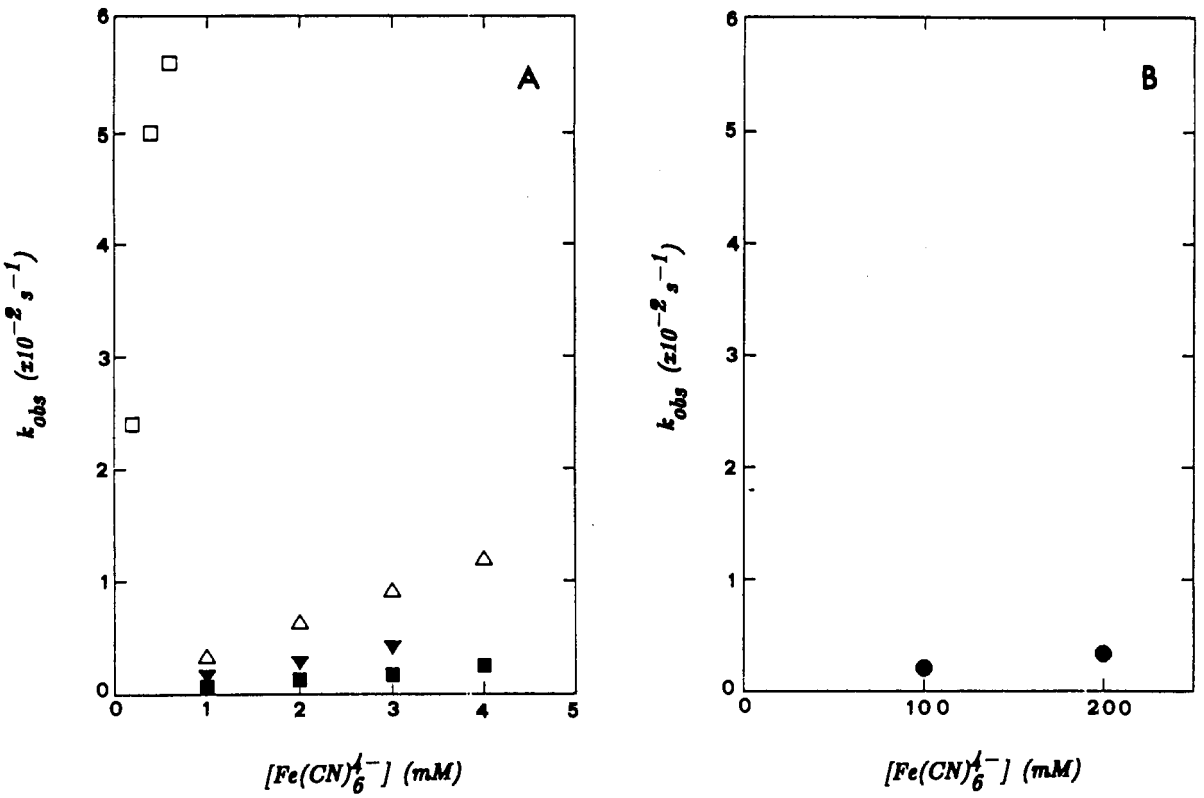


Figure 1. Relationship between $k_{obs}$ and concentration of $Fe(CN)_6^{4-}$. Wild type SOD and the mutants are $10^{-5}$–$10^{-4}$ M, in 0.02 M MES, 0.14 M phosphate, pH 6.0: (A) wild type SOD (△), Glu133–Gln (□), Lys136–Gln (■), and Thr137–Ser (▼); (B) Arg143–Ile (●).

group produces an enzyme which contains little zinc, and the fully zinc-depleted derivative does not show any tendency to migration of copper,[23] as happens in the zinc-depleted form of the wild type.[24]

## Experimental Section

Kinetic studies were done by monitoring at 420 nm the appearance of the band typical of the species $Fe(CN)_6^{3-}$ with a Shimadzu UV-3100 spectrophotometer, which was thermostated at 25 ± 0.5 °C with a NESLAB RTE-110 instrument. Potassium ferrocyanide was dissolved in 0.02 M MES (2-morpholinoethanesulfonic acid) (pH 6.0) containing 0.14 M $NaH_2PO_4$. Such a large anion concentration is used to keep the ionic strength constant. Phosphate is known to bind to Arg-143.[14,25] However, the effect of this anion on the electron-transfer rate was found to be much lower than that exerted by perchlorate and chloride. In order to protect the ferrocyanide from oxidation by oxygen, the reaction was carried out under nitrogen atmosphere. $^{13}C$ enriched $K_3Co(CN)_6$ was synthesized as reported in ref 26.

The human wild type SOD was obtained from yeast;[27,28] the mutants were expressed and isolated from *Escherichia coli* as reported elsewhere.[18,23] The apoproteins were obtained through extensive dialysis against 10 mM EDTA, in 50 mM acetate buffer at pH 3.8.[1] The chelating agent was removed through dialysis against 100 mM NaCl in the same buffer and then against acetate buffer alone.[29] Apoprotein concentration was determined through the Coomassie method.[30] The $Cu_2E_2$ derivative of wild type SOD and Asn-124 mutant was obtained through addition of solution of $Cu^{2+}$ in stoichiometric amount to the apoenzyme, at pH 3.8. The $Zn_2Zn_2$SOD derivative, used as a blank, was prepared from apo human wild type SOD in 20 mM phosphate at pH 6.0, through addition of stoichiometric amounts of $ZnSO_4$ solution.[31]

Electronic spectra were obtained with a Cary 17D spectrophotometer. EPR spectra were recorded on a Bruker ER200 operating at 9.8 GHz (X-band). $^{13}C$ NMR spectra were recorded on a Bruker AMX-400 spectrometer operating at 100.61 MHz. The spectra have been recorded with the following parameters: spectral width, 5 KHz; data points, 32K; pulse delay, 4 s; pulse width, 6 μs (40° pulse); collected number of scans, 500–20000; line broadening, 2 Hz. All runs were performed at 25 °C. The $^{13}C$ NMR spectrum of $Co(^{13}CN)_6^{3-}$ is an octet, due to the coupling of the cyanide carbons with the $I = {}^7/_2$ $^{59}Co$ nucleus. The relaxation rates of the $^{13}CN$ groups of the hexacyanocobaltate(III) complex interacting with the paramagnetic copper(II) of the protein, in a fast exchange regime on the NMR time scale, are given by[32]

$$T_1^{-1} = T_{1p}^{-1} + T_{1dia}^{-1} = f_m T_{1M}^{-1} + T_{1dia}^{-1} \quad (1)$$

$$T_2^{-1} = T_{2p}^{-1} + T_{2dia}^{-1} = f_m T_{2M}^{-1} + T_{2dia}^{-1}$$

where $T_{1dia}^{-1}$ and $T_{2dia}^{-1}$ are the intrinsic diamagnetic relaxation rates of the $^{13}C$ nucleus measured by using the $Zn_2Zn_2$ enzyme, $T_{1M}^{-1}$ and $T_{2M}^{-1}$ are the rate enhancements due to the nearby paramagnetic center, and $f_m$ is the molar fraction of $^{13}C$ nuclei interacting with the copper(II) ion. $T_2^{-1}$ equals $\pi\Delta\nu$, where $\Delta\nu$ is the line width at half peak height. $T_{2p}^{-1}$ (i.e. $T_2^{-1} - T_{2dia}^{-1}$) is therefore given by $\pi\Delta\nu'$ where $\Delta\nu'$ is now the line width at half peak height corrected for the diamagnetic effect. $f_m$ can be calculated by the following equation, with the assumption that free anion concentration is much higher than bound anion concentration:[32]

$$f_m = [E_T]/(K^{-1} + [L_T]) \quad (2)$$

Here $[E_T]$ is the total enzyme concentration, $[L_T]$ is the total ligand concentration, and $K$ is the affinity constant for ligand binding. Titration of $T_{2p}^{-1}$ as a function of $[L_T]$ yields the value of the affinity constant and

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**Table I.** Reduction Rate Constants, Affinity Constants for $N_3^-$, and Activity for $O_2^-$ Dismutation of Human Wild Type SOD and Some of its Mutants

| | $k_{obs}$ ($M^{-1}$ $s^{-1}$) | $K$ ($M^{-1}$) for $N_3^-$ ($Cu_2Co_2SOD$) | activity (%) |
|---|---|---|---|
| WT | 2.88 | 94 ± 5[a] | 100 |
| Ile-143 | 0.018 | 16 ± 1[b] | 11[c] |
| Ser-137 | 1.44 | 90 ± 7[a] | 70[d] |
| Gln-133 | 108 | 534 ± 42[b] | 222[e] |
| Gln-136 | 0.63 | 51 ± 4[a] | 65[f] |
| Asn-124 | no reduction | 137 ± 13[a] | 81[g] |

[a] Values have been measured at pH 7.5, in 10 mM Hepes buffer. [b] Values have been measured at pH 5.5, in 50 mM acetate buffer. [c] References 15 and 16. [d] Reference 20. [e] Reference 18. [f] Reference 22. [g] Reference 19.

of $T_{2M}$ [1]

$$T_{2p}^{-1} = T_{2M}^{-1}([E_T]/(K^{-1} + [L_T])) \quad (3)$$

Wild type SOD and $Zn_2Zn_2SOD$ ($7.5 \times 10^{-5}$ M, pH 6.0) were titrated with $Co(^{13}CN)_6^{3-}$ under the same conditions. Ligand concentration varied in the range $7.5 \times 10^{-4}$ to $5 \times 10^{-2}$ M. Calculations were carried out by using $\Delta\nu$ values averaged on the eight peaks of the multiplet. $T_1$ values were determined with the inversion recovery sequence[32] using Bruker standard software. Errors are within 1.5%.

## Results and Discussion

The reduction rate constants for the mutants are summarized in Table I, together with the value of the wild type enzyme. In Figure 1 the linear relationships between $k_{obs}$ and the concentration of $Fe(CN)_6^{4-}$ are shown. Table I indicates that the effects of mutations on the reduction rate constants are similar to the effects on the affinity for $N_3^-$ and to the effects on the enzymatic activity for $O_2^-$. Indeed, substitution of Arg at position 143 with the neutral residues like Ile causes a dramatic decrease of the reduction rate constant as compared to the wild type (from 2.88 to 0.018 $M^{-1}$ $s^{-1}$) as well as a sharp decrease in the activity[15] and in the affinity constant for $N_3^-$, the latter being measured through an $^1H$ NMR technique on the $Cu_2Co_2$ derivative.[16] Moreover, the substitution of Thr-137 with Ser produces a decrease of the reduction rate of only a factor of 2, in agreement with the affinity for $N_3^-$ and with the activity, that is slightly affected by this modification.[20] Slightly stronger effects are observed with the 136 mutant. On the contrary the removal of the negative Glu-133 and its substitution with a neutral group like Gln produce an enzyme with a much more efficient electron-transfer process: the rate is increased by a factor of about $10^2$. The mutated enzyme is more active toward the natural substrate $O_2^-$ than the native superoxide dismutase and also has higher affinity for azide (Table I).

The electron-transfer rate constant of $Fe(CN)_6^{4-}$ is deeply affected when the modification involves charged residues, especially when they play relevant roles in tuning the electrostatic field in the active channel, like Glu-133. This indicates that the electron-transfer reaction is strongly controlled by electrostatic effects. This is not unexpected for an anion with four negative charges.

Most important, the above data point out that these electrostatic effects influence in the same sense superoxide dismutase activity of the enzyme and azide binding. Hence most probably $Fe(CN)_6^{4-}$ approaches the active cavity. Docking the $Fe(CN)_6^{4-}$ ion at the cavity (Figure 2) results in the bulk anion being able to enter the cavity only partially, with a distance from the terminal N atom to copper of about 4 Å. This distance is short enough to allow the electron jump.[33] This hypothesis has been confirmed by $^{13}C$ NMR data. $Cu_2Zn_2SOD$ and the diamagnetic species $Zn_2Zn_2$-SOD have been titrated with $^{13}C$-enriched $Co(CN)_6^{3-}$, a structural analog of $Fe(CN)_6^{4-}$. The eight $^{13}C$ NMR lines of the ligand interacting with the copper enzyme show a significant broadening as compared to the zinc enzyme control. Figure 3 shows the dependence of the transverse relaxation rate enhancement ($T_{2p}^{-1}$) on ligand concentration. This behavior is typical of a ligand exchanging rapidly between "free" and "bound" states. This is also confirmed by the temperature-independence of the line width. Magnetic field inhomogeneity does not contribute to the present line widths, which range between 20 and 70 Hz. These checks make the measurements of the affinity constant through line width measurements quite reliable. From the best fit of the data to eq 3 an affinity constant of 444 ± 23 $M^{-1}$ has been estimated. Such a binding of $Co(CN)_6^{3-}$ to Cu,ZnSOD does not affect the electronic spectra of the enzyme. Furthermore, a control

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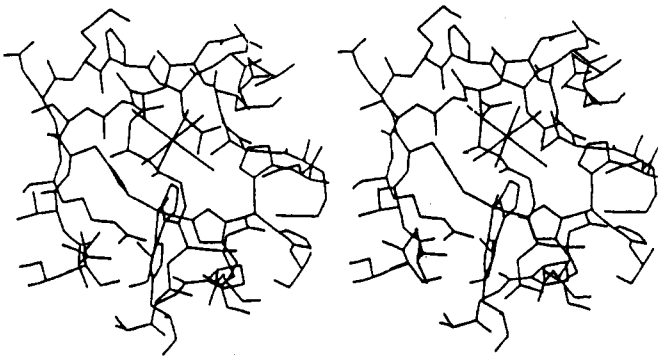

**Figure 2.** Stereoview of the approach of $Fe(CN)_6^{4-}$ to the active cavity.

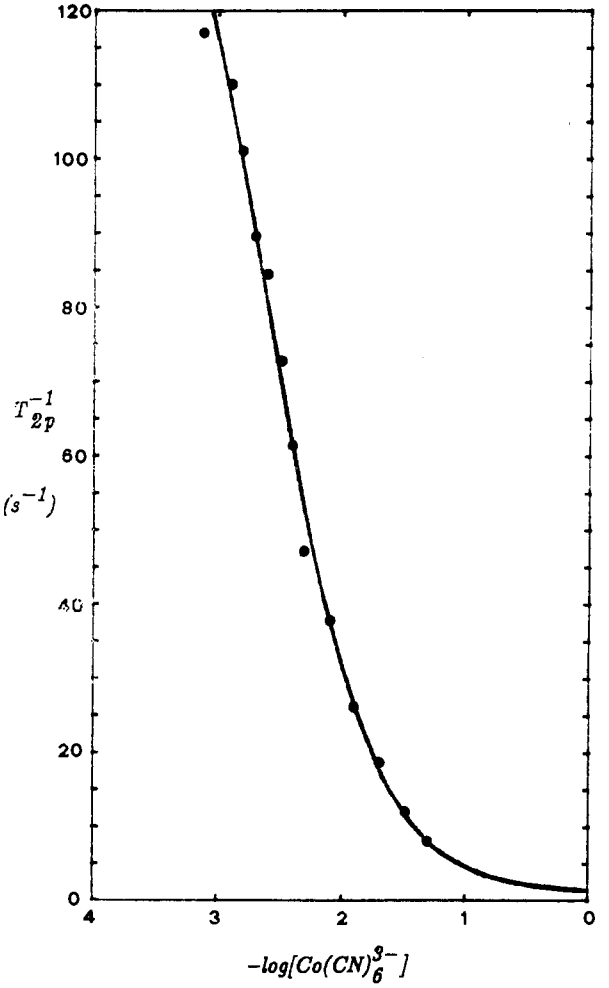


**Figure 3.** $T_{2p}^{-1}$ values of the $^{13}C$ nucleus of the $Co(CN)_6^{3-}$ group in the presence of $7 \times 10^{-5}$ M Cu,Zn human wild type SOD, as a function of hexacyanocobaltate concentration. The values have been obtained at 400 MHz and 298 K. The protein is in 0.02 M phosphate solution, at pH 6.0. The error on $T_{2p}^{-1}$ is ±3 $s^{-1}$.

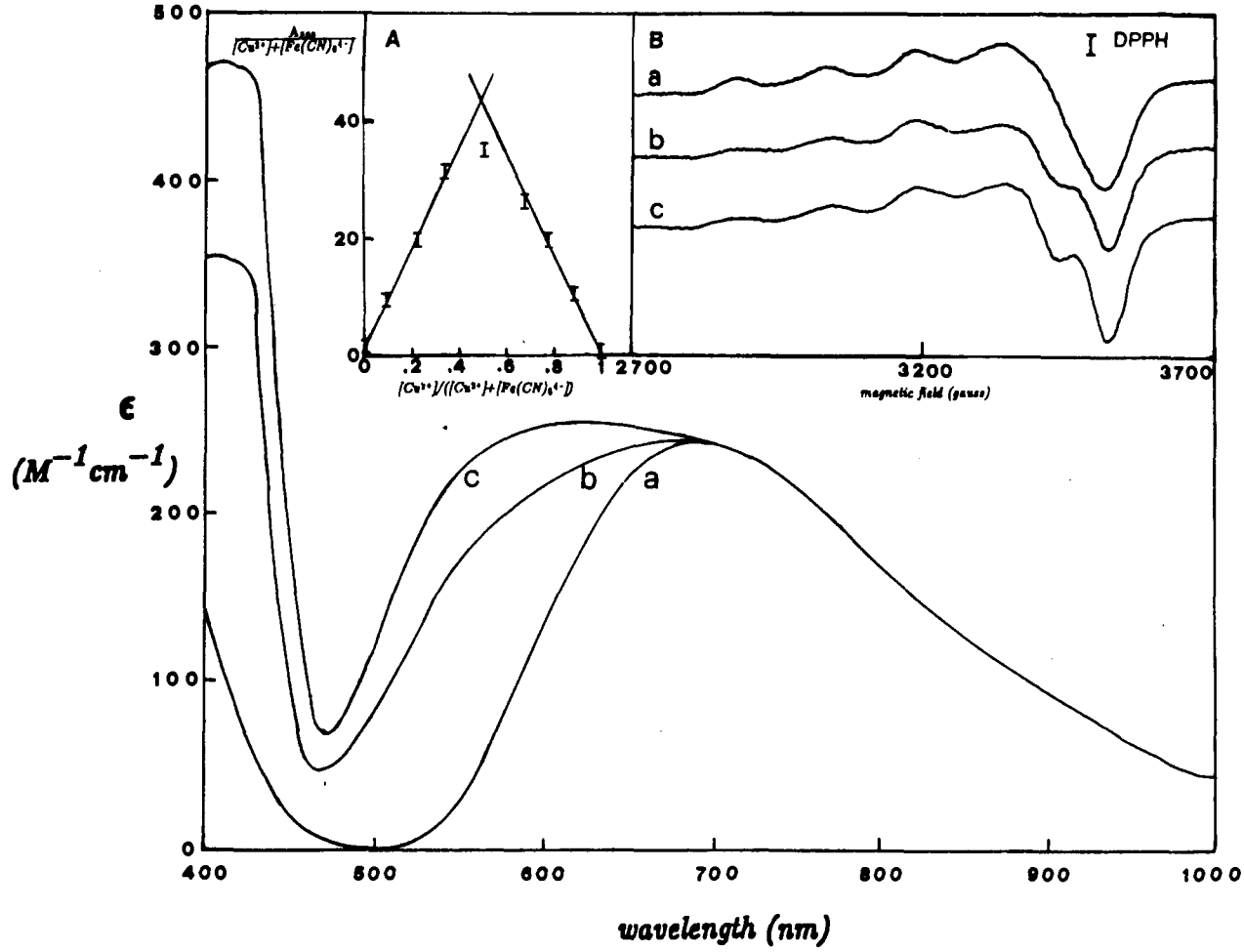


**Figure 4.** Optical titration of $Cu_2E_2$ wild type SOD with $Fe(CN)_6^{4-}$. The ratios $Fe(CN)_6^{4-}/Cu^{2+}$ are the following: (a) 0; (b) 1.0; (c) 2.0. The band at 420 nm is due to a small amount of $Fe(CN)_6^{3-}$ (10% of the initial $Fe(CN)_6^{4-}$) formed probably as effect of reduction of some impurities ($Cu_2Zn_2$SOD or other reducible species). Molar absorbance is expressed for mole of protein. Inset A: Job's plot of the optical titration. Inset B: EPR titration of $Cu_2E_2$ wild type SOD with $Fe(CN)_6^{4-}$. The ratios $Fe(CN)_6^{4-}/Cu^{2+}$ are the following: (a) 0; (b) 1.0; (c) 2.0. The protein, in millimolar concentration, is in 0.05 M acetate buffer at pH 6.0.

at the end of the NMR titration shows no evidence of the cyanide–SOD derivative. A band typical of the cyanide–SOD adduct, which falls at 550 nm, begins to appear for a ligand concentration higher than 40 mM in the presence of 10-fold more concentrated enzyme, most probably as a result of the hydrolysis of the hexacyanocobaltate complex.

From the affinity constant and $T_{1p}^{-1}$ measurements a longitudinal relaxation rate for the copper-bound carbon nucleus of the cyanide group of $Co(CN)_6^{3-}$ ($T_{1M}^{-1}$) of 224 ± 3 $s^{-1}$ was obtained. The Solomon equation relates such value with the metal–nucleus distance:[34]

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left[\frac{\tau_c}{1+(\omega_I-\omega_s)^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_I+\omega_s)^2\tau_c^2}\right]$$

Using a correlation time of $2.2 \times 10^{-9}$ s, as obtained through water proton NMRD (nuclear magnetic relaxation dispersion) measurements,[35] a Cu(II)–$^{13}$C distance of 5.0 Å is obtained. Since the C–N bond length is 1.15 Å,[36] a Cu–N distance of about 3.9 Å is obtained. This value indicates that the $Fe(CN)_6^{4-}$ ion enters the cavity, approaches the copper ion and a direct electron transfer between the terminal nitrogen and copper occurs, through a small jump. This picture is consistent with the conclusions of a redox study on the enzyme which points out the low electron-transfer rate.[37]

As we can observe from Table I the mutations have more dramatic effects on the electron transfer rate constant of $Fe(CN)_6^{4-}$ than on the affinity for $N_3^-$ and on the activity. The superoxide dismutation process is controlled by the rate of access to the catalytic center which on its turn is controlled by the electrostatic charges.[15,16,38] The affinity for $N_3^-$ is also controlled by the charges,[16,39] even if not exactly in the same way. The reduction rate of copper by $Fe(CN)_6^{4-}$ depends much more on the electric charges in the active cavity. This can be accounted for by considering that the observed rate constant, $k_{obs}$, is function of the formation rate of the enzyme-reductant intermediate and of the electron-transfer rate. The latter depends on several factors including $\Delta G°$ of the electron transfer process and nitrogen to copper distance.[40] $\Delta G°$ cannot account for the differences observed with the mutants because, through redox potential measurements,[41] we have shown that $\Delta G°$ is not related to the charges in the catalytic cavity. However the charges might be

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relevant in the senses that they can affect the actual Cu–N distance, as well as the thermodynamic stability of the precursor.

$Fe(CN)_6^{4-}$ is unable to reduce Zn-deprived SOD. Addition of $Fe(CN)_6^{4-}$ affects the d–d transition of the oxidized copper(II) ion in the electronic spectra of the $Cu_2E_2$ wild type (E = empty) (Figure 4); indeed, the addition of $Fe(CN)_6^{4-}$ produces a further absorption maximum at 17 800 $cm^{-1}$). The Job's plot[42] obtained by measuring the intensity at 17 800 $cm^{-1}$ (560 nm) (inset A of Figure 4) shows that the stoichiometry of interaction between copper(II) and $Fe(CN)_6^{4-}$ is 1:1. We have then investigated the $Cu_2E_2$Asn-124 and we have obtained the same results. The EPR spectra are significantly affected by the addition of $Fe(CN)_6^{4-}$: $g_\parallel$ is significantly smaller than that in the absence of $Fe(CN)_6^{4-}$ (2.16 versus 2.26) as is the $A_\parallel$ value ($143 \times 10^{-4}$ versus $154 \times 10^{-4}$ $cm^{-1}$) (Figure 4, inset B). This indicates that no reduction of copper(II) occurs, although hexacyanoferrate(II) affects the electronic levels of copper(II). Direct interaction between $Fe(CN)_6^{4-}$ and bovine $Cu_2Zn_2$SOD was previously observed at low pH (3.0), when the His bridge is presumably broken.[43] We have independently shown that the redox potential of $Cu_2E_2$SOD is lower than $Cu_2Zn_2$SOD (0.28 V instead of 0.45 V) at the pH value of the present experiments.[41] The reduction of copper by $Fe(CN)_6^{4-}$ is therefore disfavored by zinc depletion. Moreover, $Fe(CN)_6^{4-}$ is a stronger ligand than $Fe(CN)_6^{3-}$, and copper(II) is a better acceptor than copper(I), so it is tempting to suggest that the establishment of the coordination bond between copper(II) and $Fe(CN)_6^{4-}$ through the cyanide group further stabilizes copper as copper(II) ion. The possibility of direct binding of the anion to copper(II) in $Cu_2E_2$SOD could be justified by the possibility of copper(II) to move. In the presence of zinc, the bridging histidinato immobilizes copper and anchors it to the depth of the protein cavity. $Fe(CN)_6^{4-}$ could interact with copper(II) in an axial position, possibly semicoordinated. We have checked the possibility that free $CN^-$, coming from $Fe(CN)_6^{4-}$, binds $Cu_2E_2$SOD. The electronic spectrum of the $CN^-$ derivative shows a maximum at 18 200 $cm^{-1}$ (550 nm) and practically no absorption at 14 700 $cm^{-1}$ (680 nm). Even the EPR spectra are quite different from those shown in the inset of Figure 4. In particular, the spectral features are consistent with the presence of two species: the major species shows a $g_\parallel$ value of 2.20 and a $A_\parallel$ value of $182 \times 10^{-4}$ $cm^{-1}$. The minor one can be tentatively attributed to the $Cu_2Cu_2$SOD derivative formed by copper migration into the empty site. However, the characterization of the $CN^-$ derivative of $Cu_2E_2$SOD is beyond the scope of the present work.

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## Concluding Remarks

We have shown in this research that electron transfer between $Fe(CN)_6^{4-}$ and $Cu_2Zn_2$SOD occurs with a rate which depends on the presence of positive charge in the active cavity. Since no direct complex-to-copper bond is established, although the two are close enough to each other, it is reasonable to propose an electron jump of about 4 Å from nitrogen to copper. The range of the electron-transfer rate is consistent with this jump.[33] The bulkiness of the ligand does not permit a direct nitrogen–copper bond. The rate of the approach of the anion toward the depth of cavity is important; indeed, the rate has been found to be dramatically dependent on the charge of the residues inside the active cavity. Charged residues may also tune the copper–nitrogen distance. Residues that favor the superoxide dismutation rate also favor the electron-transfer rate. The reverse is also true. The activity of $O_2^-$ dismutation measured under nonsaturating conditions is probably determined by $k_{on}$, the rate constant for the approach of $O_2^-$ to the protein's active site.[4] The presence of charged residues in the cavity also affects the thermodynamics of the azide binding to copper. A comparison of all of these data provides a clear picture of the function of the enzyme.

In the zinc-depleted enzyme no redox reaction occurs whereas the electronic levels of copper(II) are perturbed. The possibility of direct binding between $Fe(CN)_6^{4-}$ and copper(II) is discussed.

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