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The Binding of Copper Ions to Copper-Free Bovine Superoxide Dismutase

COPPER DISTRIBUTION IN PROTEIN SAMPLES RECOMBINED WITH LESS THAN STOICHEIOMETRIC COPPER ION/PROTEIN RATIOS

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Samples of superoxide dismutase containing less than stoicheiometric amounts of Cu^{2+} were obtained by either partial re-addition of Cu^{2+} to the Cu^{2+}-free protein or partial removal of Cu^{2+} by controlled CN^- treatment. In these samples the distribution of the metal between the two identical sites on the two subunits was studied by quantitative gel electrophoresis and found to be statistical only in the process of copper removal by CN^-. In the other case the distribution fits a model of co-operative interaction between the two sites, where the sites are equivalent for the binding of the first Cu^{2+} ion, but the occupation of the first site lowers the activation energy of the binding of the second Cu^{2+} ion. This indicates that binding of Cu^{2+} ion at its site on one subunit brings about conformational changes that facilitate Cu^{2+} binding on the other subunit. These results may relate to possible intersubunit interactions during the catalytic activity.

Bovine superoxide dismutase (EC 1.15.1.1) is a dimeric enzyme, made of identical subunits, each containing a Cu^{2+} and a Zn^{2+} ion (Fridovich, 1974). Studies on the catalytic mechanism of the enzyme have shown that 50% of the protein-bound Cu^{2+} is involved in catalysis (Fielden *et al.*, 1974). On the other hand, spectroscopic measurements were not able to detect any difference between the two Cu^{2+} sites (Fielden *et al.*, 1974). Therefore the understanding of the catalytic mechanism of this enzyme requires a re-evaluation of the equivalence of the two Cu^{2+} sites. In particular, possible differences in the behaviour of the Cu^{2+} sites of superoxide dismutase might be revealed by studying samples of the enzyme with different [Cu^{2+}]/[protein] ratios in terms of enzymic activity, and spectroscopic properties as a function of Cu^{2+} distribution.

The distribution of Cu^{2+}, i.e. the fraction of enzyme molecules containing zero, one or two Cu^{2+} ions, could depend on the method and experimental conditions used to prepare samples with a given [Cu^{2+}]/[protein] ratio. In particular two different situations will be considered: (*a*) Cu^{2+} is added to the Cu^{2+}-free enzyme; (*b*) Cu^{2+} is removed from the holoprotein by treatment with CN^-.

In the present paper we report on the Cu^{2+} distribution in samples of bovine superoxide dismutase containing different amounts of Cu^{2+} up to the stoicheiometric content of two Cu^{2+} ions per enzyme molecule, prepared by either of the above procedures. The aim of this work was to ascertain whether the Cu^{2+} distribution between the two sites is statistical or not.

Theory

Distribution of Cu^{2+} between the sites after addition of Cu^{2+} to the Cu^{2+}-free protein

Since the enzyme contains two Cu^{2+} sites per molecule the binding of the first Cu^{2+} ion can or cannot affect the binding of the second one. In other words the two sites are or are not interacting in the recombination process.

Theory (I): two sites interact in the binding process. In this case we consider the possibility that the binding of the first Cu^{2+} ion to the Cu^{2+}-free enzyme causes a rearrangement of the protein tertiary structure, so that the energy involved in the binding of the second ion is different from that involved in the co-ordination of the first one.

The working hypotheses that allows one to calculate the distribution of Cu^{2+} among enzyme molecules with different [Cu^{2+}]/[protein] ratios are: (*a*) the two sites of the P_0 molecules are equivalent in the binding of the first Cu^{2+} ion; (*b*) P_0 and P_1 bind Cu^{2+} with different rates, v_0 and v_1 respectively; (*c*) after the Cu^{2+} sites have attained the native conformation the process $P_0+P_2 \rightleftharpoons 2P_1$ does not occur. P_0, P_1, P_2 indicate enzyme molecules with zero, one and two Cu^{2+} ions per molecule.

On this basis the probability p_0 that a Cu^{2+} ion will bind to a P_0 molecule is given by

$$p_0 = \frac{v_0}{v_0 + v_1} = \frac{2N_0k_0}{2N_0k_0 + N_1k_1} = \frac{2N_0}{2N_0 + N_1\frac{k_1}{k_0}} \quad (1)$$

where N_0 and N_1 are the fractions of P_0 and P_1 molecules respectively, and k_0 and k_1 are the specific rate constants of the binding of a Cu^{2+} ion with P_0 and P_1. More precisely, in this case, k_0 and k_1 refer to the irreversible binding of Cu^{2+} with a given molecule.

It can safely be assumed that the difference in the rate constants k_0 and k_1 is mainly due to different values of activation energy E_0 and E_1 for the binding process of Cu^{2+} with P_0 and P_1 respectively. As a consequence

$$\frac{k_1}{k_0} = e^{-(E_1 - E_0)/kT}$$

where k is the Boltzman constant and T is the temperature (K), and the probability p_0 can be rewritten as

$$p_0 = \frac{2N_0}{2N_0 + N_1 e^{-(E_1 - E_0)/kT}}$$

If $[Cu^{2+}]/[\text{protein}] = N_c = N_1 + 2N_2$, where N_2 is the fraction of P_2 molecules, the addition of a small quantity, dN_c, of Cu^{2+} to an enzyme partially recombined with Cu^{2+} will decrease the fraction of P_0 molecules by a quantity dN_0 given by

$$-dN_0 = p_0\,dN_c \quad (2)$$

Integration of this equation by using an integrating factor gives

$$N_c = \frac{2-4p_0}{1-3p_0}[N_0 - N_0^{(1-p_0)/2p_0}] + 2[1 - N_0^{(1-p_0)/2p_0}] \quad (3)$$

which relates N_c to N_0. As $N_0 + N_1 + N_2 = 1$, N_1 and N_2 can be calculated to be

$$N_1 = 2 - 2N_0 - N_c \quad (4)$$

$$N_2 = \frac{N_c - N_1}{2} \quad (5)$$

The distribution curves, calculated from eqns. (3)–(5), for different values of $E_1 - E_0$ are very sensitive to small differences in activation energies. In particular, varying $E_0 - E_1$ from $2kT$ (4.2 kJ/mol) to $-2kT$, the maximum of the N_1 distribution curves increases from 0.15 to 0.8 and its position moves from $N_c \approx 0.5$ to $N_c \approx 1.1$. Therefore N_1 appears to be the most sensitive probe of the difference in activation energies for the binding process of the two Cu^{2+} ions.

Theory (II): two sites are different and do not interact in the binding process. In this case each enzyme molecule contains two different Cu^{2+} sites, namely (a) and (b), which do not interact in the binding process. Therefore the probability p_a that a Cu^{2+} ion will enter site (a) is

$$p_a = \frac{k_a(1-N_a)}{k_a(1-N_a) + k_b(1-N_b)} \quad (6)$$

where N_a and N_b are the fraction of sites (a) and (b) occupied by Cu^{2+}, and k_a and k_b are the specific rate constants of Cu^{2+} binding with the sites (a) and (b).

The addition of a small quantity, dN_c, of Cu^{2+} will increase the fraction of site (a) occupied by Cu^{2+} by a quantity

$$dN_a = p_a\,dN_c \quad (7)$$

Assuming

$$\frac{k_b}{k_a} = e^{-(E_b - E_a)/kT}$$

substituting for p_a in eqn. (7)

$$dN_a = \frac{1-N_a}{(1-N_a) + (1-N_b)e^{-(E_b - E_a)/kT}} \cdot dN_c \quad (8)$$

Solution of this differential equation in the variables N_a and N_b (with $N_b = N_c - N_a$) gives

$$N_c = 1 + N_a - \frac{1-N_a}{(1-N_a)^{1/[1+e(E_a - E_b)/kT]}} \quad (9)$$

from which the fraction N_a and N_b can be calculated for different values of N_c. Moreover, because the two sites are not interacting in the binding process the following hold:

$$N_2 = N_a N_b$$

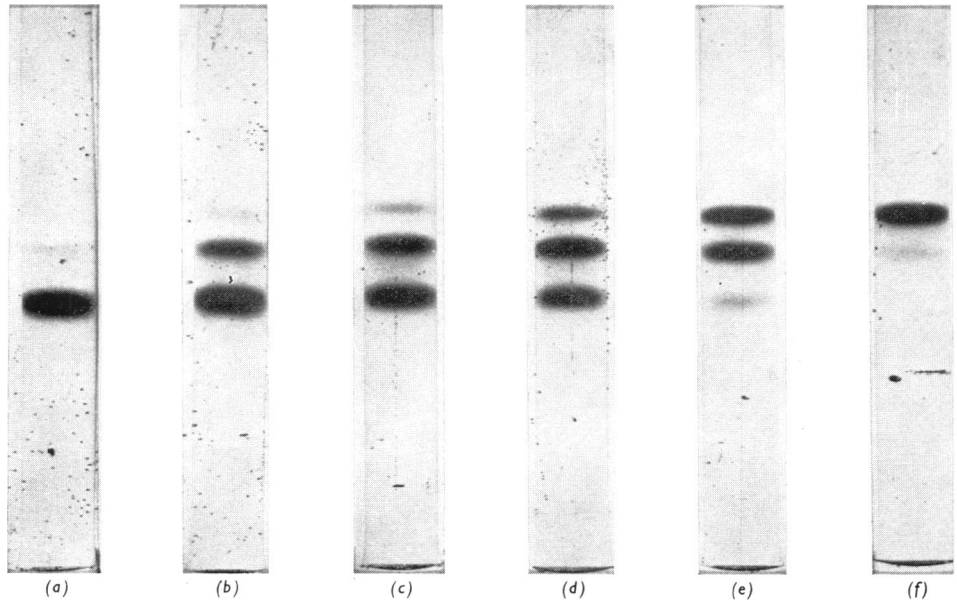
$$N_1 = N_c - 2N_2$$

$$N_0 = 1 - (N_1 + N_2)$$

In this case the value of the N_1 maximum is ≥ 0.5 regardless of the $E_b - E_a$ value. Further, the distribution curves for $E_b - E_a = 0$ are identical with those for $E_1 - E_0 = 0$ as described in Theory (I).

Distribution of Cu^{2+} between the sites after partial removal of Cu^{2+} from the holoprotein

The models and the hypotheses we have considered for the recombination of Cu^{2+} to the Cu^{2+}-free protein, are also valid for the removal of Cu^{2+} from the holoprotein. Since in this case we start from the holoprotein, the distribution curves of N_0, N_1 and N_2 as a function of the $[Cu^{2+}]/[\text{protein}]$ ratio, N_c, in the Cu^{2+} recombination process correspond to the distribution curves of N_2, N_1 and N_0 respectively as a function of the $[Cu^{2+}]/[\text{protein}]$ ratio, N_c', where $N_c' = 2 - N_c$, in the Cu^{2+}-removal process.



EXPLANATION OF PLATE 1

Polyacrylamide-disc-gel electrophoresis of samples with different [Cu^{2+}]/[*protein*] *ratios*

[Cu^{2+}]/[protein] ratios: (*a*) 0.24; (*b*) 0.70; (*c*) 1.15; (*d*) 1.35; (*e*) 1.53; (*f*) 1.84. The recombination was performed in sodium borate, pH 8.0 ($I = 0.02$ mol/litre); 20 μg of protein was applied to each gel.

Experimental

Bovine superoxide dismutase was prepared by the method of McCord & Fridovich (1969). Protein samples with different [Cu^{2+}]/[protein] ratios were prepared in two different ways.

(*a*) Known amounts of Cu^{2+} were added to solutions of 10mM-Cu^{2+}-free enzyme buffered with sodium borate, pH >7. The recombination mixtures were then incubated at 25°C for 24h to attain the equilibrium distribution. The Zn^{2+}-containing Cu^{2+}-free enzyme was obtained by the method of Rotilio *et al.* (1972*a*) with the following modification: the dialysis against 0.05M-KCN after ferrocyanide reduction was performed in 0.1M-potassium phosphate buffer, pH6.0, for 8–10h. With this modification, the protein reproducibly retains all its Zn^{2+} content and loses more than 95% of its Cu^{2+}. Samples with different [Cu^{2+}]/[protein] ratios were obtained by multiple additions of Cu^{2+}, so as to approximate as much as possible to the assumption that Cu^{2+} is added in infinitesimally small quantities, dN_c. This could be particularly important if the binding process of Cu^{2+} to the enzyme is very fast.

(*b*) Cu^{2+} ions are removed from the holoprotein by treatment with CN^- as described in method (*a*) for different times ranging from 1 to 8h.

The concentration of the Cu^{2+}-free protein was determined by a biuret method (Goa, 1953) standardized against a solution of native superoxide dismutase, whose concentration was known from its A_{680} (McCord & Fridovich, 1969), and its Cu^{2+} content as measured by atomic-absorption spectroscopy (Hilger and Watts Atomspek, model H1170).

Polyacrylamide-disc-gel electrophoresis of samples with different [Cu^{2+}]/[protein] ratios was performed by the method of Davis (1964) with some modifications. The gels contained 10% (w/v) polyacrylamide, 2.5% (w/v) *NN'*-methylenebisacrylamide, 0.06% *NNN'N'*-tetramethylethylenediamine and 0.07% ammonium persulphate in 0.05M-Tris/0.38M-glycine buffer, pH8.4. The same buffer was used for the electrode compartments. Electrophoresis of the samples was started after a 2h pre-run to remove excess of ammonium persulphate. The gels were stained with Amido Black. Densitometric analysis of the electrophoretograms was made with a CGA Cellomatic densitometer equipped with a CGA AT3 recorder.

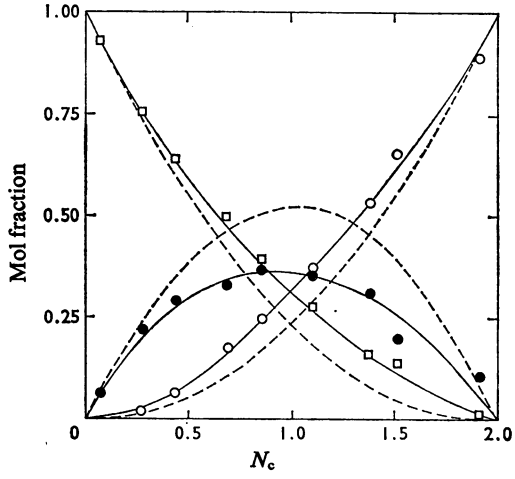


Fig. 1. *N_0, N_1 and N_2 values obtained from a recombination experiment in sodium borate, pH*8.0 (*$I = 0.02$ mol/litre*)
The protein concentration was 0.3mM. ——, Theoretical plots for two interacting sites with $E_1 - E_0 = -1.0kT$; ----, theoretical plots for two non-interacting sites with the same ΔE. □, N_0; ●, N_1; ○, N_2.

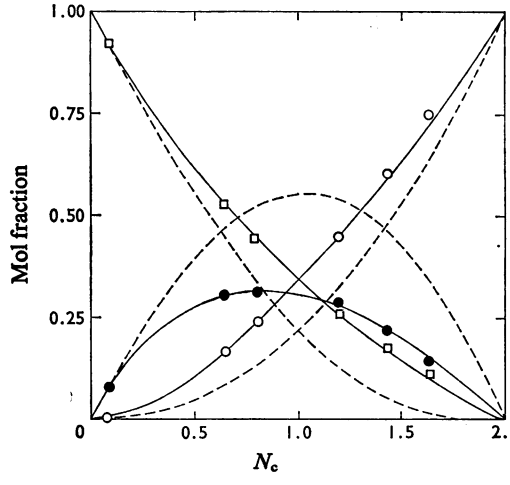


Fig. 2. *N_0, N_1 and N_2 values obtained from a recombination experiment in sodium borate, pH*9.72
Details were as for Fig. 1, except that $E_1 - E_0 = -0.7kT$.

Results and Discussion

Plate 1 shows the results of a polyacrylamide-disc-gel electrophoresis of protein samples with different [Cu^{2+}]/[protein] ratios. The majority of gels display three bands. From comparison with the electrophoretic mobility of the native and Cu^{2+}-free proteins, these bands can confidently be assigned to the enzyme molecules with zero, one and two Cu^{2+} ions per molecule. Further, the electrophoretogram of an incubated mixture of native and Cu^{2+}-free proteins does not show the band with intermediate mobility. These facts support the assumption of irreversible binding used to calculate the Cu^{2+}

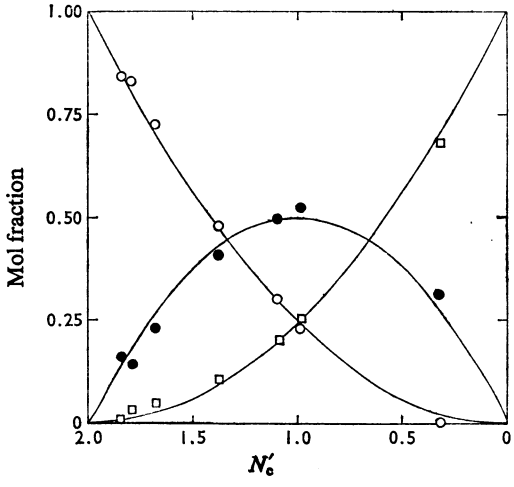


Fig. 3. *Experimental values of N_0, N_1 and N_2 obtained after gradual Cu^{2+} removal from holoprotein by controlled CN^- treatment*

——, Theoretical plots for a statistical model of Cu^{2+} removal ($E_0-E_1=0$). □, N_0; ●, N_1; ○, N_2.

distribution curves. The experimental values of N_0, N_1 and N_2 calculated from densitometric analysis of the electrophoretograms of protein samples recombined with Cu^{2+} at pH 8.0 and 9.7 are shown in Figs. 1 and 2. The good agreement between these data and the theoretical plots of Theory (I) indicates that the two sites interact co-operatively in the process of Cu^{2+} binding to the Cu^{2+}-free protein. In particular it appears that the two sites are equivalent in the binding of the first Cu^{2+} ion and that this equivalence is abolished after the first site is occupied. In fact according to theoretical plots the activation energy of the binding of the second Cu^{2+} ion is approx. 2.5–1.7 kJ/mol lower than that of the first one. The difference appears to decrease slightly at higher pH. This change in activation energy should reflect a modification in the enzyme structure brought about by the binding of the first Cu^{2+} ion at its site, which facilitates the binding of the second Cu^{2+} ion. On the contrary the experimental values of N_0, N_1 and N_2 for Cu^{2+} removal by CN^- from the holoprotein, which are shown in Fig. 3, fit a statistical model of Cu^{2+} removal, i.e. theory (I) or (II) with $\Delta E=0$. This indicates no kinetic difference in the process of removal of a Cu^{2+} ion between enzyme molecules containing two or one Cu^{2+} ions. This behaviour, as compared with the co-operative process of binding, could be due to the binding of a strong ligand such as CN^-, which would decrease the energy difference of P_1 and P_2 molecules. The statistical binding of CN^- to bovine superoxide dismutase has already been described (Rotilio *et al.*, 1972*b*).

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