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

PROPERTIES OF THE PROTEIN RECOMBINED WITH INCREASING AMOUNTS OF COPPER IONS

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(Received 3 June 1976)

1. E.p.r. (electron-paramagnetic-resonance), proton-relaxation and u.v.-absorption parameters, and enzyme activity of samples of Cu^{2+} -free bovine superoxide dismutase recombined with different amounts of Cu^{2+} up to the stoichiometric $[\text{Cu}^{2+}]/[\text{protein}]$ ratio were investigated after attainment of equilibrium in the recovery process. 2. The e.p.r. spectra were identical with the spectrum of the native protein at all $[\text{Cu}^{2+}]/[\text{protein}]$ ratios. The relaxation rate of the water protons (T_1) and the u.v. absorption increase as linear functions of the added Cu^{2+} . 3. On the other hand, in recombination experiments in the range pH 7.6–10.5 the enzyme activity shows a non-linear increase as the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio rises. The experimental curves can be interpreted in terms of the model of co-operative binding of Cu^{2+} to the two sites proposed on the basis of the electrophoretic analyses of the samples, and show that the specific activity of the molecules containing only one Cu^{2+} ion is twice as high as that of the molecules with two Cu^{2+} ions. 4. These results support the hypothesis of an anti-co-operative interaction between the two sites during the activity, which allows only one Cu^{2+} ion to function in catalysis.

In the preceding paper (Rigo *et al.*, 1977) we have shown that re-addition of Cu^{2+} to Cu^{2+} -free bovine superoxide dismutase results in a non-statistical distribution of Cu^{2+} between the two Cu^{2+} sites. In particular, the process of Cu^{2+} binding appears to be co-operative, as the binding of the second Cu^{2+} ion is favoured with respect to the binding of the first one. In the present paper we report on various properties displayed at equilibrium by samples recombined with different amounts of Cu^{2+} . These data show further aspects of the interdependence between the active sites of the enzyme, which could relate to the half-sites mechanism of action of the enzyme (Fielden *et al.*, 1974). In particular we show that the recovery of the spectral properties is a linear function of the added Cu^{2+} , whereas the enzyme activity is restored in a non-linear fashion, which can be interpreted as the Cu^{2+} ion bound first being more active than that bound second.

Materials and Methods

The procedures for the preparation of superoxide dismutase, Cu^{2+} -free protein, and protein samples

with different Cu^{2+} content for the determination of protein concentration and metal content and for gel electrophoresis, are as described in the preceding paper (Rigo *et al.*, 1977). Enzyme activity was determined polarographically, as previously reported (Rigo *et al.*, 1975).

Optical absorption measurements were done with a model 635 Varian spectrophotometer; X-band e.p.r. (electron-paramagnetic-resonance) spectra were recorded on a Varian V-4502 spectrometer. The spin-lattice relaxation time, T_1 , of the water protons was measured at 25°C by the 90°– τ –90° pulse sequence on a Polaron pulsed n.m.r. (nuclear-magnetic-resonance) apparatus working at 16.0 MHz. pH measurements were made with a model 331 Amel pH-meter.

Results

T_1 measurements

In Fig. 1 the normalized value of

$$\frac{1}{T_{1P}} = \frac{1}{T_1} - \frac{1}{T_{1,0}}$$

Fig. 1. Normalized values of the paramagnetic contribution to the nuclear magnetic relaxation of water protons (○) and of the activity (△) of bovine superoxide dismutase as a function of the Cu^{2+} content of protein samples recombined with Cu^{2+} at pH 8.0 in borate buffer

The normalized values are the ratios between the measured values and that of the native holoprotein. The ionic strength of the buffer was 0.02 mol/litre and the temperature was 25°C.

is plotted as the function of the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio, where $1/T_{1p}$ is the paramagnetic contribution to the proton relaxation rates, and $T_{1,0}$ and T_1 are respectively the measured proton spin-lattice relaxation times of the Cu^{2+} -free enzyme solution before and after the Cu^{2+} addition. A linear increase of $1/T_{1p}$ in the $[\text{Cu}^{2+}]/[\text{protein}]$ range 0–2.1 is observed, the final point ranging between 1.8 and 2.1 depending on different protein batches. At values of $[\text{Cu}^{2+}]/[\text{protein}] > 2$ the plot is still linear, but the slope is negligible. This behaviour is independent of pH in the range 3.5–10.5.

U.v. absorption

As previously reported (Rotilio *et al.*, 1972; Fee, 1973) the u.v. absorption of Cu^{2+} -free superoxide dismutase is much less than that of the holoprotein. Addition of Cu^{2+} to the Cu^{2+} -free enzyme results in a gradual increase of the absorbance in the region 250–360 nm. The plot of A_{258} versus $[\text{Cu}^{2+}]/[\text{protein}]$ ratio is shown in Fig. 2 and is linear in the range 0–1.8. An abrupt change of the slope occurs around $[\text{Cu}^{2+}]/[\text{protein}] = 2$ and is likely to reflect spurious binding outside the active sites.

Activity measurements

Measurements of the activity constant were made on samples recombined at various pH values. In recombination experiments at pH 3.5 and 5.2 a linear increase of k with the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio, was observed. On the other hand in the pH range 7.6–10.5 the recombination experiments displayed a non-linear increase in the k versus $[\text{Cu}^{2+}]/[\text{protein}]$ plot. The data of an experiment at pH 8.0 are shown in Fig. 1. Identical plots were obtained either by single

or multiple Cu^{2+} additions. These results indicate that the specific activity constants of the Cu^{2+} sites occupied first are higher than those occupied second. On the other hand, the e.p.r. spectra were identical with those of the native protein for all the $[\text{Cu}^{2+}]/[\text{protein}]$ ratios in the range 0.2–1.8 at any pH between 3 and 10. The reconstituted enzyme samples containing stoichiometric amounts of Cu^{2+} always showed 90–100% of the activity of the native protein, indicating full recovery of enzyme activity by addition of Cu^{2+} to the Cu^{2+} -free enzyme. Increasing the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio over 2 gives a much smaller linear increase of the activity constant, which in the range $[\text{Cu}^{2+}]/[\text{protein}] = 2$ –4 corresponds to 5–10% of the activity of the native enzyme. The same result was obtained by adding Cu^{2+} to the native holoprotein.

Distribution of Cu^{2+} between the two sites and relative enzyme activity of the two Cu^{2+} ions

The molar fractions N_0 , N_1 and N_2 , that is the fractions of molecules which in the recombination process contain zero, one and two Cu^{2+} ions per protein molecule respectively, were determined by gel electrophoresis as previously described (Rigo *et al.*, 1977). The results of experiments performed at two different pH values above pH 7 are shown in Tables 1 and 2, which also include the values of the activity constants, k_n , for each sample. From these data it appears that the maximum value of N_1 is lower than 0.5, which agrees with the proposed model of co-operative Cu^{2+} binding to the Cu^{2+} -free enzyme, and that the first Cu^{2+} is approximately twice as active as the second one. A similar set of results was obtained with samples where less than stoichiometric $[\text{Cu}^{2+}]/[\text{protein}]$ ratios were obtained by Cu^{2+} removal from the holoprotein (Rigo *et al.*, 1977). Also, in this case

Fig. 2. Normalized A_{258} increments as a function of the Cu^{2+} added to Cu^{2+} -free bovine superoxide dismutase, at pH 7.6. The experiments were done in borate buffer, pH 7.6 ($I = 0.02$ mol/litre), and the temperature was 25°C.

Table 1. Mol fractions N_0 , N_1 and N_2 of superoxide dismutase molecules with zero, one and two Cu^{2+} ions respectively, in samples of enzyme recombined with various amounts of Cu^{2+} up to $[\text{Cu}^{2+}]/[\text{protein}] = 2$

The recombination process was performed at 25°C, in borate buffer, pH 8.0 ($I = 0.02$ mol/litre). k values are expressed as the ratios between the measured values and those of the native holoprotein. The mean value of $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ is 2.01.

N_0	N_1	N_2	$N_c = N_1 + 2N_2$	k_n	$k_{\text{Cu}_1}/k_{\text{Cu}_2}$
0.92	0.08	—	0.08	0.09	2.16
0.54	0.31	0.17	0.65	0.44	1.81
0.45	0.31	0.24	0.79	0.53	1.87
0.26	0.29	0.45	1.19	0.73	1.93
0.17	0.22	0.61	1.44	0.82	1.95
0.12	0.11	0.77	1.65	0.90	2.36
0	0.01	0.99	1.99	1.00	—

Table 2. Experiment as for Table 1, but with pH 8.6 buffer
All details were as for Table 1. The mean value of $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ is 1.92.

N_0	N_1	N_2	$N_c = N_1 + 2N_2$	k_n	$k_{\text{Cu}_1}/k_{\text{Cu}_2}$
0.99	0.01	0	0.01	0.02	—
0.93	0.07	0	0.07	0.08	2.36
0.84	0.14	0.01	0.17	0.16	2.13
0.68	0.23	0.09	0.41	0.33	2.08
0.39	0.35	0.26	0.87	0.55	1.66
0.31	0.34	0.35	1.04	0.69	2.01
0.19	0.31	0.50	1.31	0.76	1.69
0.10	0.25	0.65	1.55	0.85	1.50
0.08	0.16	0.76	1.68	0.99	—
0.03	0.01	0.96	1.92	1.00	—

Table 3. Mol fractions of superoxide dismutase with different extents of residual Cu^{2+} after controlled CN^- treatment
The mean value of $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ is 1.61.

Time of CN^- treatment (h)	N_0	N_1	N_2	$N_1 + 2N_2$	$[\text{Cu}^{2+}]/[\text{protein}]$	$10^{-9} \times k \text{ (M}^{-1} \cdot \text{s}^{-1})$	$k_{\text{Cu}_1}/k_{\text{Cu}_2}$
0	0	0.16	0.84	1.84	1.80	2.53	1.61
2	0.03	0.14	0.93	1.80	1.75	—	—
4	0.05	0.23	0.72	1.67	1.69	—	—
6	0.11	0.41	0.48	1.37	1.50	2.87	1.71
8	0.20	0.50	0.30	1.10	1.20	3.28	1.43
14	0.25	0.52	0.23	0.98	0.80	3.28	1.62
24	0.67	0.33	0	0.33	0.30	3.70	1.61

it is evident (see Table 3) that the activity constant, calculated on the basis of the Cu^{2+} content, increases as the Cu^{2+} content decreases. It is also evident that there is fairly good agreement between the value of the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio obtained from protein and Cu^{2+} determinations and the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio $= N_1 + 2N_2$, calculated from densitometric analysis of the gels.

Discussion

The results obtained in this work with samples of superoxide dismutase containing less than stoicheio-

metric $[\text{Cu}^{2+}]/[\text{protein}]$ ratios, although confirming the spectroscopic equivalence of the Cu^{2+} sites (Fielden *et al.*, 1974), show a non-linear correlation between the rate constant, k_1 , and the Cu^{2+} content of the enzyme. Further, electrophoretic data on Cu^{2+} distribution (Rigo *et al.*, 1977) indicate an equivalence both between the free sites of the protein in the process of Cu^{2+} binding to the Cu^{2+} -free protein, and between the native Cu^{2+} sites in the process of Cu^{2+} removal from the holoprotein. Three questions arise. (1) How many types of enzyme molecules with different Cu^{2+} activity are present? (2) How much does each type of molecule contribute to the cata-

lytic process? (3) Are the activity differences an intrinsic characteristic of the Cu^{2+} sites or do they appear when the enzyme is working in catalysis?

As far as question (1) is concerned, only two types of Cu^{2+} -containing molecules, namely those containing one Cu^{2+} ion (P_1) and those containing two Cu^{2+} ions (P_2), should be present in the samples under investigation. In fact it appears quite improbable that two types of P_1 molecules, characterized by different activities, are present, because of the spectroscopic equivalence of the Cu^{2+} ions in the resting enzyme and the equivalence of the Cu^{2+} sites in the Cu^{2+} -free protein and in the holoprotein.

As to question (2), from the non-linear plots of k against $[\text{Cu}^{2+}]/[\text{protein}]$ it necessarily follows that the specific Cu^{2+} activity in P_1 , k_{Cu_1} , differs from that in P_2 molecules, k_{Cu_2} . A way to evaluate the ratio $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ is to compare the experimental plots, normalized k against $[\text{Cu}^{2+}]/[\text{protein}]$, with a series of theoretical plots, $N_1(k_{\text{Cu}_1}/k_{\text{Cu}_2}) + N_2$ against $N_1 + 2N_2$, obtained by giving different tentative values to the ratio $k_{\text{Cu}_1}/k_{\text{Cu}_2}$, and where N_1 and N_2 refer to the different patterns of Cu^{2+} distribution we have considered in the preceding paper (Rigo *et al.*, 1977). Such theoretical plots, together with the non-linear experimental curve of Fig. 1, are shown in Figs. 3 and 4 for different values of the activation energies involved in the processes of Cu^{2+} binding or removal. It appears that the experimental data are close to the curves (b) and (c) of Fig. 3 and to the curve (a) of

Fig. 3. Plots of normalized activity, k , against Cu^{2+} content of bovine superoxide dismutase

—, Theoretical curves calculated assuming that the P_1 molecules have the same activity as the P_2 molecules and the two Cu^{2+} sites interact in the recombination process. $E_1 - E_0$ values are: (a) $-2kT$; (b) $-kT$; (c) 0; (d) kT ; (e) $2kT$. Δ , Values obtained in a recombination experiment performed in borate buffer, pH 7.6 ($I = 0.02 \text{ mol/litre}$).

Fig. 4. Plots of normalized activity, k , against Cu^{2+} content of bovine superoxide dismutase

—, Theoretical curves calculated assuming that the P_1 molecules have the same activity as the P_2 molecules and each enzyme molecule contains two non-interacting sites, (a) and (b), which bind Cu^{2+} with different activation energies. $E_b - E_a$ values are: (a) 0; (b) kT ; (c) $2kT$. Δ , Values obtained in a recombination experiment performed at pH 7.6.

Fig. 5. Plot ($\circ$) of normalized activity, k , against N_c values obtained from electrophoretic data in a recombination experiment performed at pH 7.3

—, Curve (b) of Fig. 3 for comparison.

Fig. 4 if $k_{\text{Cu}_1}/k_{\text{Cu}_2} = 2$. A best fit between experimental and theoretical plots can be obtained if the experimental values of N_1 and N_2 are known from electrophoretic measurements. Fig. 5 shows a plot of the normalized k values against $N_1 + 2N_2$ (Table 2) together with the curve (b) of Fig. 3. The good fit between the experimental and theoretical curves of

Fig. 5 seems to support the hypotheses that $k_{\text{Cu}_1}/k_{\text{Cu}_2} \approx 2$, and, independently, the model of co-operative binding of Cu^{2+} in the reconstitution process proposed on the basis of distribution curves of Cu^{2+} (Rigo *et al.*, 1976). However, the comparison of the theoretical plots of Figs. 3 and 4 with the experimental data, as reported in Figs. 3, 4 and 5, does not determine the $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ value unequivocally. In fact very similar theoretical plots can be obtained assuming different values of $k_{\text{Cu}_1}/k_{\text{Cu}_2}$, different Cu^{2+} binding processes and different activation energies. Another way to evaluate $k_{\text{Cu}_1}/k_{\text{Cu}_2}$, independently of the Cu^{2+} -removal or -binding process, is to calculate its average value in the experiments where N_1 , N_2 and k are known. This gives:

$$\frac{1}{2}N_1 \frac{k_{\text{Cu}_1}}{k_{\text{Cu}_2}} + N_2 = k_n$$

where k_n is the normalized experimental k value. From Tables 1–3 it appears that the mean value of $k_{\text{Cu}_1}/k_{\text{Cu}_2}$ is very near to 2.

As to the question (3), the decrease of specific activity with the increasing filling of the second Cu^{2+} site, in spite of the spectroscopic identity of the two sites, is in favour of the hypothesis that the catalytic act itself brings about a non-equivalence of the two active sites. The detailed mechanism of such an anti-cooperative interaction is not known, but may be related to that observed in the binding of Cu^{2+} to

the Cu^{2+} -free protein (Rigo *et al.*, 1977). This appears to be a co-operative process (Rigo *et al.*, 1977) and should involve some kind of interaction between the two subunits.

In this context, it should be recalled that isolated monomers of a superoxide dismutase from wheat germ, which is very similar to the bovine enzyme, are practically inactive and recover the enzyme activity on reassociation of dimers (Marmocchi *et al.*, 1974).

On the other hand, from the results presented above, a single Cu^{2+} ion on a subunit located inside the intact dimer structure seems to be fully active. It would therefore be interesting to look at the protein containing only one Cu^{2+} ion.

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