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

KINETIC ASPECTS

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The kinetics of reconstitution of bovine superoxide dismutase from Cu^{2+} and the copper-free enzyme have been studied by activity, u.v.-absorption, electron-paramagnetic-resonance and pulsed-nuclear-magnetic-resonance measurements. The process appears to be first-order up to 80% completion in most conditions, and is pH-dependent, with an apparent pK of 6.5. U.v.-absorption and solvent proton relaxation rate measurements show that fast binding of Cu^{2+} occurs, and the initial ligands are likely to be, at least in part, those of the native active site. The recovery of the native activity and spectroscopic properties is a slow process with activation energies of 92 kJ/mol at pH 5.3 and 8.4 kJ/mol at pH 8.1 and can be described as a rearrangement of the site around the bound metal. The rate of this process is lower in partially recombined protein samples, probably because of intersubunit interactions.

In previous papers of this series (Rigo *et al.*, 1977a,b) we reported on the equilibrium properties of samples of superoxide dismutase reconstituted from Cu^{2+} and the copper-free protein. From the distribution of Cu^{2+} among the protein molecules and the specific activity of samples recombined with Cu^{2+} to different extents, it was concluded that the copper-binding sites of different subunits interact with each other. In particular the process of binding appears to be co-operative, that is the binding of Cu^{2+} at one site facilitates the binding at the other site (Rigo *et al.*, 1977a); on the other hand the catalytic involvement of one site seems to prevent the second site from participation in catalysis (Rigo *et al.*, 1977b).

In the present paper we report on the kinetics of the restoration of catalytic and spectral properties of bovine superoxide dismutase after re-addition of Cu^{2+} to the copper-free protein. The results give information on the molecular rearrangement taking place at the active site in the process of copper-binding and add further evidence for subunit interaction in this enzyme.

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Materials and Methods

Most of the experimental procedures used throughout the present work have been described previously (Rigo *et al.*, 1977a,b). The spin-lattice relaxation rate of the water protons is expressed as relaxivity (Terenzi *et al.*, 1974). For kinetic studies of activity restoration after addition of Cu^{2+} to the copper-free protein in conditions of fast recovery, sampling at different reaction times was carried out by addition of portions of the solution of recombining protein to 10 vol. of 0.1 M-Tris adjusted to pH 9.5 with NaOH. In fact, Tris stopped the restoration of activity, probably because of its chelating properties.

Results

Kinetics of the recovery of activity after re-addition of stoichiometric amounts of Cu^{2+} to the copper-free enzyme

Addition of stoichiometric amounts of Cu^{2+} to a solution of copper-free superoxide dismutase fully restored the native enzymic activity (Rigo *et al.*, 1977b). The kinetic features of this process were studied at various values of pH, temperature and $[\text{Cu}^{2+}]/[\text{protein}]$ molar ratio at constant 10.02.

Fig. 1. pH-dependence of the rate constant, k (h^{-1}), of reconstitution of native bovine superoxide dismutase from the copper-free protein

$[\text{Cu}^{2+}]/[\text{protein}]$ ratio was 1.8; temperature, 25°C .

Δ , Rates from A_{258} measurements (slow phase); $\circ$, rates from activity measurements.

The rate of activity reappearance was found to follow first-order kinetics over 80–95% of the time course in the pH range 4.4–10.5. At pH 3.5 the kinetics were first-order only in the final part. The value of the rate constant was a function of pH, with a minimum around pH 5.3 at lower pH values, but it is pH-independent above pH 7.5 (Fig. 1). The activation energies, measured by Arrhenius plot in the range 9 – 37°C , were 92 kJ/mol at pH 5.3 and 8.4 kJ/mol at pH 8.1.

The rate constants of the recovery process were independent of the ratio $[\text{Cu}^{2+}]/[\text{protein}]$ in the range 1.8–9 and of protein concentration between 37 and $0.86\text{ }\mu\text{M}$.

Kinetics of the u.v.-absorption increase on re-addition of stoichiometric amounts of Cu^{2+}

Addition of stoichiometric amounts of Cu^{2+} to the copper-free enzyme gives rise to a considerable increase of absorbance in the near-u.v. region with a maximum at 258 nm (Rotilio *et al.*, 1972). The total absorption change, the extent of which is pH-independent in the range pH 3.3–10.0, was found to occur in at least two steps, an abrupt one lasting less than 1 s , and a much slower recovery up to the final absorption value. The relative intensities of the two absorption changes at various pH values was complementary (Fig. 2), that occurring in the fast step being greater at alkaline pH. The apparent pK of the process was approx. 6.5.

The slow increase of absorption followed first-order kinetics in the range pH 5.5–10.5, with rate con-

stants identical with those for the process of activity recovery (Fig. 3). Below pH 5.5 the time course could be analysed in terms of more than one first-order step, the slowest having the same rate constant as the activity-recovery process at the same pH. The rate constants for the slow phase were pH-independent in the range pH 7.5–10.5 and fell below pH 7.8 in parallel with the rates of the activity recovery (Fig. 1).

Fig. 2. pH-dependence of fast ($\circ$) and slow (Δ) increase of A_{258} after re-addition of Cu^{2+} to copper-free bovine superoxide dismutase

$[\text{Cu}^{2+}]/[\text{protein}]$ ratio was 1.8. $\Delta A_{\text{inst.}}$ and $\Delta A_{\text{tot.}}$ are the fast and the slow A_{258} changes respectively, with $\Delta A_{\text{tot.}}$ normalized to 1. The values on the ordinate refer to the fast change, the slow change being the complement to 1 at each pH value.

Fig. 3. Time course of restoration of activity ($\circ$) and u.v. absorption (Δ) by copper-free bovine superoxide dismutase after re-addition of Cu^{2+}

$[\text{Cu}^{2+}]/[\text{protein}]$ ratio was 1.8 at protein concentrations of $0.86\text{ }\mu\text{M}$ ($\circ$) and $37\text{ }\mu\text{M}$ (Δ). The experiment was done in 0.05 M -sodium acetate buffer, pH 5.75. The symbol A refers both to activity and to absorbance.

E.p.r. spectra

The restoration of the native e.p.r. signal after addition of approximately stoichiometric amounts of Cu^{2+} to the copper-free protein was investigated at various pH values by low-temperature e.p.r. spectroscopy. Below pH 5.5 the earliest spectra showed the presence of a species with e.p.r. spectrum similar to that of the free hydrated Cu^{2+} . This species was not observed at higher pH where more than one protein-bound copper species spectrally different from the Cu^{2+} of the native protein were present at the earliest time. Below pH 5.5 the reconstitution process could be followed by room-temperature (25°C) e.p.r. spectroscopy, and the spectra obtained in these conditions were particularly suitable for kinetic analysis, as the initial and final copper signals overlapped much less than in the low temperature spectra (Fig. 4).

The time course of the reappearance of the e.p.r. signal typical of the native protein, as followed by room-temperature e.p.r. spectroscopy, was found to parallel the recovery of the u.v. absorption at the same pH, with more than one first-order step, the last one having the same rate constant as the activity recovery.

Solvent proton relaxation rate measurements

The spin-lattice relaxation rate T_1^{-1} , of the water protons of a solution of copper-free enzyme increases after addition of stoichiometric amounts of Cu^{2+} (Rigo *et al.*, 1977b). The equilibrium value of relaxi-

vities (0.05M-sodium acetate buffer, pH 5.2) was $1.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the free Cu^{2+} ion and $4.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the native and recombined proteins, but it was much lower ($3 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) when Cu^{2+} was added to either the native protein or the recombined protein above the stoichiometric $[\text{Cu}^{2+}]/[\text{protein}]$ ratio. This change of relaxivity is due to different accessibility of water to the co-ordination sphere of the protein-bound metal and indicates that Cu^{2+} binds to different sites in the copper-free protein and in the protein where the copper-binding sites typical of the native enzyme are fully occupied. Kinetic experiments were done at pH 5.2, where the half-life of the activity recovery is about 2h, to permit precise T_1 measurements, which take at least 1 min. After addition of Cu^{2+} ($[\text{Cu}^{2+}]/[\text{protein}]$ molar ratio = 1.8) the relaxivity values were 4.15×10^3 , 4.25×10^3 , $4.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ after 1, 12 and 27 min respectively and reached the constant value of $4.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, typical of the native protein, after 40 min. From these measurements it appears that the final value of the spin-lattice relaxation rate of the water protons was approached in less than 1 min even under conditions of slow recovery of activity, u.v. absorption and e.p.r. spectrum.

Kinetics of binding of less-than-stoichiometric amounts of Cu^{2+} to the copper-free enzyme

The addition of a stoichiometric amount of Cu^{2+} in more than one step resulted in different values of the first-order rate constants. The kinetic constants relative to the restoration, under these conditions, of the u.v. and activity properties of the native enzyme at different pH and $[\text{Cu}^{2+}]/[\text{protein}]$ values are reported in Table 1. It appears that at both pH values the rate

Fig. 4. E.p.r. spectra of the reconstitution of 0.5 mM copper-free bovine superoxide dismutase on re-addition of 1.8 equiv. of Cu^{2+} in 0.05M-sodium acetate buffer pH 5.4: (a) after 2 min incubation at room temperature; (b) after 12 h incubation at room temperature

The broken line is the spectrum of the free Cu^{2+} salt in the same conditions. E.p.r. conditions were: modulation amplitude, 1 mT; microwave power, 50 mW; microwave frequency, 9.24 GHz; temperature, 25°C. The relative amplification factors were 1 for the broken line and 3.2 for (a) and (b).

Table 1. Rate constants of the reconstitution of bovine superoxide dismutase, as monitored either by activity (pH 5.9) or u.v.-absorption (pH 10.5) measurements, at various $[\text{Cu}^{2+}]/[\text{protein}]$ values

For the $[\text{Cu}^{2+}]/[\text{protein}]$ values each interval indicates the copper content of the protein before the addition and the total copper content obtained after the addition. The reactions were followed up to completion and were strictly first-order for approximately 80% of the time course.

pH	$[\text{Cu}^{2+}]/[\text{protein}]$	k (h^{-1})
5.9	0–0.6	9.2
5.9	0.6–1.2	5.1
5.9	1.2–1.8	3.5
5.9	0–1.8	3.0
10.5	0–0.45	2280
10.5	0.45–0.9	1640
10.5	0.9–1.35	650
10.5	1.35–1.8	640
10.5	0–1.8	380

constant of recombination decreased as the $[\text{Cu}^{2+}]/[\text{protein}]$ ratio approached the stoichiometric value and was always higher than that measured when the stoichiometric amount of Cu^{2+} was added in a single step.

Discussion

The kinetics of recombination of Cu^{2+} to the Cu-free superoxide dismutase can be followed by monitoring the time course of the restoration of different parameters such as enzyme activity, u.v. absorption, e.p.r. and n.m.r. properties of the native copper binding site. Our results show a strong pH-dependence between pH 5 and 8 of the rate of recovery of activity, u.v. absorption and e.p.r. spectrum. This is reflected by a tenfold decrease in the measured activation energy at the higher pH value. This fact indicates that the rearrangement of the protein molecules, to give the specific copper-binding site responsible for the catalytic, optical and e.p.r. properties of the native enzyme, is a relatively slow process depending on the protonation state of some groups on the protein. On the other hand the recovery of the relaxivity value of the native enzyme is relatively fast even at the most unfavourable pH. Apparently the enhancement of the relaxation rate of the solvent protons is not a good probe of the configuration of the copper-binding site in the native enzyme, but still indicates that fast binding of copper by the protein takes place.

This initial type of binding is not clearly resolved in the e.p.r. spectra at pH values lower than pH 5.5 (Fig. 4). The spectrum of the free hydrated ion seems to be predominant in these conditions, although the T_1 measurements clearly indicates that the copper is protein-bound. The presence of this species is likely to be related to the multistep kinetic patterns displayed by the reconstitution process below pH 5.5, as monitored by u.v. absorption and e.p.r. The initial copper binding is certainly reflected by the rapid initial change in A_{258} , the extent of which increases with pH (Fig. 2). It is evident from the pH-dependence of this initial change (Fig. 2) that protonation of protein groups with an apparent pK of 6.5 is involved in the binding.

This pK is likely to describe also the pH-dependence of the rates of activity recovery, though in this case the curve does not level out at low pH values (Fig. 1). Thus the same type of ligands, probably the imidazole group of histidine residues (Richardson *et al.*, 1975), seem to be involved in the initial and final type of binding, and the overall process can be described as a slow rearrangement of the site around the bound metal. Probably the invariance of the T_1 value in the various phases detected by other methods in the recovery process reflects the fact that copper is

always bound to the same ligands (the histidine residues and the water of the active site) although in different configurations. This is also supported by the very different T_1 value of the copper that binds to different protein ligands above the stoichiometric $[\text{Cu}^{2+}]/[\text{protein}]$ ratio. An important corollary of the results reported above is that e.p.r., u.v.-absorption and activity measurements probe the rearrangement of ligands to give the active site in a parallel fashion. This demonstrates that the dismutase activity, at least as monitored by polarography, is a specific property of the copper-binding site in the native enzyme and is not displayed, or only to a very low extent, by the copper bound to the same site but in a configuration different from that present in the native enzyme. This is in contrast with previous statements (Fee *et al.*, 1973) and allows a confident use of e.p.r., u.v. and activity to quantify the reappearance of native enzyme molecules in a reconstitution study.

The involvement of subunit interactions in the recombination process is revealed by the fact that activity and u.v. absorption are restored much more slowly when some sites have already fully recombined. In fact, the results shown in Table 1 give direct evidence for interaction between copper-binding sites and not necessarily between subunits, but, since the two copper-binding sites of the dimer are 3.4 nm (34 Å) apart (Richardson *et al.*, 1975), the only reasonable explanation of the observed facts is to assume that rearrangement of one subunit to give the native site slows down the same step in the other subunit. This interpretation can be related to the half-the-sites mechanism of action proposed for this enzyme (Fielden *et al.*, 1974) and to other evidence for inter-subunit interaction derived from equilibrium measurements (Rigo *et al.*, 1977a,b).

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