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The Involvement of the Bridging Imidazolate in the Catalytic Mechanism of Action of Bovine Superoxide Dismutase

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The pulse-radiolysis method has been used to study the catalytic mechanism of $O_2^{\cdot -}$ dismutation by the Co(II)-substituted bovine erythrocyte superoxide dismutase, EC 1.15.1.1. Catalysis is accompanied by spectral changes that may be interpreted in terms of rapid protonation and deprotonation of the Cu-facing nitrogen atom of the imidazolate that bridges the Cu(II) and the Co(II) [or Zn(II)] in the oxidized enzyme. This rapid change permits the possibility that the imidazole is a proton donor in the catalytic reduction of $O_2^{\cdot -}$.

The catalytic mechanism of the (Cu,Zn)- (copper-and-zinc-containing) superoxide dismutase (EC 1.15.1.1) has been shown to involve alternate oxidation and reduction of the copper by $O_2^{\cdot -}$ (Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974):

In reaction (2), $O_2^{\cdot -}$ is directly involved in proton transfer as well as electron transfer; this is not so in reaction (1). By analogy with the known pH-dependence of the uncatalysed dismutation of $O_2^{\cdot -}$, Fee & Ward (1976) suggested that the transition state of the enzyme-catalysed reduction (2) must contain at least one proton. The high value of the catalytic rate constant ($k_{+2} = k_{+1}$) (Fielden *et al.*, 1974) and its pH-independence (pH 5–10) (Rotilio *et al.*, 1972) preclude hydronium ions from the bulk of the solvent as a proton source (Fee & DiCorleto, 1973). Hodgson & Fridovich (1975) found that enzyme activity, as measured by its ability to inhibit $O_2^{\cdot -}$ -mediated reduction of cytochrome *c*, was the same in 2H_2O as in H_2O , and concluded that proton transfer is not rate-limiting. If a proton is involved in the transition state then it must come from a source

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that is near to the active site and is capable of fast proton transfer.

Fee & Ward (1976) suggested that reaction (2) proceeds through a cupric-peroxide complex and that protons are donated from water molecules in the region of the superoxide bound to the copper. An alternative scheme involves the acid form of the bridging imidazolate of the histidine-61 residue (Richardson *et al.*, 1975) as the proton source (Hodgson & Fridovich, 1975; Rotilio *et al.*, 1977). According to the latter hypothesis, reduction of the Cu(II) by $O_2^{\cdot -}$ leads to (or is accompanied by) release of the bridging imidazolate and protonation of the formerly Cu-bound nitrogen atom; re-formation of the Cu–N bond during oxidation of the Cu(I) form of the enzyme releases the bound proton, which participates directly in the transition state leading to reduction of $O_2^{\cdot -}$:

In the present paper we show that protonation and deprotonation of the bridging imidazolate in the Co(II)-substituted dismutase occurs simultaneously with the catalytic dismutation.

Materials and Methods

(Cu,Zn)-superoxide dismutase was isolated from bovine erythrocytes by the method of McCord & Fridovich (1969) and the Co-substituted enzyme was prepared as described previously (Calabrese *et al.*, 1972). Enzyme concentrations were calculated assuming $\epsilon_{680} = 300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The cobalt content was determined by parallel visible-absorbance measurements and atomic-absorption spectroscopy. These measurements gave an $\epsilon_{597.5} (\lambda_{\text{max}})$ of $450 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the protein-bound Co (expressed per mol of Co) having allowed for the absorbance of the protein-bound Cu at this wavelength ($\epsilon = 195 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, expressed per mol of protein) by comparison with the spectrum of the (Cu,Zn)-superoxide dismutase.

The use of the pulse-radiolysis method to measure rate constants and to determine absorption changes occurring during catalysis was described previously (Fielden *et al.*, 1974). Experiments were performed at room temperature (approx. 25°C) in either aerated or oxygenated buffer (2 mM-sodium pyrophosphate/100 μM -EDTA) containing 0.1 M-ethanol and adjusted (with H_2SO_4) to pH 9.

Results and Discussion

Cobalt (II) can be partially substituted for zinc without markedly affecting the structure and properties of Cu-superoxide dismutase (Calabrese *et al.*, 1972; Rotilio *et al.*, 1973). The turnover rate constants determined in the present study (Table 1) show that catalytic activity is fully retained in the Co-substituted enzyme. The optical spectrum associated with the Co(II) is sensitive to the state of the nearby copper site (Calabrese *et al.*, 1976). In particular, the spectrum is identical in the Cu-free protein and in the Cu(I),Co(II)-protein obtained by reduction with H_2O_2 or $\text{K}_4\text{Fe}(\text{CN})_6$. Redox titration has demonstrated the uptake of a proton during the reduction of each Cu(II) centre in the (Cu,Zn)-enzyme (Fee & DiCorleto, 1973), and the changes observed in the Co(II) spectrum of the substituted enzyme are consistent with the breaking of the Cu(II)-imidazolate bond and protonation of the formerly Cu(II)-bound nitrogen atom (reaction 3) (Rotilio *et al.*, 1977).

To determine whether or not the above alteration of the Co(II) environment occurs during catalysis, we recorded the spectral changes seen under turnover conditions (see the legend to Table 1) over a time

Table 1. Rate constants and relative intensities of spectral changes at 601 nm and 680 nm for the (Cu,Zn)- and the (Cu,Co)-superoxide dismutases of two of the batches (B and C) of enzyme

Second-order rate constants under turnover conditions (k) were estimated by analysis of the exponential decrease of the absorbance at 250 nm due to $\text{O}_2^{\cdot -}$ (approx. $3 \mu\text{M}$) in the presence of enzyme (approx. $0.6 \mu\text{M}$). Rate constant (k_λ) and total changes in absorbance (ΔA_λ) under initial rate conditions were obtained by analysing the change, at a given wavelength, in the absorbance due to the superoxide dismutase when an excess of the enzyme (approx. $35 \mu\text{M}$) in the oxidized (ox) or reduced (red) form was exposed to $\text{O}_2^{\cdot -}$ (approx. $4 \mu\text{M}$). The enzyme was reduced by exposure to approx. $300 \mu\text{M}$ - H_2O_2 for about 1 min; this produced no inactivation. Absorbance changes under turnover conditions were obtained by subjecting an oxygenated solution of the enzyme (approx. $20 \mu\text{M}$) to a train of closely spaced radiation pulses each producing approx. $25 \mu\text{M}$ - $\text{O}_2^{\cdot -}$; the reduced state was achieved by exposure to the H_2O_2 (approx. $300 \mu\text{M}$ for 1 min) generated during turnover (see Fielden *et al.*, 1974). Results marked * are means $\pm$ s.d. for the numbers of determinations given in parentheses. For results marked †, duplicate determinations of k_λ differed by up to 30% from each other, a variation attributable partly to the poor quality of the oscillograms obtained under such conditions. The average values (those quoted) are lower than those measured under turnover conditions. This difference is equally apparent for both the (Cu,Zn)-enzyme and the (Cu,Co)-enzyme. However, duplicate estimates of ΔA_λ , either under initial-rate conditions or at turnover, agreed within 5%, and therefore the main conclusions drawn in the text are not affected by the apparent differences between k and k_λ .

Enzyme	$10^{-9} \times$ Turnover rate constant (k) ($\text{M}^{-1} \cdot \text{s}^{-1}$)*	$10^{-9} \times$ Initial rate constants (k_λ) ($\text{M}^{-1} \cdot \text{s}^{-1}$)†			$\Delta A_{601}/\Delta A_{680}^\dagger$	
		k_{601}	k_{680}	k_{601}/k_{680}	Initial-rate conditions	Turnover conditions
Cu,Zn (B)	3.15 ± 0.14 (4)	1.67 (ox)	2.12 (ox)	0.79	0.64	0.61
		1.90 (red)	2.13 (red)	0.89	0.71	0.71
Cu,Zn (C)	3.14 ± 0.13 (5)	1.80 (ox)	2.28 (ox)	0.79	0.67	0.62
		2.46 (red)	2.88 (red)	0.85	0.82	0.82
Cu,Co (B) (1.13 g-atom of Co/mol)	3.21 ± 0.08 (3)	2.39 (ox)	2.19 (ox)	1.09	1.34	1.33
		2.23 (red)	1.93 (red)	1.16	1.40	1.45
Cu,Co (C) (0.53 g-atom of Co/mol)	3.25 ± 0.14 (5)	2.04 (ox)	1.86 (ox)	1.10	0.90	0.89
		2.31 (red)	2.19 (red)	1.05	1.10	1.09

Fig. 1. Optical spectra of (Cu,Co)-superoxide dismutase. Enzyme (610 μ M) containing 0.93 g-atom of Co/mol (batch A) in sodium pyrophosphate buffer (20 mM), pH 8.9 (upper curve), was reduced by approx. 30% by the addition of 140 μ M- H_2O_2 . The resulting spectrum (lower curve) was recorded immediately, and neither inactivation nor re-oxidation of the enzyme occurred during this period. The light-path was 1 cm. The closed circles (●) represent the absorbances observed under turnover conditions (see the legend to Table 1) during pulse radiolysis of a solution of the oxidized (Cu,Co)-superoxide dismutase from the same enzyme preparation. For comparison, these absorbances have been corrected because different enzyme concentrations were used in the two sets of experiments.

Fig. 2. Difference spectrum for (Cu,Co)-superoxide dismutase: the effect of going from oxidized enzyme to enzyme at turnover

The continuous line (—) was calculated from the difference between the spectra of the oxidized and the partially reduced (H_2O_2) enzyme (batch A) shown in Fig. 1. The points refer to absorbance changes observed under turnover conditions (see the legend to Table 1) during pulse radiolysis of solutions of the oxidized enzyme. Batch A (●), 0.93 g-atom of Co/mol; batch B (□), 1.13 g-atom of Co/mol; batch C (○), 0.53 g-atom of Co/mol; batch D (Δ), 0.99 g-atom of Co/mol. The absorbance changes have been expressed relative to those observed at 680 nm for a given enzyme sample. The results obtained under turnover conditions for batch A can be directly compared with the continuous line. Because of the different Co content of the other batches of enzyme, corrections have been applied to the data before plotting so that they too may be compared with the continuous line. The broken line (----) represents the best-fit line obtained for analogous pulse-radiolysis experiments with (Cu,Zn)-superoxide dismutase, and is in agreement with predictions from simple spectrophotometric experiments (similar to those of Fig. 1).

range during which there was neither reduction nor inactivation by H_2O_2 . The observed changes were in close agreement with those expected if the enzyme alternated between the oxidized state [Cu(II),-Co(II)] and a reduced state [Cu(I),Co(II)] identical with that produced by reduction with H_2O_2 (Fig. 1). The identity of the spectral changes is more clearly illustrated by the difference spectrum in Fig. 2. Analogous results were obtained when the H_2O_2 -reduced enzyme was present initially. If the environment of only the Cu was affected during catalysis then the observed changes would be expected to be similar to those seen for the (Cu,Zn)-superoxide dismutase (----, Fig. 2); the difference spectrum clearly indicates that the data are compatible with a concomitant change in the Co(II) and the Cu environments.

To establish conclusively that the observed spectral changes occur at the same rate as the catalytic step, as determined by the decrease in absorbance [starting with Cu(II),Oo(II)enzyme] or by the increase in absorbance [starting with the Cu(I),Co(II)enzyme] of the visible absorption bands, parallel measurements were made at 601 nm, where the difference spectrum has a maximum, and at 680 nm, where the Cu band has a broad maximum. Under conditions

where initial rates were observed (i.e. with an excess of enzyme over substrate), the second-order rate constant characterizing the ΔA_{601} (k_{601}) was the same as that characterizing ΔA_{680} (k_{680}) (Table 1). The ratio of the overall change at the two wavelengths

($\Delta A_{601}/\Delta A_{680}$) was higher for the (Cu,Co)-superoxide dismutase than for the (Cu,Zn)-enzyme. This indicates that the Co(II) chromophore is involved in the observed change. Moreover, for the Co(II)-containing enzyme, $\Delta A_{601}/\Delta A_{680}$ is the same as that found under turnover conditions, implying that the change in the Co(II) environment is indeed occurring at the same time as the catalytic dismutation. The results imply also that, in the Co(II)-substituted protein, the Cu(II),Co(II) centres and the remaining Cu(II),Zn(II) centres are equally reactive towards substrate, as would be expected from the known lack of effect on activity of Co(II) substitution (Table 1).

The results indicate that a concomitant change in the Co and Cu environments occurs during catalysis, and that the environment of the Co(II) in the Cu(I),-Co(II) form of the enzyme is identical with that obtained by reduction. In view of the evidence (discussed above) that protonation of the Cu-facing nitrogen atom of the bridging imidazolate is the most likely cause of this type of spectral change, the results support the hypothesis that protonation and deprotonation of this bridging ligand occur at the same rate as reduction or oxidation of the Cu centre. Of course, this does not prove that the proton attached to the imidazole nitrogen atom in the [Cu(I),Co(II)]-enzyme is indeed responsible for protonating the precursor of HO_2^- . However, it is clear that the transfer of that proton and the accompanying conformational changes are sufficiently rapid at least, to be considered, as catalytically important. It can be suggested that re-oxidation of the [Cu(I),-Co(II)]-enzyme involves three simultaneous processes: (i) electron transfer from the Cu(I) to O_2^- ; (ii) proton transfer from the imidazole to the O_2^- ; (iii) formation of a Cu-N bond (i.e. the imidazole-bound proton participates directly in the transition state leading to HO_2^-).

The catalytic oxidation of O_2^- (step 1) involves also rapid protonation of the imidazolate, although in this case the product is O_2 , and there is no need to speculate about the juxtaposition of substrate and the bridging imidazolate. Nevertheless the rate of this reaction shows clearly that there must be other proton sources (presumably solvent molecules) able to react extremely rapidly. The pK of the pyrrole hydrogen in the simple Co(Im)_4^{2+} (Im = imidazole) is about 12.5 (Sundberg & Martin, 1974) and the first pK of H_2O_2 is about 11.6 (*Handbook of Chemistry and Physics*, 1970). Since the nitrogen of the bridging imidazolate protonates very rapidly during the reduction of the [Cu(II),Co(II)]-enzyme (step 1), it is likely that in the re-oxidation (step 2) there is a proton source available close to the active site that could

rapidly transfer a second proton to convert HO_2^- into H_2O_2 .

Conclusion

The imidazole-bound proton seems the most likely proton to be involved in the transition state leading to the formation of HO_2^- . However, it is clear that there are solvent molecules in the region of the active site available to participate in equally rapid proton transfer. Even if these water molecules are not involved in the first step of the reduction of O_2^- , they may rapidly protonate HO_2^- to give H_2O_2 . In view of the complete retention of activity when Co(II) is partially substituted for Zn(II) in the (Cu,Zn)-superoxide dismutase, it can be assumed that the above arguments apply equally well to the parent enzyme.

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