Evidence for catalytic dismutation of superoxide by cobalt(II) derivatives of bovine superoxide dismutase in aqueous solution as studied by pulse radiolysis

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By using the technique of pulse radiolysis to generate $O_2^{\cdot-}$, it is demonstrated that Co(II) derivatives of bovine superoxide dismutase in which the copper alone and both the copper and zinc of the enzyme have been substituted by Co(II), resulting in (Co,Zn)- and (Co,Co)-proteins, are capable of catalytically dismutating $O_2^{\cdot-}$ with 'turnover' rate constants of $4.8 \times 10^6 \text{ dm}^3 \cdot \text{s}^{-1} \cdot \text{mol}^{-1}$ and $3.1 \times 10^6 \text{ dm}^3 \cdot \text{s}^{-1} \cdot \text{mol}^{-1}$ respectively. The activities of the proteins are independent of the pH (7.4–9.4) and are about three orders of magnitude less than that of the native (Cu,Zn)-protein. The rate constants for the initial interaction of $O_2^{\cdot-}$ with the Co-proteins were determined to be $(1.5\text{--}1.6) \times 10^9 \text{ dm}^3 \cdot \text{s}^{-1} \cdot \text{mol}^{-1}$; however, in the presence of phosphate, partial inhibition is apparent [$k \sim (1.9\text{--}2.3) \times 10^8 \text{ dm}^3 \cdot \text{s}^{-1} \cdot \text{mol}^{-1}$]. To account for the experimental observations, two reaction schemes are presented, involving initially either complex-formation or redox reactions between $O_2^{\cdot-}$ and Co(II). This is the first demonstration that substitution of a metal into the vacant copper site of (Cu,Zn)-protein results in proteins that retain superoxide dismutase activity.

Native superoxide dismutases contain either Cu and Zn or Mn or Fe. In the (Cu,Zn)-enzyme the copper is catalytically active. From previous studies it has been suggested that no other metal can substitute the native, active one of each class and give an enzymically active derivative (McCord & Fridovich, 1969; Beem *et al.*, 1977; Ose & Fridovich, 1976).

Cobalt substitution has been successfully carried out for zinc in the (Cu,Zn)-superoxide dismutase and the (Cu,Co)-derivative is fully active (Calabrese *et al.*, 1972, 1976; Rotilio *et al.*, 1973; McAdam *et al.*, 1977a). More recently, Co(II) has been bound to the vacant Cu-site of the copper-free, zinc-containing protein (Calabrese *et al.*, 1979a).

The new derivative (Co,Zn) has specific optical and e.s.r. spectra, which are indicative of tetra- or penta-hedral co-ordination and are sensitive to the presence of phosphate (Calabrese *et al.*, 1979b). The (Co,Co)-protein (Calabrese *et al.*, 1979a) has analogous properties.

Preliminary activity tests gave inconclusive results, as the putative activity was very low and comparable with that expected for small amounts of residual (Cu,Zn)-protein. Therefore, a pulse-radiolysis investigation of the (Co,Zn)- and (Co,Co)-proteins was undertaken to establish whether cobalt(II), specifically bound to superoxide dismutase, is able to dismutate $O_2^{\cdot-}$ catalytically.

Experimental

The metal content of the superoxide dismutases is represented by the symbol (M,N), where M and N represent the metal ion in the copper and zinc sites of the native protein respectively, and therefore (Cu,Zn) represents the native protein.

The native (Cu,Zn)-protein was prepared as described by McCord & Fridovich (1969) and the apo-protein and the (—,Zn) proteins were prepared as previously described (Fee, 1973; Rigo *et al.*, 1977). The preparation and properties of the

(Co,Zn)- and also the (Co,Co)-proteins have previously been described (Calabrese *et al.*, 1979a). Metal contents were determined by using atomic-absorption spectroscopy (Hilger and Watts Atomspek, model H1170). The stock solution of the (Co,Zn)-protein contained $1.4 \text{ mmol of Co} \cdot \text{dm}^{-3}$ and $8.5 \pm 0.11 \mu\text{mol of residual Cu} \cdot \text{dm}^{-3}$. The corresponding control protein, (—,Zn), obtained before addition of Co, contained $10.2 \pm 0.07 \mu\text{mol of residual Cu} \cdot \text{dm}^{-3}$. The stock solution of the (Co,Co)-protein contained $3.4 \text{ mmol of Co} \cdot \text{dm}^{-3}$, of which $1.4 \text{ mmol of Co} \cdot \text{dm}^{-3}$ is in the Cu site of the protein. The (Co,Co)-protein and the corresponding control apoprotein both contained $3.0 \pm 0.12 \mu\text{mol of residual Cu} \cdot \text{dm}^{-3}$. All concentrations of the Co proteins are expressed in terms of [Co]. In the case of the (Co,Co)-protein, the protein concentration is based upon the [Co] in the Cu-site. All other chemicals were of 'AnalaR' grade for use in the radiolysis experiments.

The pulse-radiolysis equipment and ancillaries have been described (McAdam *et al.*, 1977b). All solutions were prepared using triple-distilled water and contained $0.1 \text{ mol of ethanol} \cdot \text{dm}^{-3}$, $100 \mu\text{mol of EDTA} \cdot \text{dm}^{-3}$ and phosphate or pyrophosphate buffer ($7 \text{ mmol} \cdot \text{dm}^{-3}$) to which the appropriate aliquot of protein was then added. For the control proteins the concentration was adjusted so that the [Cu] in the control solution was equivalent to that in the solutions containing the Co-proteins. Before irradiation, solutions were saturated with O_2 . In this system the radiolytically produced primary species (e_{aq}^- , $\text{OH}^\bullet$ and H atoms) were converted into $\text{O}_2^{\bullet-}$, the concentration of which was determined by assuming $\epsilon_{250}^{\text{O}_2^{\bullet-}} = 200 \text{ m}^2 \cdot \text{mol}^{-1}$ (Rabani & Nielson, 1969).

Solutions, contained in an optical cell of path length 0.07 or 0.2 dm, were irradiated with electron pulses of $1.6 \mu\text{s}$ duration. Light filters were used when necessary to minimize photochemical effects.

Results and discussion

Assay of the Co-proteins for catalytic activity: $[\text{O}_2^{\bullet-}]$ much greater than [Co]

The (Co,Zn)- and (Co,Co)-proteins were assayed for superoxide dismutase activity by observation of the optical absorption changes at 250 nm, a wavelength at which $\text{O}_2^{\bullet-}$ is known to absorb, at pH 7.4 and 9.4, in the presence of phosphate and pyrophosphate respectively.

In Fig. 1, typical optical absorption changes at 250 nm with time are shown for the (Co,Zn)-protein and the corresponding 'Cu-free' protein, which was used as the source material for Co-substitution. The latter was used as the control to take into account the residual copper-containing protein in both of

Fig. 1. Transient optical-absorption changes with time at 250 nm in the presence and absence of Co-protein for initial $[\text{O}_2^{\bullet-}] > 70 \mu\text{mol} \cdot \text{dm}^{-3}$

Oxygen-saturated aqueous solutions containing $0.1 \text{ mol of ethanol} \cdot \text{dm}^{-3}$ and $100 \mu\text{mol of EDTA} \cdot \text{dm}^{-3}$ were irradiated with electron pulses of $1.6 \mu\text{s}$ duration in an optical cell of path length 0.07 dm . The decay of $\text{O}_2^{\bullet-}$ ($80\text{--}90 \mu\text{mol} \cdot \text{dm}^{-3}$) was monitored at 250 nm. (a) $20 \mu\text{mol of (Co,Zn)-protein} \cdot \text{dm}^{-3}$ with phosphate buffer ($7 \text{ mmol} \cdot \text{dm}^{-3}$), pH 7.4; (b) (—,Zn)-protein with phosphate buffer ($7 \text{ mmol} \cdot \text{dm}^{-3}$), pH 7.4; (c) $20 \mu\text{mol of (Co,Co)-protein} \cdot \text{dm}^{-3}$ with pyrophosphate buffer ($7 \text{ mmol} \cdot \text{dm}^{-3}$), pH 9.4; and (d) (—,—)-protein with pyrophosphate buffer ($7 \text{ mmol} \cdot \text{dm}^{-3}$), pH 9.4. The (—,Zn)- and apoprotein concentrations were adjusted so that the residual [Cu] was equivalent to that in the solutions containing Co-proteins. In the presence of the Co-proteins (traces a and c) a 'residual' absorption is observed after removal of $\text{O}_2^{\bullet-}$.

these samples (see the Experimental section). As shown in Fig. 1, the lifetime of $\text{O}_2^{\bullet-}$ in the presence of catalytic amounts of the (Co,Zn)-protein is less than in the presence of the control protein under conditions of $[\text{O}_2^{\bullet-}] > [\text{Co}]$. Similar observations were made in the presence of the (Co,Co)-protein,

whereby the apo-protein was used as the control in order to take into account the presence of residual Cu-protein in both of these samples. Dismutase activity due to impurities in the buffer solution is also taken into account by the use of the apo-proteins as the controls for Co-proteins.

The dismutase activity observed in the Co-substituted proteins could be ascribed to the adventitious presence of copper only if the Cu content of the (Co,Zn)- and (Co,Co)-samples was greater than that of the controls. The ratio of [Cu] in the Co-samples to their controls would have to be 1.5:1 for the (Co,Zn)-protein and 7:1 for the (Co,Co)-protein at pH 9.4. At pH 7.4 the natural lifetime of $O_2^{\cdot-}$ is considerably shorter and contributes significantly to the observed decay in both the Co-sample and the control. However, even under these conditions, the ratio of excess copper in the samples to the controls would have to be 1.2:1 and 2.3:1 for the (Co,Zn)- and (Co,Co)-proteins respectively. Such differences in the copper levels are well outside the limits of accuracy of the measured values for Cu contamination in the samples, which were diluted to give a 1:1 ratio for [Cu] in the sample to [Cu] in the control.

It is therefore suggested, on the basis of the enhanced rate of decay of $O_2^{\cdot-}$ in the presence of these Co-proteins as compared with that in the presence of the corresponding control proteins, that the (Co,Zn)- and (Co,Co)-proteins catalytically dismutate $O_2^{\cdot-}$. Furthermore, the lifetime of $O_2^{\cdot-}$ is unaffected when a second pulse, producing $O_2^{\cdot-}$, is delivered to the system, from which it is inferred that the proteins are not inactivated because of processes occurring after the first pulse and that the catalytic activity of the proteins is retained.

The decay at 250 nm of $O_2^{\cdot-}$ ($80\text{--}180\mu\text{mol}\cdot\text{dm}^{-3}$) was determined to be first-order and dependent upon the [Co]. The 'turnover' rate constants (k_0), corrected for the contribution from

the control protein to take into account the activity of the residual Cu in the samples, are presented in Table 1.

It was found that the (—,Co)-protein does not enhance the rate of decay of $O_2^{\cdot-}$ as compared with that obtained when using the apoprotein as control. Furthermore, Co-EDTA and Co-carbonic anhydrase both interact with $O_2^{\cdot-}$, but not catalytically under conditions when $[O_2^{\cdot-}] > [\text{Co}]$, demonstrating, in the systems studied, that only the Co-proteins in which Cu of superoxide dismutase is substituted by Co, are capable of catalytically dismutating $O_2^{\cdot-}$. It has previously been demonstrated that Co(III)-porphyrins may also catalytically dismutate $O_2^{\cdot-}$ (Pasternack & Skowronek, 1979; 1979; Pasternack & Halliwell, 1979).

As shown in Fig. 1 for the (Co,Zn)-protein, a longer-lived absorption is observed after completion of the reaction of $O_2^{\cdot-}$ (see below). The yield of this absorption was found to be independent of the initial $[O_2^{\cdot-}]$, and therefore of the $[\text{H}_2\text{O}_2]$ produced during dismutation of $O_2^{\cdot-}$, and it decays with a first half-life of $\geq 0.2\text{ s}$. A kinetic analysis of the decay was not performed, owing to the instability of the monitoring lamp on this time scale.

Interaction of $O_2^{\cdot-}$ with the Co-proteins under stoichiometric conditions: $[O_2^{\cdot-}]$ approximately equal to [Co]

The optical absorption changes at 250 nm with time in the presence of $22\mu\text{mol}$ of (Co,Zn)-protein $\cdot\text{dm}^{-3}$ at pH 7.4 in the presence of 7 mmol of phosphate $\cdot\text{dm}^{-3}$, and at pH 9.4 in the presence of 7 mmol of pyrophosphate $\cdot\text{dm}^{-3}$, are shown in Fig. 2 for an initial $[O_2^{\cdot-}]$ of $\sim 32\mu\text{mol}\cdot\text{dm}^{-3}$. After the initial formation of $O_2^{\cdot-}$ is complete ($<10\mu\text{s}$), its reaction with the protein results in a further increase in absorption at 250 nm. From the first-order rate of formation of this absorption (see Fig. 2), rate constants for the reaction of $O_2^{\cdot-}$ with (Co,Zn)- and

Table 1. Second-order rate constants for reaction of Co-proteins with $O_2^{\cdot-}$ during turnover at pH 7.4 and 9.4

The reaction of Co-proteins with $O_2^{\cdot-}$ ($32\text{--}180\mu\text{mol}\cdot\text{dm}^{-3}$) was monitored at 250 nm at pH 7.4 and 9.4 in the presence of phosphate and pyrophosphate buffer ($7\text{ mmol}\cdot\text{dm}^{-3}$) respectively. The concentration of Co in the Co-proteins was $10\text{--}32\mu\text{mol}\cdot\text{dm}^{-3}$ and the concentrations of the control proteins were chosen so that the copper concentration in the control proteins and the Co-proteins are equivalent. (a) The turnover rate constants corrected for the contribution from the control proteins; (b) rate constants determined from first-order rate of formation of optical absorption.

Protein (P)	pH ...	$k(O_2^{\cdot-} + P)(\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1})$	
		7.4	9.4
(Co,Zn)		$4.8 (\pm 0.5) \times 10^6$ (a)	$4.8 (\pm 0.5) \times 10^6$ (a)
(Co,Co)		$2.9 (\pm 0.3) \times 10^6$ (a)	$3.3 (\pm 0.3) \times 10^6$ (a)
(Co,Zn)		$2.3 (\pm 0.3) \times 10^8$ (b)	$1.6 (\pm 0.2) \times 10^9$ (b)
(Co,Co)		$1.9 (\pm 0.3) \times 10^8$ (b)	$1.5 (\pm 0.2) \times 10^9$ (b)

Fig. 2. Transient optical-absorption changes with time at 250 nm in the presence of (Co,Zn)-protein when $[O_2^{\cdot-}] \approx [Co]$

Oxygen-saturated aqueous solutions containing 0.1 mol of ethanol $\cdot dm^{-3}$ and 100 μmol of EDTA $\cdot dm^{-3}$ were irradiated with electron pulses of 1.6 μs duration in an optical cell of path length 0.07 dm. The transient optical-absorption changes with time were monitored at 250 nm in the presence of 22 μmol of (Co,Zn)-protein $\cdot dm^{-3}$. Trace (a), pH 9.4, in the presence of pyrophosphate buffer (7 mmol $\cdot dm^{-3}$), $[O_2^{\cdot-}] = 28 \mu mol \cdot dm^{-3}$, $\Delta A = 6.06\%$, $t = 20 \mu s$; trace (b), pH 7.4, in the presence of phosphate buffer (7 mmol $\cdot dm^{-3}$), $[O_2^{\cdot-}] = 32 \mu mol \cdot dm^{-3}$, $\Delta A = 3.03\%$, $t = 200 \mu s$. The initial rapid increase in optical absorption ($< 10 \mu s$) is due to the formation of $O_2^{\cdot-}$, and the subsequent increase in optical absorption is due to the interaction of $O_2^{\cdot-}$ with the (Co,Zn)-protein to produce a transient product whose absorption coefficient is greater than that of $O_2^{\cdot-}$.

(Co,Co)-proteins were determined and are presented in Table 1.

It is suggested that Co in the Cu-site interacts with $O_2^{\cdot-}$, irrespective of the metal in the zinc site. Furthermore, in line with previous spectroscopic evidence (Calabrese *et al.*, 1979a), which has shown that the optical absorption spectra of the proteins are modified by the presence of phosphate, the rate constants for initial reaction of $O_2^{\cdot-}$ with the Co(II)-proteins are about an order of magnitude less in the presence of phosphate at pH 7.4 as compared with those determined in the absence of phosphate at pH 9.4 (see Table 1). On the other hand, activity of the proteins is independent of the presence of phosphate or of pH.

Transient changes in the Co optical absorption band

The transient optical-absorption changes with time monitored at 575 nm on reaction of $O_2^{\cdot-}$ with (Co,Zn)-protein at pH 7.4 and 9.4 are shown in Fig. 3. Bleaching of the optical absorption, within a few ms after formation of $O_2^{\cdot-}$, is only observed in the presence of phosphate and when the pH is less than

or equal to the pK of the protein-phosphate chromophore complex (Calabrese *et al.*, 1979b). In the presence of pyrophosphate at pH 7.6 and 9.4, quantitatively similar increases in optical absorption in the wavelength region 500–650 nm were observed on reaction of $O_2^{\cdot-}$ with (Co,Zn)-protein. At pH 7.4 in the presence of phosphate, restoration of the optical absorption is observed, as shown in Fig. 3. The first half-life is estimated to be ≥ 0.2 s, comparable with that observed for the rate of decay of the residual absorption at 250 nm. The rate of restoration of the optical absorption at 575 nm was also found to be independent of the $[O_2^{\cdot-}]$, $[O_2]$, $[H^+]$ and $[Co]$.

Transient optical-absorption changes were also observed in the wavelength region 250–500 nm. However, these changes were not investigated in detail owing to the quantity of protein that would be required for such studies. It should be noted from observations at 650 nm, where ϵ of the protein is more-or-less independent (Calabrese *et al.*, 1979a,b) of the presence of phosphate, that the transient absorption produced on reaction of $O_2^{\cdot-}$ with the protein is a factor of approx. 2 greater in the

Fig. 3. Transient optical-absorption changes with time at 575 nm in the presence of 22 μmol of (Co,Zn)-protein $\cdot dm^{-3}$

Transient optical-absorption changes with time in the Co optical band at 575 nm on pulse irradiation of oxygen-saturated aqueous solutions containing 0.1 mol of ethanol $\cdot dm^{-3}$, 100 μmol of EDTA $\cdot dm^{-3}$ and 22 μmol of (Co,Zn)-protein $\cdot dm^{-3}$ and a light-path of 0.2 dm. Traces (a) and (b) show the initial interaction of $O_2^{\cdot-}$ (28 $\mu mol \cdot dm^{-3}$) at pH 9.4 (pyrophosphate buffer, 7 mmol $\cdot dm^{-3}$) and at pH 7.4 (phosphate buffer, 7 mmol $\cdot dm^{-3}$) respectively. Only in the presence of phosphate is a bleaching in the Co optical absorption band observed (trace b). Trace (c) shows the time scale of restoration of the optical absorption at 575 nm after removal of $O_2^{\cdot-}$ (30 $\mu mol \cdot dm^{-3}$) is complete, for solutions containing 20 μmol of (Co,Zn)-protein $\cdot dm^{-3}$ at pH 7.4 in the presence of 7 mmol of phosphate $\cdot dm^{-3}$.

presence of phosphate at pH 7.4 than in the absence of phosphate. It is implied that the ϵ of the transient species produced at pH 7.4 in the presence of phosphate is a factor of approx. 2 greater than that observed in the absence of phosphate and, therefore, that different species are produced depending on whether phosphate is present or not.

At pH 7.4 in the presence of 7 mmol of phosphate $\cdot\text{dm}^{-3}$, the rate of bleaching of the optical absorption at 575 nm is first-order and dependent upon [Co] ($14\text{--}35\mu\text{mol}\cdot\text{dm}^{-3}$); this yields a rate constant of $2.5 \times 10^8\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$, the value of which is a good agreement with that determined at 250 nm. At pH 7.4 and 9.4 in the presence of pyrophosphate, a rate constant of $1.7 \times 10^9\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ was determined from the dependence of the first-order rate of formation of the transient absorption at 650 nm on [Co].

At pH 6 in the presence of 10 mmol of phosphate $\cdot\text{dm}^{-3}$ and $20\mu\text{mol}$ of (Co,Zn)-protein $\cdot\text{dm}^{-3}$, a rate constant of $\sim 1.0 \times 10^8\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ was established from the initial rate of bleaching of the optical absorption at 575 nm. This rate constant is suggested to represent that for the initial reaction of $\text{O}_2^{\cdot-}$ with the (Co,Zn)-protein in the presence of phosphate, since the pH is more than $2\text{p}K_a$ units lower than the $\text{p}K_a$ (Calabrese *et al.*, 1979b) of the protein-phosphate chromophore, so that the concentration of the phosphate-Co-protein is at least two orders of magnitude greater than that of the free Co-protein. Therefore the partial rate constant for interaction of $\text{O}_2^{\cdot-}$ with the Co-protein not associated with phosphate at pH 6.0 is estimated to be less than $2 \times 10^7\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$, which is about one order of magnitude less than that determined experimentally.

In the presence of phosphate at pH 7.4, the extent of bleaching of the optical absorption is not quantitative, owing to the concomitant production of a species that absorbs in the wavelength region studied. However, on multiple pulsing with an interpulse duration of 10 ms, two pulses each producing $20\mu\text{mol}$ of $\text{O}_2^{\cdot-}\cdot\text{dm}^{-3}$ are sufficient to maximize the loss of absorption at 600 nm of a solution containing $38\mu\text{mol}$ of Co-protein $\cdot\text{dm}^{-3}$.

The corresponding transient spectral changes on reaction of $\text{O}_2^{\cdot-}$ with (Co,Co)-protein are qualitatively similar to those observed on reaction of $\text{O}_2^{\cdot-}$ with the (Co,Zn)-protein; the rate constants determined for reaction of $\text{O}_2^{\cdot-}$ with (Co,Co)-protein are $1.5 \times 10^8\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ and $1.5 \times 10^9\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ at pH 7.4 in the presence of phosphate and at pH 9.4 respectively. Furthermore it is apparent from the optical-absorption changes in the wavelength region studied of 500–650 nm that a blue shift of the transient spectrum was observed with (Co,Co)-protein as compared with that with the (Co,Zn)-protein.

Reaction scheme

It is evident from the experimental observations at 250 nm that both the proteins in which Cu is substituted by Co catalytically dismutate $\text{O}_2^{\cdot-}$. It was found that the initial reaction of $\text{O}_2^{\cdot-}$ with these proteins leads to transient changes in the Co optical bands and produces, at 250 nm, a transient species whose ϵ is greater than that of $\text{O}_2^{\cdot-}$.

The following Schemes (1 and 2) are proposed to account for the experimental observations, where p-Co(II) represents the Co in the Cu-site of the protein:

Scheme 1

Previous studies have shown that the reaction of $\text{O}_2^{\cdot-}$ with Co(II) macromolecules results in the production of either a Co(II)-superoxy or a Co(III)-peroxy complex with rate constants more than $10^9\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ (Simic & Hoffman, 1977). In Scheme 1 above $(\text{p-Co} \cdots \text{O}_2)^{+\cdot}$ may formally be considered to be a Co(III)-peroxy or a Co(II)-superoxy complex, by analogy to the findings of Simic & Hoffman (1977). Co(III)-superoxy complexes have also been shown to interact with $\text{O}_2^{\cdot-}$ to produce O_2 and a Co(III)-peroxy complex (Natarajan & Raghavan, 1980).

Alternatively, a Scheme analogous to the Ping Pong-type mechanism proposed for the native protein [Fielden *et al.* (1974) and references cited therein] would also account for the experimental observations. The Ping Pong-type mechanism could proceed through Co(III) or Co(I):

Scheme 2

It has previously been established that Co(I) (Sellers & Simic, 1976) and Co(I)-macrocyclic complexes (Simic & Hoffman, 1977; Tait *et al.*, 1976) interact preferentially with O_2 so that such a route involving Co(I) may be less favoured. Owing to the inertness of Co(III) complexes to sub-

stitution, the reactions postulated in Schemes 1 and 2 involving Co(III) are thought not to proceed by inner-sphere electron-transfer processes. Outer-sphere electron-transfer processes involving Co(III), however, cannot be ruled out. For example, the one-electron reduction of Co(III) porphyrins by e_{aq}^- , $CO_2^{\cdot-}$, $(CH_3)_2\dot{C}O^-$ and one-electron-reduced Zn-porphyrins to yield Co(II) porphyrins, has been shown to have rate constants in the range 2×10^7 to $2 \times 10^{10} \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, considerably greater than the rate constants for Co(III) substitution reactions of $\leq 10^4 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ (Neta, 1981).

At present we cannot distinguish kinetically between Schemes 1 and 2, since steady-state analysis of both these Schemes results in the following equations:

$$\frac{-d[O_2^{\cdot-}]}{dt} = \frac{2k_{+1}k_{+2}}{k_{+1} + k_{+2}} [p\text{-Co}][O_2^{\cdot-}]$$

Assuming $k_{+1} \gg k_{+2}$:

$$\frac{-d[O_2^{\cdot-}]}{dt} \approx 2k_{+2}[p\text{-Co}][O_2^{\cdot-}]$$

From the kinetic analysis of the initial absorption changes at 250 nm and at 500–650 nm on reaction of $O_2^{\cdot-}$ with the Co-proteins, the determined rate constants of $1.6 (\pm 0.2) \times 10^9 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and $1.5 (\pm 0.2) \times 10^9 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ for the (Co,Zn)- and (Co,Co)-proteins respectively are assigned to k_{+1} . In the presence of phosphate at pH 7.4, k_{+1} is determined to be $2.3 (\pm 0.3) \times 10^8 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and $1.9 (\pm 0.3) \times 10^8 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ for the (Co,Zn)- and (Co,Co)-proteins respectively.

Since $k_{+1} \gg k_0$, the 'turnover' rate constants $k_0 \approx 2k_{+2}$. Thus, based on the experimentally determined k_0 values, $k_{+2} \approx 2.4 (\pm 0.3) \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and $\approx 1.5 (\pm 0.2) \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ for the (Co,Zn)- and (Co,Co)-proteins respectively, irrespective of the presence of phosphate.

On the basis of the turnover rate constants of $4.8 \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and $3.1 \times 10^6 \text{ dm}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, the superoxide dismutase activity of the Co-proteins is about three orders of magnitude less than that for the native (Co,Zn)-protein. However, the activity observed is about one order of magnitude greater than that reported for Co(III)-porphyrins (Pasternack & Skowronek, 1979; Pasternack & Halliwell, 1979).

H_2O_2 and O_2 are thought not to be involved in the catalytic scheme, since the turnover rate constants and the rate of restoration of the absorption in the Co band are both independent of the [Co] and of the $[O_2^{\cdot-}]$ and consequently of the resulting $[H_2O_2]$ produced during turnover. A 10-fold excess of $[H_2O_2]$ over [Co] has been demonstrated (G. Rotilio, D. Cocco & L. Calabrese, unpublished work) to modify the optical absorption of the

Co-band but only over a time period that is far longer than those reported here (6–10 h).

The transient increase in optical absorption at 650 nm on reaction of $O_2^{\cdot-}$ with the Co-proteins is more intense in the presence of phosphate at pH 7.4, but it is not, as yet, known whether phosphate remains associated with, or remains in the vicinity of, the active site during turnover.

The subsequent reactions of the transient species present after completion of the reactions with $O_2^{\cdot-}$ have not been investigated in detail. It appears as though the Co-proteins are essentially unchanged, since the optical absorption is restored and a second pulse producing $O_2^{\cdot-}$ about 5 s after the first shows that no loss of activity has occurred.

It is concluded that both the (Co,Zn)- and (Co,Co)-proteins are capable of catalytically dismutating $O_2^{\cdot-}$, but far less efficiently than the native (Cu,Zn)-protein. This is the first demonstration that substitution of a metal into the vacant Cu-site of (Cu,Zn)-superoxide dismutase results in a protein that retains superoxide dismutase activity. It is also apparent that phosphate partially inhibits the initial reaction of $O_2^{\cdot-}$ with the protein, and this result reinforces the hypothesis that a net positive charge in the vicinity of the active site is important for the substrate-metal interaction (Malinowski & Fridovich, 1980). If a cobalt- $O_2^{\cdot-}$ complex is formed, then the mode of catalytic dismutation is analogous to the oxidation of $O_2^{\cdot-}$ in the presence of $HO_2^{\cdot}$, whereby a proton catalysis occurs.

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