A Pulse-Radiolysis Study of the Manganese-Containing Superoxide Dismutase from *Bacillus stearothermophilus*

FURTHER STUDIES ON THE PROPERTIES OF THE ENZYME

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In the preceding paper the mechanism of catalysis of the manganese-containing superoxide dismutase from *Bacillus stearothermophilus* was shown to involve a 'fast cycle' and a 'slow cycle' [McAdam, Fox, Lavelle & Fielden, 1977 (*Biochem. J.* 165, 71–79)]. Further properties of the enzyme are considered in the present paper. Pulse-radiolysis studies, under conditions of low substrate concentration to enzyme concentration (i.e. when the fast cycle predominates), showed that enzyme activity decreases as pH increases (6.5–10.2). Activity was unaffected by the addition of H_2O_2 or NaN_3 but slightly decreased by KCN. Both H_2O_2 and the reducing radical anion $\text{CO}_2^{\cdot-}$ caused a decrease in A_{480} of the native enzyme. The rate of the fast catalytic cycle was independent of temperature (5–55°C), and as temperature increases the slow catalytic cycle becomes relatively more important. Arrhenius parameters of the rate constants were estimated. The possible identity of the various forms of the enzyme is considered.

In the preceding paper (McAdam *et al.*, 1977), the essential kinetic features of the mechanism of action of Mn- (manganese-containing) superoxide dismutase from *Bacillus stearothermophilus* were described. In the present paper further observations on the properties of the enzyme are reported and, where useful, compared with the kinetic observations. Whenever such comparisons are made, the notation used will be that of Model I (McAdam *et al.*, 1977):

Materials and Methods

Experimental procedures and enzyme samples were as described previously (McAdam *et al.*, 1977), and unless stated otherwise solutions studied by pulse radiolysis contained ethanol (0.1M), sodium pyrophosphate (2mM) and EDTA (100 μM).

Results and Discussion

Effect of composition of assay medium on measured activity

For activity determination by the pulse-radiolysis method it is necessary to maintain a relatively high

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pH to decrease the spontaneous dismutation of $\text{O}_2^{\cdot-}$. It is also desirable to convert hydroxyl radicals, primary products of the radiolysis of water, into $\text{O}_2^{\cdot-}$. These requirements demand a buffered medium containing ethanol or sodium formate (Fielden *et al.*, 1974).

When the ratio of substrate concentration to enzyme concentration ($[\text{O}_2^{\cdot-}]/[\text{E}_0]$) is low (<15), the measured decay rate reflects closely the value of k_1 and k_2 (see McAdam *et al.*, 1977). These 'assay' conditions facilitate comparison with the activity of other dismutases, and preliminary experiments were performed to check the effects of varying the composition of the medium. At pH 8.9 and 25°C ($[\text{O}_2^{\cdot-}] = 2.6 \mu\text{M}$; $[\text{E}_0] = 0.4 \mu\text{M}$) the replacement of ethanol (0.1M) by sodium formate (0.1M) resulted in an approx. 30% decrease in the fitted first-order rate constant. A similar decrease in rate has been found for bovine erythrocyte (McAdam, 1977), although in a previous study of this enzyme such a decrease was reported only at higher concentrations of formate (Fielden *et al.*, 1974).

The use of 0.5M-sodium pyrophosphate, in place of 2mM-sodium pyrophosphate, in the presence of ethanol (0.1M), resulted in a lower (approx. 15%) fitted first-order rate constant, and the use of 5mM-pyrophosphate gave a slightly higher (approx. 10%) value. Replacement of 0.5M-sodium pyrophosphate with 2mM-sodium borate, pH 9, leads to only a small change in ionic strength and resulted in no change in measured activity. Variation of the ethanol concentration from 0.1 to 0.05M likewise resulted in no change in activity.

Despite the decrease in k_1 (and k_2) in the presence of formate there is no qualitative difference in the observations made at 250nm or at 480nm during turnover experiments. This suggests that the change of medium appears to affect the values of the rate constants without altering the mechanism of dismutation. The exact cause of these effects is not known, although it is probably partly due to changes in ionic strength (see also McAdam, 1977).

Effect of KCN, NaN₃ and H₂O₂ on enzyme activity

Under conditions similar to those above ($[O_2^{\cdot-}]/[E_0] < 15$; 25°C; pH9), NaN₃ or KCN caused little or no inactivation. For an enzyme concentration of 0.4 μ M a 90-fold excess of CN⁻ produced no measurable change in activity, and with a 2000-fold excess there was a 35% loss of activity. However, a 2000-fold excess of N₃⁻ did not affect the activity. The low sensitivity of the enzyme towards cyanide is in contrast with the sensitivity of Cu-superoxide dismutase to this reagent (Rotilio *et al.*, 1972; Beauchamp & Fridovich, 1973; Weisiger & Fridovich, 1973), but, in agreement with the results for the Mn-superoxide dismutases from the mitochondria of chicken liver (Weisiger & Fridovich, 1973) and of yeast (Ravindranath & Fridovich, 1975), and from *Plectonema boryanum* (Asada *et al.*, 1975). Furthermore Fe-containing superoxide dismutases from *Plectonema boryanum* (Asada *et al.*, 1975) and *Spirulina platensis* (Lumsden & Hall, 1974) are cyanide-insensitive.

Useful comparison between the properties of the different types of dismutases comes also from the effect of H₂O₂. For the *B. stearothermophilus* Mn-superoxide dismutase used in the present work, exposure to H₂O₂ under all conditions studied resulted in no loss of activity. For instance, a solution of enzyme (100 μ M) dialysed overnight against buffer [2mM-sodium pyrophosphate/100 μ M-EDTA (pH9)], when added to 10mM-H₂O₂ in the same medium, was fully active after 1 h. Removal of peroxide from such a solution by dialysis yielded fully active enzyme. Asada *et al.* (1975) found that the Mn-superoxide dismutase from *Plectonema boryanum* was likewise insensitive to H₂O₂, whereas Keele *et al.* (1975) report that the enzyme from *Escherichia coli* is inactivated by peroxide with concomitant loss of manganese (see, however, Hodgson & Fridovich, 1975). Cu-superoxide dismutases are inactivated by peroxide (Beauchamp & Fridovich, 1973; Bray *et al.*, 1974; Asada *et al.*, 1975), as are the Fe-superoxide dismutases (Asada *et al.*, 1975; Lavelle *et al.*, 1977).

The rates of the post-irradiation rise observed at 480nm after multiple pulsing and of the zero-order decay seen at 250nm in the presence of high concentrations of substrate (McAdam *et al.*, 1977) are both unaffected by the presence of H₂O₂. This suggests that peroxide has no effect on any of the rate constants.

Effect of pH on enzyme activity

There is a gradual decrease in the rate of disappearance of O₂^{·-} with increasing pH for conditions where O₂^{·-} decay is close to exponential (Table 1). The variation of this rate with pH, reflecting the pH-dependences of k_1 and k_2 , is in contrast with the pH-independence of the rate of dismutation by bovine erythrocyte (Rotilio *et al.*, 1972). However, similar pH-dependences have been found for the Mn-superoxide dismutases from *E. coli* (Forman & Fridovich, 1973) and mitochondria (Ravindranath & Fridovich, 1975) as well as for the Fe-superoxide dismutases from *E. coli* (Forman & Fridovich, 1973) and *Photobacterium leiognathi* (Lavelle *et al.*, 1977).

Preliminary observations at 480nm suggest also that the rate of the post-irradiation rise (k_3) is approximately three times higher at pH6.6 than at pH9. Studies on the pH-dependence of the zero-order decay are difficult, owing to the more rapid spontaneous dismutation of substrate at low pH. Therefore the effect of pH on k_3 has not been established.

Effect of H₂O₂ and CO₂^{·-} radical anions on the visible absorption spectrum of the enzyme

Comparison with known spectra of Mn(III) compounds (Dingle, 1966; Davies, 1969) suggests that this is the oxidation state present in the native Mn-superoxide dismutases (Keele *et al.*, 1970, 1975; Weisiger & Fridovich, 1973; Ravindranath & Fridovich, 1975; see also Scrutton, 1971). N.m.r. (nuclear-magnetic-resonance) studies indicate also the presence of Mn(III) in the native superoxide dismutase from *E. coli* (Villafranca *et al.*, 1974).

Experiments in a Cary spectrophotometer showed that A_{480} values of the aerated solutions of the Mn-superoxide dismutase from *B. stearothermophilus* (60–120 μ M in 2mM-pyrophosphate/100 μ M-EDTA,

Table 1. Variation of measured second-order rate constant with pH

The concentration of enzyme was 2.48 μ M and that of O₂^{·-} was 15 μ M. The decay of substrate was followed at 250nm.

pH	10 ⁻⁸ × Measured second-order rate constant (M ⁻¹ ·s ⁻¹)
6.5	7.3
7.1	5.9
8.0	6.0
9.0	4.7*
10.2	1.2

* This value is lower than that reported in the preceding paper (McAdam *et al.*, 1977), since different batches of enzyme were used in the two studies.

pH9) were decreased (bleached) by between 70 and 90% of their original values by the addition of an approx. 100-fold excess of H_2O_2 . The variations cited refer to different batches of enzyme, the estimate for each batch having an error of about $\pm 5\%$. The final level of bleaching for a given sample could be attained after the addition of approx. 1 equiv. of peroxide. Removal of the peroxide either by overnight dialysis against buffer (2mM-pyrophosphate/100 μM -EDTA, pH9) or by treating the solution with catalase on a solid support (Enzygel; Boehringer, London W5 2TZ, U.K.) led to complete recovery of A_{480} and resulted in no loss of specific activity (see above). The rate of decrease of A_{480} by the H_2O_2 generated during catalysis has been determined (McAdam *et al.*, 1977).

For different batches of Mn-superoxide dismutase $\epsilon_{280}/\epsilon_{480}$ varied by only about 15% (McAdam *et al.*, 1977); however, because of variation in the shapes of the visible-absorption spectra centred on 480 nm, the ratio of $\epsilon_{480}/\epsilon_{400}$ ranged from 1 to 2. The different shapes of the visible spectra suggest the presence of an additional species in some samples. The activities of the separate samples differed by only 20%, which suggests that this species has little effect on the concentration of active enzyme measured by using A_{280} (McAdam *et al.*, 1977).

The radical anion $\text{CO}_2^{\cdot -}$ is a strong reducing agent (Neta *et al.*, 1969), and unlike H_2O_2 does not exhibit oxidizing properties. Treatment of the enzyme with $\text{CO}_2^{\cdot -}$ (Fig. 1) resulted in a 70% decrease in A_{480} , the same as that observed after treatment with peroxide. Assay of the sample, after reintroduction of oxygen,

showed that $\text{CO}_2^{\cdot -}$ had caused no inactivation of the enzyme. Since either oxidation or reduction of the native enzyme might decrease A_{480} , the similar effect of $\text{CO}_2^{\cdot -}$ and H_2O_2 does not prove that the latter is acting as a reducing agent.

Effect of temperature on measured rate constants

B. stearothermophilus is an organism which grows at a temperature of about 60°C and we decided to investigate how the measured enzyme activity varies with temperature. Incubation at 60°C for 5 min under assay conditions (approx. 1 μM -enzyme; pH9) resulted in no loss of activity, whereas incubation at 70°C for about 2 min caused a 30% loss of activity.

Under conditions where the decay of substrate was close to exponential (1.3 μM -enzyme, 11 μM - $\text{O}_2^{\cdot -}$), there was a change of less than 10% in the value of the first-order rate constant between 10 and 60°C. Therefore it would seem that k_1 and k_2 have an Arrhenius activation energy close to zero. The effect of diffusion alone would cause an approx. 3-fold increase in the bimolecular rate constant between 10 and 60°C (Moelwyn-Hughes, 1971). Preliminary results with a Mn-superoxide dismutase from a more extreme thermophile, *Thermus aquaticus* (supplied by Dr. J. I. Harris; see Sato & Harris, 1977), show that, under conditions where the decay of substrate is close to exponential, there is little (<10%) variation in measured first-order rate constant between 25 and 60°C. Thus the phenomenon does not appear to be an artifact of the particular enzyme isolation. It must be concluded that the steps characterized by k_1 and k_2 are not elementary bimolecular reactions controlled only by diffusion.

Although k_1 and k_2 are invariant with temperature, approximately linear Arrhenius plots are obtained for values of k_3 and k_5 between 5 and 55°C (Figs. 2 and 3). k_5 was calculated from the post-irradiation rise observed at 480 nm (McAdam *et al.*, 1977). No such direct method can be used to determine k_3 , which was obtained as follows. At each temperature, fixed values of k_1 , k_2 and k_5 were used in the theoretical model, and the value of k_3 was adjusted to give the best agreement between theory and experiment for conditions where the $\text{O}_2^{\cdot -}$ decay was zero-order (e.g., Fig. 4). For this calculation k_1 and k_2 were taken as $5.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ at all temperatures and k_5 was evaluated by using the line fitted to the data points in Fig. 3. The use of the experimental values of k_5 will cause only small changes in the deduced values of k_3 .

As temperature increases, the rapid decay at the beginning of the zero-order trace becomes less marked (Fig. 4). This is because k_3 increases with temperature, whereas k_1 and k_2 remain constant, thus decreasing the amount of $\text{O}_2^{\cdot -}$ removed before zero-order kinetics are established. At 55°C, k_3 is approx. $2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ and so k_3/k_1 is about 1:3

Fig. 1. Effect of the reducing radical $\text{CO}_2^{\cdot -}$ on A_{480} of the native enzyme

The enzyme solution (5.7 μM) at pH9 containing formate (0.1 M) in place of ethanol was deoxygenated and saturated with N_2O by gently bubbling with the gas for about 30 min. This procedure caused no inactivation of the enzyme. The solution was then transferred anaerobically to an irradiation cell (path-length 7.5 cm) at room temperature (about 25°C). A train of 300 pulses (the pulses were separated by 20 ms), each producing 1.2 μM - $\text{CO}_2^{\cdot -}$, caused a decrease in A_{480} . The decrease was complete after about 100 pulses. A second train of pulses applied 45 s after the first train caused no further change in A_{480} .

Fig. 2. Arrhenius plot for the second-order rate constant k_3

$\ln(k_3)$ is plotted against $1/T$. The gradient of the line leads to the empirical activation energy (E_a) for entry into the slow cycle (Table 2). k_3 was obtained at each temperature as follows. Values of $k_1 = k_2 = 5.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ and k_5 , as determined from the line fitted to the Arrhenius plot in Fig. 3, were used in the computer calculation of substrate decay under experimental conditions. k_3 was varied until the computed curve was in best agreement with the data at each temperature. The values of the rate constant so determined (and hence, E_a) would be only slightly altered if Model II were used in place of Model I in the calculation. The zero-order decay curve at 5°C is in poor agreement with computer predictions; therefore the value of k_3 at this temperature has not been used in calculating the straight line plotted.

Fig. 3. Arrhenius plot for the first-order rate constant k_5 . The plot shows the variation with temperature of the rate constant for the post-irradiation rise as observed at 480nm . The gradient of the line leads to the empirical activation energy (E_a) for exit from the slow cycle (Table 2). The value of k_5 (and hence E_a) comes directly from observations and so is unaltered if Model II is considered instead of Model I (i.e. $k_5' = k_5$).

Fig. 4. Effect of temperature on the observed decay of O_2^- at high ratio of substrate concentration to enzyme concentration

The enzyme solution ($0.4 \mu\text{M}$) at pH9 was exposed to a pulse of radiation producing approx. $65 \mu\text{M} \cdot \text{O}_2^-$. The optical path-length was 2cm . The decay at 15°C (a) is clearly slower than the decay at 45°C (b). In addition, the rapid decrease in absorption at the start of the decay is almost absent at the higher temperature (see the text).

compared with 1:12 at 25°C . The increase in this ratio might be expected to alter the rate of the approximately exponential decay observed at low substrate concentrations. However, computer predictions show that the effect on this decay of increasing temperature up to 55°C is negligible.

From the Arrhenius activation parameters for k_3 and k_5 the enthalpies and entropies of activation ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) for steps (3) and (4) have been calculated (Table 2) by using transition-state theory (Schaleger & Long, 1963). This theory leads to a value of $-88 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for the entropy of activation of steps (1) and (2). This can be compared with a value of close to zero for step (3). According to Model I, steps (2) and (3) involve the same reactants (i.e. enzyme E_B and O_2^-) and therefore the difference between the entropies of activation should reflect the difference between the entropies of the two transition states. Thus the transition state of step (3) is less ordered than that of step (2). This could be due to an unfolding of the protein structure (Laidler & Bunting, 1973) which may include, more specifically, displacement of a ligand bound to the manganese (e.g. the replacement of a water molecule).

Table 2. *Activation parameters for the enzyme reactions*

The values ($\pm$ s.d.) of empirical activation energies (E_a) were calculated from the measured gradients of Figs. 2 and 3. If all points are included in calculating the straight line of Fig. 2, the activation energy for entry into the slow cycle becomes $29.8 \pm 3.3 \text{ kJ} \cdot \text{mol}^{-1}$, which modifies ΔS^* to $-2.7 \pm 5.4 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Values of ΔS^* were calculated by using $k = 5.67 \times 10^{10} \text{ Texp}(\Delta S^*/R) \exp(-E_a/RT)$ (Schaleger & Long, 1963), where k is obtained from the line fitted to the corresponding Arrhenius plot. The errors quoted for ΔS^* reflect only the standard deviation in the measured E_a . The use of Model II instead of Model I alters only slightly the activation parameters for the entry into the slow cycle and does not affect the parameters for the exit from the slow cycle (see legends to Figs. 2 and 3). ΔH^* and ΔS^* were calculated for 25°C and by using a standard state of 1 mol/l .

	Entry to the slow cycle	Exit from the slow cycle
$E_a \text{ (kJ} \cdot \text{mol}^{-1})$	36.8 ± 1.7	63.2 ± 2.1
$\Delta H^* \text{ (kJ} \cdot \text{mol}^{-1})$	34.3 ± 1.7	60.7 ± 2.1
$\Delta S^* \text{ (J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1})$	8.8 ± 2.9	-5.9 ± 7.5

If a pre-equilibrium is involved either between forms E_B and E_B^* in Model I or between forms E_A' and $E_A'^*$ in Model II (McAdam *et al.*, 1977), then variations of the zero-order decay rate with temperature will be affected by the temperature-dependences of both k_3 and the equilibrium constant K . Separate evaluation of these dependences is not possible from the present measurements. It should be noted that since the magnitude of the change in A_{480} observed after the first pulse of a train of pulses is the same for both the native and the H_2O_2 -treated enzyme at all temperatures then $[E_B^*] < [E_B]$. Thus the existence of such a pre-equilibrium could not account for the temperature-independence of k_1 and k_2 .

Optical changes at different wavelengths during and after turnover

The involvement of the manganese in catalysis is shown by changes in A_{480} during and after turnover (McAdam *et al.*, 1977). At this wavelength the molar extinction coefficients of forms E_A , E_B and E_C are in the order $\epsilon_A > \epsilon_B \approx \epsilon_C$. Observations at wavelengths other than 480 nm support the involvement of one or more forms of the enzyme different from E_A and E_B . For instance, at 400 nm (Fig. 5) $\epsilon_C > \epsilon_A > \epsilon_B$.

Substrate decay is normally studied at 250 nm and there are changes which occur at this wavelength other than those directly attributable to $\text{O}_2^{\cdot -}$ (Fig. 6), implying that $\epsilon_A > \epsilon_B > \epsilon_C$. The A_{250} after the post-irradiation rise is greater than the A_{250} due to form E_A . Only about 50% of this difference is due to the generated H_2O_2 ; the reason for the remaining difference is unknown. Over a longer time (approx. 5s)

reduction by H_2O_2 occurs, and the A_{250} of the enzyme corresponds to that expected for form E_B .

The measured difference between ϵ_A and ϵ_B at 250 nm is about $450 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ compared with an ϵ_{250} of the enzyme of about $28000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Thus spectrophotometric confirmation of the difference is difficult. As with other Mn-superoxide dismutases (Weisiger & Fridovich, 1973), the enzyme from *B. stearothermophilus* exhibits a shoulder in its u.v. spectrum at about 230 nm . The results at 250 nm support the idea that this shoulder is associated with the active centre of the enzyme.

Chemical nature of the enzyme species involved during catalysis

As discussed above, comparison with the spectra of known manganese compounds suggests that Mn-superoxide dismutases possess Mn(III) in the native state. For the Mn-superoxide dismutase from *B. stearothermophilus*, both the native and the peroxide-treated enzyme participate in the fast catalytic cycle. In all discussions it has been assumed that the manganese in the native superoxide dismutase is reduced by H_2O_2 to Mn(II). An e.p.r. (electron-paramagnetic-resonance) spectrum might be expected for a Mn(II) species (see, for example, Haffner *et al.*,

Fig. 5. *Changes in A_{400} during and after turnover* Enzyme ($7 \mu\text{M}$) at pH9 was subjected to a train of 16 radiation pulses (2.5 ms between pulses) each producing about $17 \mu\text{M} \cdot \text{O}_2^{\cdot -}$. The arrow marks the sixteenth pulse. The path-length of the optical cell was 7.5 cm and the experiments were carried out at ambient temperature (about 30°C). Trace (a) refers to native enzyme and trace (b) to peroxide-reduced enzyme (approx. $180 \mu\text{M} \cdot \text{H}_2\text{O}_2$).

Fig. 6. Changes in A_{250} during and after turnover. Enzyme ($7\mu\text{M}$) at pH9 was subjected to a train of 16 radiation pulses (2.5 ms between pulses) each producing about $17\mu\text{M-O}_2^{\cdot-}$. The path-length of the optical cell was 0.7 cm and the experiments were carried out at ambient temperature (about 30°C). (a) Native enzyme; (b) enzyme reduced by the H_2O_2 (approx. $180\mu\text{M}$) generated during the first train of pulses. Owing to the absorption of $\text{O}_2^{\cdot-}$ at this wavelength, it is easier to present the output of the transient recorder as displayed on the oscilloscope rather than as recorded on the chart-recorder (cf. Fig. 5).

1974). Our preliminary e.p.r. experiments have given no evidence for Mn(II) in the peroxide-treated enzyme, although line broadening may cause considerable decrease in amplitude of the manganese signal (Coleman, 1971; Reed & Ray, 1971; Reed & Cohn, 1973).

For the Mn-superoxide dismutase from *E. coli*, n.m.r. studies of proton exchange suggest that there is a water molecule bound directly to the Mn(III) in the native enzyme (Villafranca *et al.*, 1974). These authors conclude that an outer-sphere electron transfer must be important catalytically, since the water-exchange rate is too low to be involved in the mechanism. If the *B. stearothermophilus* and the *E. coli* enzyme have similar ligands attached to the manganese, then the fast cycle may involve outer-sphere electron transfer.

There is no clear indication of the detailed chemical changes involved in the slow cycle. If attack of $\text{O}_2^{\cdot-}$ occurs at the same centre (e.g. the manganese) in both fast and slow cycles, then relative activation entropies suggest that ligand substitution, such as solvent replacement, could occur during entry into the slow cycle. The fast cycle may be a redox reaction proceeding by an outer-sphere mechanism, whereas the slow cycle may involve an inner-sphere substitution. Thus k_2 ($5.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) would be the rate constant for

outer-sphere oxidation of the Mn(II) in the enzyme, whereas k_3 would represent the rate constant for inner-sphere substitution. [Results obtained in a study of the reaction between $\text{Mn}^{2+}_{\text{aq}}$ and $\text{O}_2^{\cdot-}$ were interpreted in terms of formation of a complex (Pick-Kaplan & Rabani (1976).] A value of $k_3 = 4.8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ is not unreasonable for inner-sphere substitution at Mn(II) (Hague, 1971). The first-order rate constant k_5 (70 s^{-1}) would be associated with the loss of the peroxide ion from the Mn(III) centre. Even though in this comparison the same reactants are involved, there is no definite correlation between entropies of activation and mechanism of redox reactions (see Taube, 1970; Wilkins, 1974). Nevertheless, there are a few examples in the literature of competing inner- and outer-sphere reaction pathways where activation parameters have been studied. For instance, in the reaction of IrCl_6^{2-} with $\text{Cr}(\text{H}_2\text{O})_6^{2+}$ an inner-sphere mechanism becomes relatively more important as the temperature is increased (Sykes & Thorneley, 1970), which is observed also in the present case. It must be remembered, however, that, for a protein, concomitant changes in conformation will also contribute to $\Delta S^\ddagger$. If the reactants involved in the fast and slow cycles are different (e.g. a pre-equilibrium is involved), then comparison of entropies of activation are less likely to give useful mechanistic information. It is possible also that, in the formation of the unreactive form of the enzyme, $\text{O}_2^{\cdot-}$ does not attack at the same active site as is involved in fast catalysis.

If water is attached to a trivalent metal, then the possibility of hydrolysis has to be considered. $\text{Mn}^{3+}_{\text{aq}}$ is the strongest acid of the terpositive ions of the first transition series (Wells, 1965; Wells & Davies, 1965); the acidic and basic forms have visible absorption spectra of similar shape, although the latter has a 3-fold higher extinction coefficient at the maximum, 470 nm (Davies, 1969). The pK_a of the *trans*-1,2-diaminocyclohexane tetra-acetate of Mn(III) is 8.1 and the visible spectrum of the basic form is similar in intensity to that of the acidic form, with a shift in the maximum from 510 to 450 nm (Hamm & Suwyn, 1967). The A_{480} of the native enzyme used in the present work varies little with pH or temperature. However, it may be possible to detect small differences in absorption occurring at this and other wavelengths by using higher concentrations of enzyme. During catalysis the precursors of H_2O_2 , including the highly basic O_2^{2-} , are obviously close to the manganese and may be involved in reactions with protons of the ligands. Any hydrolysis at the metal centre, which can be represented as:

would affect the lability towards substitution of other ligands attached to the manganese (Wilkins, 1974; Davies, 1974) and may also affect the rate of redox reactions at the metal centre (Wilkins, 1974).

The Mn-superoxide dismutase from *B. stearothermophilus*, like those from *E. coli*, *Streptococcus mutans* and chicken liver mitochondria (Weisiger & Fridovich, 1973), contain one Mn per two subunits. The subunits appear to be identical (Bridgen *et al.*, 1975) and manganese release is achieved only under conditions that cause dissociation of the dimeric enzyme (Brock *et al.*, 1976). Knowledge of the exact environment of the manganese, which should come from a continuation of X-ray-crystallographic studies (Bridgen *et al.*, 1976; Beem *et al.*, 1976), will enable proposed mechanisms of catalysis to be more realistically considered.

Conclusions

The properties of the Mn-superoxide dismutase from *B. stearothermophilus* are similar to those found for other Mn-superoxide dismutases. The thermal stability of the enzyme permits its study over a wide range of temperature and it is clear that the slower secondary catalytic cycle becomes faster and relatively more important at high temperatures. The exact cause of the temperature-dependences of the different processes is not clearly understood and further studies are necessary to establish whether or not pre-equilibria are an essential feature of the mechanism. Although zero-order kinetics will probably not be approached in the organism, it is clear that at 60°C both the fast and the slow cycles will be involved in O₂^{-•} dismutation. There seems to be no simple explanation, at least in terms of the dismutation reaction, as to why two pathways are available for catalysis.

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