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

A KINETIC MODEL FOR THE ENZYME ACTION

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The enzymic reaction mechanism of a manganese-containing superoxide dismutase from *Bacillus stearothermophilus* was studied by using pulse radiolysis. During catalysis (pH 8.9; 25°C), changes occurring in the kinetics of substrate disappearance and in the visible absorption of the enzyme at 480 nm established that the simple two-step mechanism found for copper- and iron-containing superoxide dismutases is not involved. At a low ratio (<15) of substrate concentration to enzyme concentration the decay of $O_2^{-\bullet}$ is close to exponential, whereas at much higher ratios (>100) the observed decay is predominantly zero-order. The simplest interpretation of the results invokes a rapid one-electron oxidation–reduction cycle ('the fast cycle') and, concurrently, a slower reaction giving a form of the enzyme that is essentially unreactive towards $O_2^{-\bullet}$ but which undergoes a first-order decay to yield fully active native enzyme ('the slow cycle'). The fast cycle involves the native enzyme E_A and a form of the enzyme E_B which can be obtained also by treating the form E_A with H_2O_2. Computer calculations made with such a simple model predict behaviour in excellent agreement with the observed results.

Superoxide dismutases have been isolated from a large number of eukaryotic and prokaryotic organisms and found to contain copper, iron or manganese as the functional metal of the enzyme (see reviews by Fridovich, 1974, 1975). Generation of the substrate, $O_2^{-\bullet}$, by pulse radiolysis has enabled mechanistic studies of the Cu- (copper-containing) superoxide dismutases isolated from bovine and human erythrocytes (Rotilio *et al.*, 1972; Klug *et al.*, 1972; Bannister *et al.*, 1973; Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974), the Mn- (manganese-containing) superoxide dismutase from *Escherichia coli* (Pick *et al.*, 1974) and the Fe- (iron-containing) superoxide dismutase from *Photobacterium leiognathi* (Lavelle *et al.*, 1977). For the bovine erythrocuprein and the Fe-superoxide dismutase, disappearance of substrate was found to be first-order (i.e. the decay of the A_{250} of $O_2^{-\bullet}$ was exponential) for all ratios of substrate concentration to enzyme concentration reported (Fielden *et al.*, 1974; Lavelle *et al.*, 1977). By following changes in the optical absorption band associated with the particular metal centre it was deduced that the catalytic mechanism involved alternate one-electron oxidation and reduction by substrate:

$$E_A + O_2^{-\bullet} \xrightarrow{k_1} E_B + O_2 \quad (1)$$

$$E_B + O_2^{-\bullet} \xrightarrow[2H^+]{k_2} E_A + H_2O_2 \quad (2)$$

E_A represents native oxidized enzyme and E_B reduced enzyme. Second-order rate constants, k_1 and k_2, were determined experimentally by the use of an excess of enzyme (either E_A or E_B) over substrate and following the rate of change of the absorption at the wavelength maximum of the metal absorption band. k_1 and k_2 were identical and equal to the characteristic second-order rate constant measured under turnover conditions.

For the Mn-superoxide dismutase isolated from *E. coli* such a simple scheme appears not to be applicable (Pick *et al.*, 1974). It was suggested that, not two, but four oxidation–reduction reactions are involved, each characterized by a different second-order rate constant; three oxidation states of manganese were postulated, the relative concentrations of these present during dismutation depending on the relative amounts of substrate and enzyme.

In the present work, pulse radiolysis has been used to study the mechanism of action of the Mn-super-

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oxide dismutase isolated from *Bacillus stearothermophilus*. Not only has the decay of substrate been studied at 250nm, but also the involvement of the manganese has been directly demonstrated by following changes in the A_{480} which occur during dismutation.

Materials and Methods

General

Purified enzyme samples, dissolved in a 30%-satd. $(NH_4)_2SO_4$ solution, were kindly supplied by Dr. J. I. Harris. The methods of isolation and purification (J. I. Harris, personal communication) are outlined elsewhere (Atkinson *et al.*, 1972; Bridgen *et al.*, 1976; Brock *et al.*, 1976). The enzyme (mol.wt. 40000) consists of two identical subunits and contains 1 g-atom of Mn/mol (Bridgen *et al.*, 1975, 1976).

Before use, samples were dialysed overnight against buffer containing sodium pyrophosphate (2mM) and EDTA (100μM) adjusted to pH9 (H_2SO_4) and centrifuged (15000*g*; 30min). Absorption spectra were measured with a Cary 15 spectrophotometer and enzyme concentrations were determined by using $A_{280}^{1\%} = 13.2$* (J. I. Harris, personal communication) and the known molecular weight. The visible spectrum has a $\lambda_{max.}$ at 480nm and deduced ε_{480} values were in the range 590–710 litre·mol^{-1}·cm^{-1} for the different batches of enzyme studied. For the sample used for most of the present work the latter was the measured value.

Triple-distilled water was used throughout and all chemicals were of the highest purity available.

Pulse radiolysis

The basic pulse-radiolysis system and its use for studying superoxide dismutase have been described elsewhere (Fielden *et al.*, 1974). Samples were contained in a thermostatically controlled (25°C) high-purity silica cell of path-length 2cm. Unless stated otherwise, reaction media contained sodium pyrophosphate (2mM), EDTA (100μM) and ethanol (0.1M) and were at pH8.9. For irradiations involving generation of large quantities of $O_2^{-\cdot}$ (>20μM), either by a single high-dose electron pulse or by a series of closely spaced lower-dose pulses, buffer was saturated with oxygen by bubbling and then the required small volume of enzyme stock solution was added.

Data analysis

A transient recorder (Data Laboratories, 28 Wates Way, Mitcham, Surrey, U.K.) which stores the magnitude of the transmitted light intensity at 1024 equally spaced time intervals was used to monitor absorption changes occurring during and after irradiation. The stored data were either displayed on an oscilloscope screen, where they could be photographed, or transmitted to an X–Y recorder. In addition the data from the transient recorder were fed directly into a computer, where they were stored on a magnetic disc for subsequent analysis. The changes in transmitted light intensity were converted into absorbances which could be fitted to expressions that were exponential or linear in time, by using a least-squares method to evaluate the parameters of best fit (Brandt, 1970).

Changes in absorption occurring over times longer than 10s were recorded by using only an oscilloscope and a camera.

* More recent determinations lead to $A_{280}^{1\%} = 14.2$ (J. I. Harris, personal communication). The use of this value alters the calculated rate constants only slightly.

Results and Discussion

Effect of H_2O_2

For the copper- and iron-containing superoxide dismutases studied by pulse radiolysis, reactions (1) and (2) are sufficient to explain their modes of action (Fielden *et al.*, 1974; Lavelle *et al.*, 1977). In both cases, H_2O_2 acts as a reducing agent towards the metal centre in the native enzyme, causing bleaching (decrease in intensity) of the characteristic chromophore, and this provides one method of producing form E_B. For the *B. stearothermophilus* Mn-superoxide dismutase, H_2O_2 causes a similar bleaching of the A_{480} and this may be assumed to correspond to a reduction of the metal centre from Mn(III) to the Mn(II), as is discussed in the following paper (McAdam *et al.*, 1977). However, unlike the Cu- and Fe-containing superoxide dismutases (Bray *et al.*, 1974; Lavelle *et al.*, 1977), the Mn-superoxide dismutase from *B. stearothermophilus* was not inactivated by H_2O_2 (McAdam *et al.*, 1977).

Since H_2O_2 is a product of the dismutation reaction, it is necessary to consider here the extent of bleaching of A_{480} by H_2O_2. For different batches of enzyme the addition of an approx. 20-fold excess of H_2O_2 to the enzyme (approx. 100μM) caused between 70 and 90% decrease in A_{480} as measured in the Cary spectrophotometer. For the sample used to test the kinetic model (see below), all measurements of the decrease in A_{480} made with the spectrophotometer were within the range 75–85%. An oscilloscope with a slow time-base was used to follow the decrease in A_{480} occurring immediately after irradiation due to reaction of the enzyme with the H_2O_2 generated during turnover. Decreases measured in this way were within 65–75% of the absorbance expected for the native enzyme, as calculated from the known ε_{480} and enzyme concentration. The remaining absorbance that is unaffected by H_2O_2 may be due to form E_B or to adventitious impurities; whichever is the case does not affect kinetic deductions. However, when com-

paring computer predictions with observations (e.g. Figs. 1 and 5), the amplitude of the predicted absorbance changes have been adjusted by the necessary small amounts to fit the amplitude of the observed changes. Such adjustments do not alter the derived values of the rate constants.

Reaction of enzyme with substrate: disagreement with a simple two-step mechanism

On the assumption that the Mn-superoxide dismutase would act similarly to its Cu- and Fe-containing counterparts (i.e. via a simple two-step mechanism), experiments analogous to those carried out with the latter enzymes (Fielden *et al.*, 1974; Lavelle *et al.*, 1977) were performed. The results which indicated that such a model was not applicable are presented, and then discussed in a more detailed comparison with the predictions of the proposed model.

By using an excess of enzyme over substrate and following the changes in A_{480}, the rate of reaction of the native enzyme E_A and of the peroxide-treated enzyme E_B were determined (Fig. 1). Such measurements on three separate enzyme samples gave the mean ratio of these rates as 1.04 ± 0.09. Thus, within experimental error, if the two-step mechanism were operative, $k_1 = k_2$, as was found for the previously studied dismutases (Fielden *et al.*, 1974; Lavelle *et al.*, 1977). It should be noted that, even for a simple two-step mechanism, under conditions where enzyme concentration is only about 10-fold greater than substrate concentration (i.e. $[O_2^{-\bullet}]/[E_0] \simeq 0.1$, where $[E_0]$ is the concentration of either E_A or E_B), the observed absorbance changes are not expected to be truly exponential. Deviation from exponentiality arises because there is a significant reaction between substrate and the generated complementary form of the enzyme. However, the noise level of the observed traces prohibits detection of this deviation. A least-squares computer fit of the closest exponential to the predicted trace gives a rate constant which is about 6% greater than the true k_1 (or k_2); again such a small difference is not detectable on the observed trace.

At lower enzyme concentrations the decay of the substrate can be followed at 250nm. For the simple two-step model such a decay should be a true exponential with rate constant k_1 ($=k_2$). The decay of $O_2^{-\bullet}$ was found to be exponential when $[O_2^{-\bullet}]/[E_0] < 15$ (Fig. 2), although, as this ratio increased within this range, the goodness of fit decreased, as did the apparent first-order rate constant. For $[O_2^{-\bullet}]/[E_0] > 15$, deviations from first-order decay became obvious, and at very high ratios the decay was nearly zero-order (Fig. 3) with a rate given approx. by $1800[E_0]\text{M}\cdot\text{s}^{-1}$, where $[E_0]$ is the total concentration of enzyme. Under either exponential or zero-order conditions, the rate of dismutation was first-order in enzyme concentration.

Thus, as found by Pick *et al.* (1974) for the Mn-superoxide dismutase isolated from *E. coli*, the simple two-step mechanism does not explain the observations. In the present case, the zero-order decay kinetics imply a substrate-independent rate-limiting step and suggest the involvement of some unreactive form of the enzyme (i.e. one which does not react with $O_2^{-\bullet}$).

The absorption changes seen in the manganese band when the enzyme solution is subjected to a train of closely spaced electron pulses, each pulse producing approx. 3equiv. of $O_2^{-\bullet}$, show the involvement of this unreactive form (Fig. 5). A simple two-step model predicts that a state comprising almost equal concentrations of forms E_A and E_B results after just one pulse, and this state should be virtually unchanged by subsequent pulses, as is seen for the Cu- and Fe-containing superoxide dismutases (Fielden *et al.*, 1974; Lavelle *et al.*, 1977). However, in the present case (Fig. 5) subsequent pulses further lower the absorption until a steady level is reached after about 12 pulses (for the conditions shown); the fraction of enzyme present as E_A has therefore been decreased to below one-half. After irradiation there is a dramatic exponential increase in the absorption back to almost its initial value. This rise is characterized by a first-order rate constant of $70\,\text{s}^{-1}$ which was independent of the concentration of enzyme, oxygen or H_2O_2.

Observation of substrate decay at 250nm during a similar train of 16 pulses shows that the rate of decay decreases with successive pulses (Fig. 7). For the given conditions all of these decays are approximately exponential and the observed first-order rate constant changes by a factor of 2.5 between the first and the sixteenth decay. Comparison with the results at 480nm shows that the decrease in A_{480} parallels the decrease in the fitted rate constant, both reaching a steady value after about 12 pulses. Thus there exists a slower secondary cycle in the operation of the enzyme which becomes more significant as a result of the continued presence of $O_2^{-\bullet}$, and which, when the $O_2^{-\bullet}$ concentration has been depleted, regenerates the native enzyme E_A.

The simplest model for the mechanism of action of the enzyme

The simplest scheme which will at least qualitatively describe the observations is the following (neglecting any possible involvement of solvent species in steps 3 and 4):

Model I

$$E_A + O_2^{-\bullet} \xrightarrow{k_1} E_B + O_2 \qquad (1)$$

$$E_B + O_2^{-\bullet} \xrightarrow[2H^+]{k_2} E_A + H_2O_2 \qquad (2)$$

$$E_B + O_2^{-\cdot} \xrightarrow{k_3} E_C \quad (3)$$

$$E_C \xrightarrow{k_5} E_A \quad (4)$$

The use of k_5 in eqn. (4) facilitates comparison with Model II which agrees equally well with the data and is presented below. However, the above represents the scheme containing the minimum number of reactions compatible with the experimental observations. It has been assumed that the native enzyme E_A, the species absorbing most strongly at 480nm, is reduced by both $O_2^{-\cdot}$ and H_2O_2 to form E_B; the kinetic considerations are unaffected if, instead, oxidation is occurring in both cases.

From the foregoing discussion it is to be expected that $k_3 \ll k_1 = k_2$. k_5 should be about $70\,s^{-1}$, representing the conversion of the unreactive form of the enzyme E_C into E_A (i.e. the post-irradiation rise of Fig. 4). Eqns. (1)–(4) give rise to a set of simultaneous differential equations for the concentrations of forms E_A, E_B and E_C and $O_2^{-\cdot}$ as a function of time. Under steady-state conditions, when:

$$\frac{d[E_A]}{dt} \simeq \frac{d[E_B]}{dt} \simeq \frac{d[E_C]}{dt} \simeq 0 \quad (5)$$

then, if $k_1 = k_2$:

$$-\frac{d[O_2^{-\cdot}]}{dt} \simeq \frac{2k_1k_5(k_1+k_3)[E_0][O_2^{-\cdot}]}{k_5(2k_1+k_3)+k_1k_3[O_2^{-\cdot}]} \quad (6)$$

where $[E_0]$ is the total concentration of enzyme. Such a steady state is closely approached at high concentrations of $O_2^{-\cdot}$ when eqn. (6) can be approximated to:

$$-\frac{d[O_2^{-\cdot}]}{dt} \simeq \frac{2k_5(k_1+k_3)[E_0]}{k_3} \quad (7)$$

and, since $k_3 \ll k_1$:

$$-\frac{d[O_2^{-\cdot}]}{dt} \simeq \frac{2k_1k_5}{k_3}[E_0] \quad (8)$$

Hence the expected zero-order decay velocity of ubstrate is not $k_3[E_0]$, which would be the case if the enzyme operated only through a scheme analogous to eqns. (3) and (4), forming an unreactive state on every encounter with $O_2^{-\cdot}$. Instead, the predicted zero-order rate is approximately $2k_1/k_3$ times this value. Thus a zero-order decay rate of $1800[E_0]\,M\cdot s^{-1}$ is consistent with $k_5 = 70\,s^{-1}$, if $k_3/k_1 = 12$. The model presented is capable of quantitatively correlating the observed post-irradiation rise with the observed zero-order decay kinetics.

Eqn. (6) would lead to a straight line on a Lineweaver–Burk plot. However, under the experimental conditions the ratio of substrate concentration to enzyme concentration is too small for the steady-state approximation to be completely justified. The procedure described above leads to a first estimate of k_3 for use in computer calculations.

For a closer examination of the model it is necessary to solve the simultaneous differential equations as a function of time, rather than to choose a particular solution. This has been done by using a computer program. The rate constants have been chosen to give the best agreement between the data and the computer predictions for all experimental measurements. Variation of the values of any of these constants by 10% makes a significant difference to the closeness with which the theoretical model fits the data. However, different enzyme isolations gave variations in these rate constants of up to 20%, and consequently, so that the model could be rigorously tested, the results referred to here correspond to a single isolation. For this isolation, the values of rate constants giving the best fit for results at 25°C are k_1 $(=k_2) = 5.6\times10^8\,M^{-1}\cdot s^{-1}$, $k_3 = 4.8\times10^7\,M^{-1}\cdot s^{-1}$, $k_5 = 70\,s^{-1}$. The comparison can now be considered in more detail.

Reaction of substrate with an excess of native or reduced enzyme

The traces obtained under conditions where $[O_2^{-\cdot}]/[E_0] \simeq 0.1$ are predicted to be non-exponential, not only because of the reason discussed above but also because of the presence of a secondary cycle in the mechanism. However, noise level does not permit distinction between the observed trace and an exponential, and the experimental data in Fig. 1 could be fitted with an exponential having a

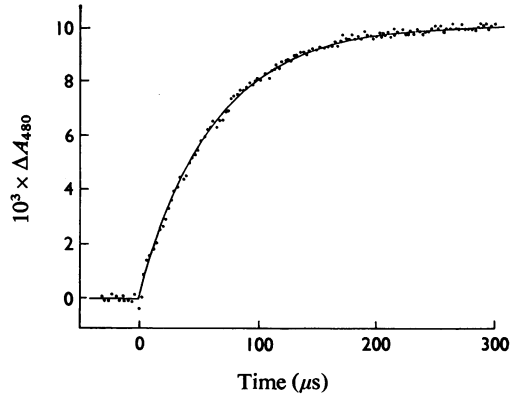

Fig. 1. *Change in A_{480} when an excess of reduced enzyme is exposed to substrate*

The enzyme (25μM) had been reduced by H_2O_2 (approx. 200μM for 4min), and at zero time a single pulse of radiation produced approx. 2.4μM-$O_2^{-\cdot}$. Every fifth data point has been plotted (·). The solid curve is obtained directly from the theoretical Model I; the total absorbance change has been adjusted to fit the observed absorbance change (see the text).

first-order rate constant of $(1.55\pm0.03)\times10^4 s^{-1}$. Fig. 1 includes a curve predicted by Model I with the above values of the rate constants. As in the case of the experimental data, this curve could be closely fitted by an exponential.

The theory predicts that, if $k_1 = k_2$, then the rate constant measured by the above procedure should be 8% higher starting from reduced enzyme (as in Fig. 1) than starting from the native enzyme; this is due to the small influence of step (3). In fact, the latter rate constant appears to be slightly greater than the former, a ratio of 1.04 ± 0.09 being found (see above). It is clear that precision of measurement does not permit any positive conclusion; if the difference were proved to be genuine it would be easy to modify the theory by allowing a small inequality between k_1 and k_2.

It must be stressed that it is incorrect to evaluate k_2 by dividing by $[E_0]$ the first-order rate constant as obtained above. The value so obtained is $(6.20\pm0.12)\times10^8 M^{-1}\cdot s^{-1}$ for the fit to the experimental data points. This can be compared with $5.6\times10^8 M^{-1}\cdot s^{-1}$, which is the rate constant used for k_1 and k_2 in all of the theoretical calculations.

Reaction of substrate with enzyme: $[O_2^{-\cdot}]/[E_0]<15$

There is good agreement between theory and experiment under conditions where the decay of $O_2^{-\cdot}$ is close to exponential (Fig. 2). In contrast with the decays observed under similar conditions for the Cu- and Fe-superoxide dismutases (see above), this decay is not truly exponential, because of the involvement of the secondary cycle. However, the predicted deviation from exponentiality is small and an exponential fit to the data of Fig. 2 gives a first-order rate constant of $(2.20\pm0.08)\times10^2 s^{-1}$. Division of this number by $[E_0]$ yields an approximate second-order rate constant of $(5.5\pm0.2)\times10^8$ $M^{-1}\cdot s^{-1}$. This is very close to $5.6\times10^8 M^{-1}\cdot s^{-1}$, the value used for k_1 and k_2 in calculating the predicted curve. The effect of the secondary cycle in the theoretical model is to increase the initial rate relative to the final rate of decay; however, for relatively low $[O_2^{-\cdot}]/[E_0]$ the major influence arises from k_1 and k_2.

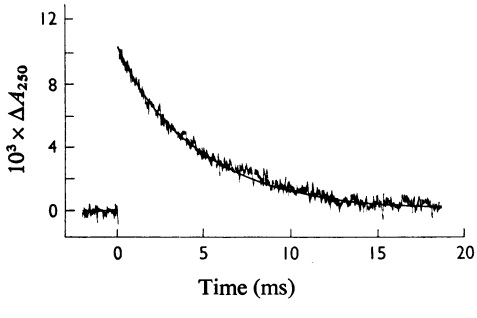

Fig. 2. *Decay of substrate in the presence of enzyme: low ratio of substrate concentration to enzyme concentration*
The experimental trace shows the decrease in the A_{250} of $O_2^{-\cdot}$ (2.6 μM) in the presence of enzyme (0.4 μM). The smooth curve is the prediction of the theoretical model (Model I).

Reaction of substrate with enzyme: $[O_2^{-\cdot}]/[E_0]>15$

For higher $[O_2^{-\cdot}]/[E_0]$ ratios the decay becomes non-exponential, and for values >100 it becomes predominantly zero-order (Fig. 3). The rapid decay at the start of this trace arises because initially most of the enzyme participates in the fast cycle [eqns. (1)

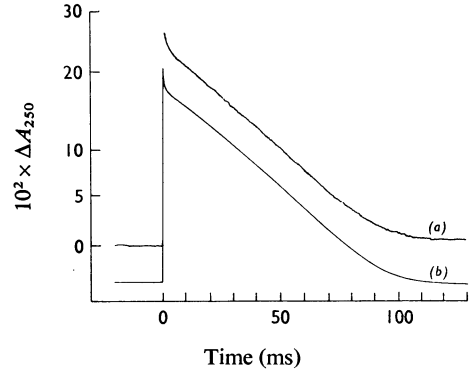

Fig. 3. *Decay of substrate in the presence of enzyme: high ratio of substrate concentration to enzyme concentration*
Trace (*a*) shows the decay of the transient A_{250} of $O_2^{-\cdot}$ (66 μM) in the presence of enzyme (0.4 μM). Trace (*b*) shows the theoretical curve predicted by Model I. Trace (*b*) has been displaced vertically for clarity. Since data are recorded as changes in transmitted light intensity the absorbance scale is non-linear; this becomes apparent only at high absorbance.

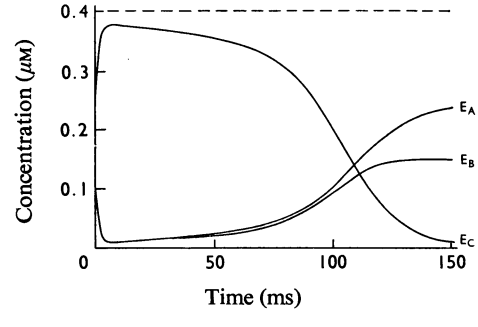

Fig. 4. *Theoretical predictions of concentrations of forms E_A, E_B and E_C according to Model I for* $[O_2^{-\cdot}]/[E_0]\gg15$
The predictions refer to conditions identical with those in Fig. 3. ----, Total enzyme concentration.

and (2)], then, as a greater proportion of the enzyme is converted into form E_C, the observed decay approaches zero-order. Towards the end of the decay the $O_2^{-\bullet}$ concentration is too low to maintain a sufficiently large proportion of the enzyme in the E_C state and the decay deviates from zero-order. Fig. 4 shows the theoretical predictions for the amounts of forms E_A, E_B and E_C present as a function of time.

The final absorbance is slightly higher than that observed before irradiation (Fig. 3). The presence of H_2O_2 generated during dismutation accounts for only 30% of this residual absorbance. Because of this, precise agreement between theory and experiment is difficult to attain.

Multiple-pulsing experiments

The effect of multiple pulsing on the A_{480} (Fig. 5) is in accord with the theoretical model. The measured absorbance reached after the first pulse of a train is the same, within 10%, whether native or reduced enzyme is present initially. This illustrates that all of the manganese present, which has an A_{480} bleachable by H_2O_2, is simultaneously involved in catalysis. This is in contrast with the findings for bovine erythrocuprein (Fielden *et al.*, 1974), but similar to the results obtained with Fe-superoxide dismutase from *Photobacterium leiognathi* (Lavelle *et al.*, 1977).

In fitting the theoretical model (Fig. 5) the rate of reduction of form E_A by H_2O_2 has been ignored. Observation on a longer time-scale (over 50s) showed that H_2O_2 reduction under the conditions of Fig. 5 proceeds with a first-order rate constant of about $1.6 s^{-1}$; this can be neglected compared with other rates. Since about 210 μM-H_2O_2 is generated during the first train of pulses, the deduced second-order rate constant for peroxide reduction is approx. $7.5 \times 10^3 M^{-1} \cdot s^{-1}$. This rough value applies only to the stated conditions and there is no evidence that the rate is indeed strictly second-order (cf. Bray *et al.*, 1974).

Good agreement between theory and experiment is obtained by assuming that forms E_B and E_C have identical ε_{480} values and that this value is 0.35 times the ε_{480} of form E_A. In other words, in this case, H_2O_2 caused a 65% decrease in A_{480} of the native enzyme. Any observed variation between theoretical

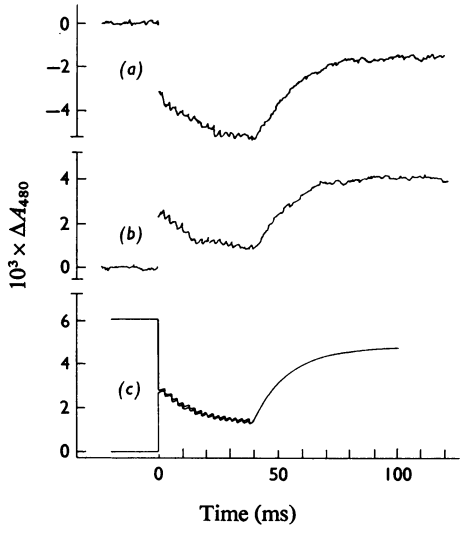

Fig. 5. *Changes in A_{480} when the enzyme is subjected to a train of pulses producing $O_2^{-\bullet}$*

Trace (*a*) shows the effect of 16 pulses (2.5ms between pulses), each producing 20 μM-$O_2^{-\bullet}$, on the A_{480} of the native enzyme (7 μM). Trace (*b*) shows the effect of a similar train of pulses on the enzyme after reduction by H_2O_2 (approx. 200 μM) generated during the first train of pulses; about 1 min was sufficient time for reduction (see the text). Traces (*c*) are the theoretical predictions (Model I) and are plotted assuming that form E_C has the same extinction coefficient as form E_B, the peroxide-reduced enzyme, at 480nm (see the text).

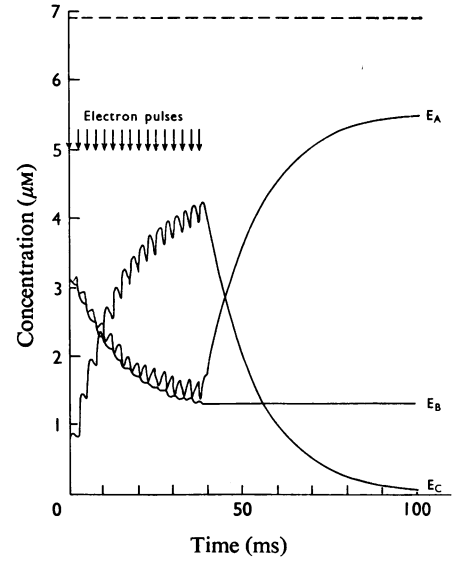

Fig. 6. *Theoretical predictions of the concentrations of forms E_A, E_B and E_C according to Model I for multiple-pulse experiment*

The predictions refer to conditions identical with those in Fig. 5. ----, Total enzyme concentration.

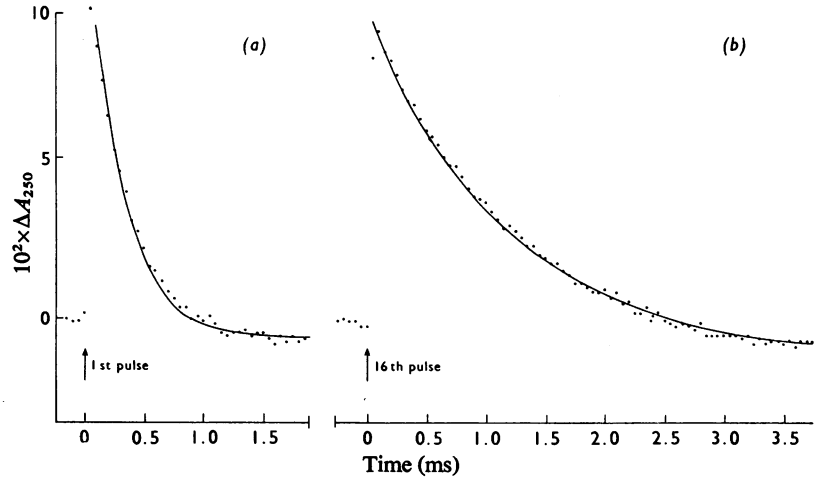

Fig. 7. *Decay of $O_2^{-\bullet}$ observed at 250nm during a train of pulses*

The dots (·) represent data points obtained for the decay of the $O_2^{-\bullet}$ (25μM) produced by the first and last pulses of a train of 16 pulses (2.5ms between pulses). The concentration of enzyme was 7μM. The zero time in (*a*) corresponds to the time of the first pulse and the zero time in (*b*) corresponds to the time of the sixteenth pulse. The theoretical curves predicted by Model I have been drawn to coincide with the experimental data at the end of each decay.

and experimental curves may be remedied by changing the extinction coefficient chosen for form E_C and, since form E_C is the major species present after several pulses (Fig. 6), small changes are sufficient. The exact shape of the theoretical curve is dependent also on the assumed concentration of $O_2^{-\bullet}$ per pulse.

Typical observations made at 250nm under similar conditions are shown in Fig. 7. The experimental results show a net decrease in absorbance after each pulse; this is almost certainly due to changes in the absorption band associated with the manganese (McAdam *et al.*, 1977). The data and the computer-calculated curves show clearly the decrease in decay rate between the first and last pulse. The exact size of this decrease, as predicted by the model, is very dependent on the precise $O_2^{-\bullet}$ concentration per pulse. A value of 25μM was chosen for this fit, being the measured average concentration generated per pulse during the train of 16 pulses, assuming $\varepsilon_{250}(O_2^{-\bullet}) = 2000$ litre·mol^{-1}·cm^{-1} (Rabani & Nielsen, 1969).

To establish that the post-irradiation recovery led to a state kinetically indistinguishable from the initial state (i.e. contained only form E_A or E_B), the solution was exposed to a second train of 16 pulses 65ms after the last pulse of the first train (i.e. at 105ms in Fig. 5). The measured first-order rate constants for the corresponding decays of the two trains agreed well with one another. The curves were fitted to exponentials, and comparison of the rate constants for decay gave ratios of 1.05±0.02 and 1.08±0.02 for the first and second pulses respectively of the two trains of pulses.

Modifications to theoretical model

(*a*) Introduction of a reverse step:

$$E_B + O_2^{-\bullet} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_C \qquad (9)$$

is an obvious modification, which would be in closer accord with many enzyme schemes if form E_C represents an enzyme–substrate complex. This would necessitate $(k_{-3}+k_5) = 70s^{-1}$, other rate constants remaining unchanged. However, the predicted post-irradiation rise (Fig. 5) would be decreased in amplitude, since some of form E_C will now yield form E_B (as well as form E_A). It seems likely therefore that $k_{-3} \ll k_5$, although it cannot be stated that it is zero.

(*b*) An alternative sequence of reactions leading into the slow cycle could involve a rapid pre-equilibrium between two forms of the enzyme:

$$E_B \underset{}{\overset{K}{\rightleftharpoons}} E_B^* \qquad (10)$$

$$E_B^* + O_2^{-\bullet} \xrightarrow{k_6} E_C \qquad (11)$$

followed by:

$$E_C \xrightarrow{k_5} E_A \qquad (12)$$

As represented, E_B and E_B^* are two forms of the reduced enzyme. Compared with the parameters of

the basic model, $k_6 = k_3/K$, where K is the equilibrium constant in reaction (10). Kinetically the distinction between the basic model and a model involving a pre-equilibrium is not possible. If a suitable property of form E_B enabled its study as a function of temperature or pH, then thermodynamic measurements may permit the existence of two equilibrating forms to be tested.

(*c*) It is possible to alter the model by increasing the complexity of reactions leading to the inactive state, as, for example, in Model II:

$$E'_A + O_2^{-\bullet} \xrightarrow{k'_1} E'_B + O_2 \quad (13)$$

$$E'_B + O_2^{-\bullet} \xrightarrow[2H^+]{k'_2} E'_A + H_2O_2 \quad (14)$$

$$E'_A + O_2^{-\bullet} \xrightarrow{k'_3} E'_D + O_2 \quad (15)$$

$$E'_D + O_2^{-\bullet} \xrightarrow{k'_4} E'_C \quad (16)$$

$$E'_C \xrightarrow{k'_5} E'_A \quad (17)$$

Step (15) does not necessarily correspond to a reduction of the metal centre. It could involve oxidation (i.e. H_2O_2 would be formed), in which case step (16) would represent reduction. As with Model I, solvent species may participate in the last three reactions of the scheme.

Model I and Model II agree equally well with all of the data. The best agreement for Model II occurs with values of k'_1 ($=k'_2$), k'_3 and k'_5 which are almost identical with k_1, k_3 and k_5 of Model I, and k'_4 is $5\times10^7\,M^{-1}\cdot s^{-1}$, although the exact value of the latter does not drastically affect the predictions. In both models the rate of the fast cycle (k_1, k_2 and k'_1, k'_2), the rate of entering the slow cycle (k_3 and k'_3) and the decomposition rate of the unreactive form of the enzyme (k_5 and k'_5) are the crucial factors affecting the predictions of the model.

After multiple-pulsing experiments, some form E'_D will be present which would be expected to modify the subsequent kinetic observations; since the amount is small, these modifications will be difficult to detect.

As in the case of Model I, it is possible to introduce a pre-equilibrium between forms E'_A and $E'_A{}^*$. In this case, however, measurements show that there is no change in the characteristic absorption of form E'_A at 480nm either as a function of pH (between 6.5 and 9.0) or as a function of temperature (between 25 and 55°C). This does not preclude any subtle changes in the environment of the manganese.

Conclusions

Both Model I and Model II are relatively simple kinetic schemes, the predictions of which agree closely with the observations. The chemical nature of the species present is not crucial to the comparison between theory and experiment, although it will be considered as an aid to defining a detailed mechanism (McAdam *et al.*, 1977). Involvement of a fast and a slow cycle during turnover has similarities with the postulated mechanism for the Mn-superoxide dismutase from *E. coli* (Pick *et al.*, 1974) and indicates a fundamental difference in the catalytic mechanism of such enzymes compared with their Cu- and Fe-containing counterparts.

The present observations suggest a form of the enzyme, E_C, which is unreactive towards substrate. Form E_C does not necessarily have to be an enzyme–substrate complex (although this is a possibility); however, it must undergo a first-order reaction to regenerate active enzyme, E_A. Full enzyme activity is regained about 50ms after the disappearance of substrate.

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References

Atkinson, A., Philips, B. W., Callow, D. S., Hones, W. R. & Bradford, P. A. (1972) *Biochem. J.* **127**, 63P–64P

Bannister, J. V., Bannister, W. H., Bray, R. C., Fielden, E. M., Roberts, P. B. & Rotilio, G. (1973) *FEBS Lett.* **32**, 303–306

Brandt, S. (1970) *Statistical and Computational Methods in Data Analysis*, p. 164, North-Holland Publishing Co., Amsterdam

Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 43–48

Bridgen, J., Harris, J. I. & Northrop, F. (1975) *FEBS Lett.* **49**, 392–395

Bridgen, J., Harris, J. I. & Kolb, E. (1976) *J. Mol. Biol.* **105**, 333–335

Brock, C. J., Harris, J. I. & Sato, S. (1976) *J. Mol. Biol.* **107**, 175–178

Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 49–60

Fridovich, I. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 35–97

Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159

Klug, D., Rabani, J. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 4839–4842

Klug-Roth, D., Fridovich, I. & Rabani, J. (1973) *J. Am. Chem. Soc.* **95**, 2786–2790

Lavelle, F., McAdam, M. E., Fielden, E. M., Roberts, P. B., Puget, K. & Michelson, A. M. (1977) *Biochem. J.* **161**, 3–11

McAdam, M. E., Lavelle, F., Fox, R. A. & Fielden, E. M. (1977) *Biochem. J.* **165**, 81–87

Pick, M., Rabani, J., Yost, F. & Fridovich, I. (1974) *J. Am. Chem. Soc.* **96**, 7329–7333

Rabani, J. & Nielsen, S. O. (1969) *J. Phys. Chem.* **73**, 3736–3744

Rotilio, G., Bray, R. C. & Fielden, E. M. (1972) *Biochim. Biophys. Acta* **268**, 605–609