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Formation of Compound I by the Reaction of Catalase with Peroxoacetic Acid

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1. The formation of Compound I by the reactions of bacterial and ox liver catalases with peroxoacetic acid was examined. In both cases the process occurs almost entirely by reaction of catalase with un-ionized peroxoacetic acid molecules. The result suggests an important role for the bound peroxidic proton in the enzyme–substrate interaction. 2. The peroxidatic properties of the Compounds I formed when peroxoacetic acid was used were examined by studying the oxidations of ethanol and formate; the results closely resemble those previously reported when H_2O_2 and alkyl hydroperoxides were used. 3. Compound I formed with bacterial catalase and peroxoacetic acid is remarkably stable in the absence of added donor and the preparation has considerable potential for detailed studies of the nature of this intermediate.

The utility of alkyl hydroperoxides as pseudo-substrates for catalase (hydrogen peroxide–hydrogen peroxide oxidoreductase, EC 1.11.1.6) was first demonstrated by Chance (1949*a*,*b*). In the reaction of catalase with alkyl hydroperoxides the catalatic intermediate, Compound I, is formed but the overall catalatic reaction does not occur. Thus, the use of these pseudo-substrates provides opportunities for examining the nature and properties of Compound I. There are, however, a number of disadvantages in the use of alkyl hydroperoxides. The rate of reaction decreases rapidly with increase in size of the alkyl group, so that only the lower members of the series (methyl and ethyl hydroperoxide) are reasonably efficient. These materials are not readily available and their reaction with catalase is complicated by the occurrence of an overall internal oxidation–reduction process. In addition the alkyl hydroperoxides are very weak acids and so do not permit studies of the role of the state of substrate protonation in the reaction.

It seemed possible that acyl hydroperoxides (peroxo acids) might provide suitable pseudo-substrates and that peroxoacetic acid might be particularly effective. This material is commercially available; the acetyl group is comparable in size with the ethyl group, but is not susceptible to oxidation; the peroxidic proton has $pK = 8.2$, which is well within the pH-stability range of the catalase protein. The thermal decomposition of aqueous peroxoacetic acid is slow at ambient temperatures (Koubek *et al.*, 1963). The formation of peroxidase Compound I by reaction with aromatic peroxo acids has been reported by Schonbaum (1970). Chloroperoxidase Compound I is formed by reaction with *m*-chloroperoxobenzoic acid (Hager *et al.*, 1971); in this case a rapid 'catalatic' decomposition of the peroxo acid ensues.

Experimental

Materials

Bacterial catalase was prepared from *Micrococcus lysodeikticus* and purified as described previously (Jones & Suggett, 1968*a*,*b*; Jones *et al.*, 1970). In this work the bacteria were obtained as a spray-dried preparation from Miles Laboratories Inc., Kanakakee, Ill., U.S.A. Although this material yielded preparations of high purity index ($E_{406}/E_{280} > 0.83$), which were homogeneous in the ultracentrifuge and showed normal sedimentation characteristics (Jones *et al.*, 1970), the catalatic activity ($4.7 \times 10^7 \pm 0.1 \times 10^7$ M$^{-1}\cdot$s^{-1} at 25°C) was about 75% of that obtained previously. This lower activity appears to be characteristic of material from this source. Twice-crystallized ox liver catalase [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was subjected to further purification, by the procedure described by Kremer (1970). The preparations obtained had a purity index of 0.85 and catalytic activity of $2.1 \times 10^7 \pm 0.1 \times 10^7$ M$^{-1}\cdot$s^{-1}, in good agreement with Kremer (1970). Peroxoacetic acid was a 36–40% (w/w) solution, supplied by Laporte Industries Ltd., General Chemicals Division, Widnes, Lancs., U.K. Detailed specifications of this material and assay procedures are described in Laporte Publication L.C.19, 'Peracetic acid'. Stock solutions

of peroxoacetic acid (approx. 5mM) were prepared by dilution with distilled water.

Phosphate buffers were prepared from AnalaR Na_2HPO_4 and KH_2PO_4, and carbonate buffers from AnalaR Na_2CO_3 and $NaHCO_3$. Buffer concentrations in the reaction solutions were 10mM and ionic strength was adjusted to 0.1M with NaCl. The pH of solutions were measured by using a Pye Dynacap pH-meter.

Ethanol (re-rectified absolute alcohol) was used without further purification. 1,1-Dideuteroethanol (98% $CH_3C^2H_2OH$) was supplied by the British Oxygen Company, London, S.W.19, U.K. Sodium formate (BDH Laboratory Reagent; BDH Chemicals Ltd., Poole, Dorset, U.K.) was used without further purification.

Spectrophotometric measurements

Spectrophotometric measurements were made at 25°C by using a Unicam SP. 1800 spectrophotometer or a Durrum D-110 stopped-flow spectrophotometer as appropriate.

Pre-treatment of peroxoacetic acid solutions

Hydrolysis of peroxoacetic acid results in the gradual accumulation of H_2O_2 in the solutions. To avoid artifacts arising from the presence of H_2O_2, we have systematically employed the following pre-treatment procedure. To a stock solution of peroxoacetic acid, catalase was added in low concentration (approx. 2nM) and the solution was left for 30min. Cerimetric analysis showed that H_2O_2 was completely removed by this procedure and that no detectable H_2O_2 was accumulated in the solutions for at least 24h. The peroxoacetic acid concentrations of the solutions were determined iodometrically.

Results

Formation of Compound I

Reaction of bacterial catalase (0.3–0.6μM) with peroxoacetic acid (10–50μM) resulted in a decrease in the extinction of catalase in the Soret-band region, in a manner characteristic of Compound I formation, although the rate of the process was much slower than that with H_2O_2 and was conveniently followed on the SP. 1800 instrument. The pseudo-first-order rate constants for this process (measured at 406nm) were directly proportional to the peroxoacetic acid concentration, but the apparent second-order constants (k_0) calculated from these results decreased markedly with increasing pH, in contrast to the established behaviour in the reaction with H_2O_2.

The form of the results suggested that the rate law for the formation of Compound I may contain two terms, involving respectively the reactions of peroxoacetic acid molecules and peroxoacetate ions with catalase, i.e.:

$$d[\text{Compound I}]/dt = k_{HA}[\text{catalase}][HA] + k_{A^-}[\text{catalase}][A^-] = k_0[\text{catalase}][HA]_0$$

where HA represents peroxoacetic acid, A^- represents peroxoacetate ion, $[HA]_0$ is the stoicheiometric peroxoacetic acid concentration, and k_{HA} and k_{A^-} are the second-order rate constants for the pathways specified by the subscripts. Assuming that equilibrium between peroxoacetic acid and peroxoacetate ions is maintained gives:

$$k_0 = \alpha(k_{A^-} - k_{HA}) + k_{HA} \quad (1)$$

where α is the degree of dissociation of peroxoacetic acid. Fig. 1 illustrates the excellent agreement between the results and the predictions of eqn. (1).

Values of α were calculated from $pK_{HA} = 8.2$ (Koubek *et al.*, 1963) and the results cover the pH range 7–9.3.

Fig. 1 also contains the results of similar experiments with ox liver catalase. These reactions were much faster than those with bacterial catalase under similar conditions and were conveniently studied with the D-110 stopped-flow spectrophotometer. In both sets of experiments the results obtained in

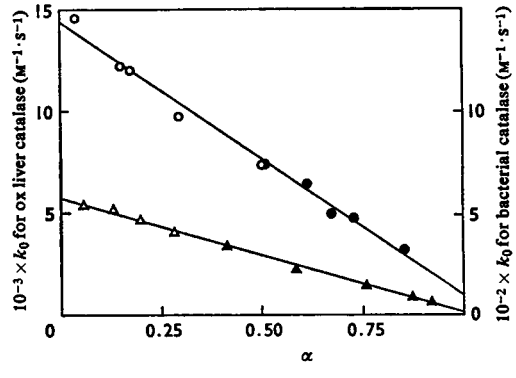

Fig. 1. *Variation of k_0 with α compared with the predictions from eqn.* (1)

Values are given for bacterial catalase in △, phosphate buffers and ▲, carbonate buffers; and for ox liver catalase in ○, phosphate buffers and ●, carbonate buffers. See the text for experimental conditions.

Table 1. *Rate constants for the formation of catalase Compound I by reaction with peroxoacetic acid (k_{HA}) and peroxoacetate ion (k_{A^-}), obtained by application of equation* (1) *as in Fig.* 1

See the text for experimental conditions. Values are given ± S.D. The value for $k_{ethylhydroperoxide}$ is given for comparison.

	Bacterial catalase	Ox liver catalase
$10^{-2} \times k_{HA}$ ($M^{-1} \cdot s^{-1}$)	5.72±0.08	144±3
$10^{-2} \times k_{A^-}$ ($M^{-1} \cdot s^{-1}$)	0.14±0.08	10±3
$10^{-2} \times k_{ethylhydroperoxide}$ ($M^{-1} \cdot s^{-1}$)	28*	200†

* Value taken from Chance & Herbert (1950).
† Value taken from Chance (1949*b*).

carbonate buffers are collinear with those in phosphate buffers, implying an absence of specific buffer effects in the processes. The rate constants obtained from the results are shown in Table 1.

These results imply that, for both types of catalase, the formation of Compound I by reaction with unionized peroxoacetic acid molecules is overwhelmingly important. It is difficult to be certain that the values of k_{A^-} are real, since they depend critically on pK_{HA}. If pK_{HA} is taken as 8.25 (rather than 8.20), k_{A^-} for bacterial catalase becomes zero within the experimental error, although k_{A^-} for ox liver catalase remains finite. Experiments at higher pH values are not feasible because of the instability of the catalase protein. Rate constants for the formation of Compound I with ethyl hydroperoxide are included in Table 1 for comparison. For ox liver catalase the values for ethyl hydroperoxide and peroxoacetic acid are very similar, whereas for bacterial catalase the reaction with peroxoacetic acid is about five times slower than with ethyl hydroperoxide.

Regeneration of catalase from Compound I

Solutions of bacterial catalase Compound I, formed with peroxoacetic acid, are remarkably stable, although a very slow regeneration of the spectrum of free catalase eventually occurs. Regeneration occurs much more slowly than in the reported case of reaction with ethyl hydroperoxide ($8.6 \times 10^{-3} s^{-1}$; Brill & Williams, 1961). At high concentration ratios of peroxoacetic acid/bacterial catalase the formation of Compound II also occurs and the overall behaviour of the system is complex. Ox liver catalase Compound I is much less stable, and we have examined the rate of regeneration of free catalase in this system by following the increase in E_{406} subsequent to the steady state. At pH7 the first-order rate constant was $1.6 \times 10^{-4} \pm 0.2 \times 10^{-4} s^{-1}$ {mean of five determinations with: [catalase] 0.27–0.61 μM, based on $\epsilon_{405} = 340$ litre·mmol^{-1}·cm^{-1} (Kremer, 1970); initial [peroxoacetic acid] 9–46 μM; initial [peroxoacetic acid]/[catalase] 17–76}. This value is much smaller than those reported for H_2O_2 and alkyl hydroperoxides (2×10^{-2}–$4 \times 10^{-2} s^{-1}$; Chance, 1949*a*). This process is sensitive to the presence of oxidizable material in the solutions (adventitious donors). The low rates obtained in the present work suggest that these intrusions are minimal, although it cannot be stated with certainty that they are entirely absent. We have demonstrated that the process is too rapid to be sustained by catalatic removal of H_2O_2 produced by hydrolysis of peroxoacetic acid, but it is possible that regeneration is controlled by a slow 'catalatic' turnover of peroxoacetic acid itself.

Peroxidatic activity of Compound I

We have examined the reactivity of the Compounds I, formed by the action of peroxoacetic acid, by studying the well-known 'peroxidatic' oxidations of ethanol and formate.

For bacterial catalase the following procedure was adopted. Solutions of Compound I were formed by mixing 5ml of catalase solution (0.3–0.6 μM, pH7) with 0.5ml of peroxoacetic acid solution (approx. 10 μM) at 25°C and allowing the system to reach the steady state (approx. 15min, monitored at 406nm). Samples (5ml) of these solutions were mixed with small volumes of substrate solution (0.025–0.25ml of 0.073M-ethanol, 0.05–0.3ml of 0.1M-deuteroethanol or 0.1–0.5ml of 0.01M-sodium formate solution) in a cuvette and the regeneration of catalase was followed at 406nm until completion. Examination of the overall spectra during the formation of Compound I and regeneration consequent to donor addition indicated recoveries of catalase of greater than 80% under these conditions. Under the conditions of these experiments the rate of reaction of ethanol or formate with Compound I greatly exceeds the rate of re-formation of Compound I by reaction of catalase with residual peroxoacetic acid in the system. The regeneration curves were first order and the pseudo-first-order rate constants were proportional to the donor concentration, as shown in Fig. 2.

Compound I of ox liver catalase and peroxoacetic acid is less stable, but solutions containing initially 0.39 μM-catalase and 9.6 μM-peroxoacetic acid (pH7) were adequately stable (for about 10min) to permit peroxidatic studies by using the D-110 stopped-flow spectrophotometer. The initial concentrations of donor in the mixed reaction solutions were varied in the range 1–5mM. The rate of regeneration of catalase was a first-order process in these experiments and the pseudo-first-order rate constants (k_r) were again proportional to the donor concentration (Fig. 2). The second-order-rate constants for the peroxidatic reactions studied are shown in Table 2.

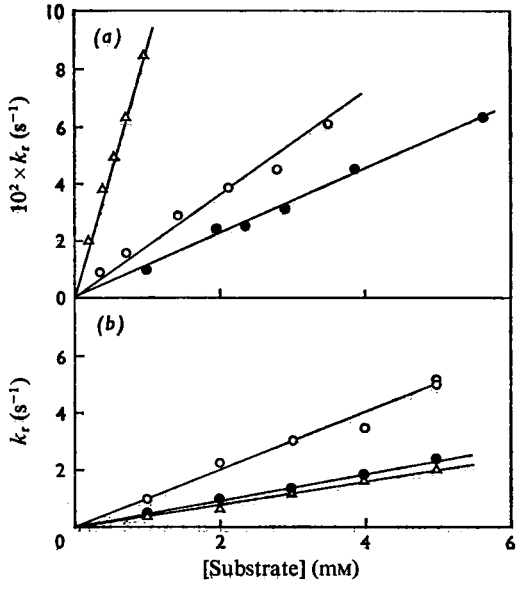

Fig. 2. *Variation of pseudo-first-order rate constants for peroxidative reactions with substrate concentration*

Substrates were: △, formate; ○, ethanol; ●, 1,1-$^2H_2$-ethanol. (*a*) Bacterial catalase; (*b*) ox liver catalase. See the text for experimental conditions.

The pattern of behaviour closely resembles that reported previously (Deisseroth & Dounce, 1970), notably in the decreased facility for formate oxidation relative to ethanol oxidation of ox liver catalase Compound I compared with the bacterial catalase compound. The kinetic-isotope effect is similar for both catalases; $k_{Ethanol}/k_{Deuteroethanol} = 1.6 \pm 0.3$ for bacterial catalase and 2.2 ± 0.3 for ox liver catalase.

Discussion

The rate constants for the formation of Compound I with peroxoacetic acid are comparable to those with ethyl hydroperoxide, in agreement with the idea that the size of the end group in a hydroperoxide is an important constraint on the ease of access to the catalase active site. A more novel result is the discrimination between peroxoacetic acid molecules and peroxoacetate anions that has been observed. This suggests the existence of an electrostatic constraint on the access of substrate to the active site, and the behaviour could be rationalized by supposing the existence of a negatively charged group (e.g. carboxylate) near to the primary Fe(III) binding site of the enzyme. This group could act as a reflector for the anionic conjugate bases of hydroperoxide substrates. It is also possible that such a group may play an intrinsically important role in the mechanism of catalase action. This hypothesis has been advanced previously on other grounds. Jones & Suggett (1968*b*) argued that the ability to utilize molecular H_2O_2 confers an important advantage on the catalase enzyme compared with the ferrihaem complexes. The latter species react via a similar mechanism (Portsmouth & Beal, 1971; Brown *et al.*, 1970; Jones, 1971; Prudhoe, 1971), except that the conjugate base of the substrate (HO_2^-) is utilized. Since the pK of H_2O_2 is approx. 12, the catalytic advantage of using molecular H_2O_2 is approx. 10^5 at pH7. The present experiments with peroxoacetic acid have permitted the role of the state of protonation of a peroxidic substrate to be examined in reactions of the catalase enzyme itself. The observed discrimination in favour of un-ionized per-acid

Table 2. *Rate constants for the peroxidatic reactions of catalase Compound I*

See the text for experimental conditions. Values in parentheses are taken from Deisseroth & Dounce (1970) and summarize the range of values obtained with Compound I formed by using H_2O_2 and methyl and ethyl hydroperoxides

	Rate constants ($M^{-1} \cdot s^{-1}$)		
Substrate ...	Ethanol	[1,1-$^2H_2$]Ethanol	Formate
Bacterial catalase	18 ± 1	11 ± 1	86 ± 4
Compound I	(11–13)		(150–180)
Ox liver catalase	980 ± 80	450 ± 30	400 ± 40
Compound I	(900–1000)		(500)

molecules is compatible with the model of the role of catalase protein in the enzyme active site suggested previously (Jones & Suggett, 1968*b*), involving an important role for the bound peroxidic proton in the enzyme–substrate interaction.

The remarkable stability of catalase Compound I formed from bacterial catalase and peroxoacetic acid opens considerable practical possibilities for detailed examination of this intermediate species. For example, it is possible that infusion of crystalline bacterial catalase with peroxoacetic acid may enable the preparation of crystalline catalase Compound I.

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