Formation of Catalase Compound I by Reaction with Peroxoacetic Acid: pH Changes in Unbuffered Systems

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Studies of the pH decreases that accompany the formation of Compound I from ox liver catalase and peroxoacetic acid in unbuffered systems imply that 1 mol of acetic acid is released/mol of catalase ferrihaem Compound I formed. This result is consistent with results previously obtained (Schonbaum & Lo, 1972) for the formation of peroxidase Compound I by using peroxo acid substrate.

Compelling evidence that the Compounds I of peroxidases contain one oxygen atom derived from the parent peroxide molecule has recently been obtained by using peroxo acid substrates. Schonbaum & Lo (1972) have studied both the changes in pH and the release of chromophoric carboxylate ion (*p*-nitrobenzoate from *p*-nitroperoxobenzoic acid) in the reaction with horseradish peroxidase. Hager *et al.* (1973) have studied by a double oxygen-isotopic labelling method (Edwards & Fleischauer, 1968) the catalytic decomposition of *m*-chloroperoxobenzoic acid by chloroperoxidase. These results imply that the reaction at the iron atom of the ferrihaem prosthetic group of the peroxidases may be formulated as:

Thus the reaction is a two-electron equivalent oxidation of peroxidase in which the formal oxidation state of iron in the oxy-cation product has increased from +III to +V. Compound I retains both oxidizing equivalents of its parent peroxide molecule. The carboxylic acids have much lower *pK* values than the peroxo acids from which they are formed, so that, in unbuffered systems at about pH 7, the ionization of these product species results in a measurable fall in pH.

The substrate selectivity of the catalase hydroperoxidases prevents the use of aromatic peroxo acids in the study of these systems. However, Compound I of ox liver catalase is readily formed by re-

action with peroxoacetic acid (Jones & Middlemiss, 1972), and measurements of pH changes accompanying this reaction in unbuffered systems are possible. The present paper reports the results of such experiments.

Experimental

The further purification of ox liver catalase [twice crystallized; Sigma (London) Chemical Co., London S.W.6, U.K.] followed the procedures described previously (Kremer, 1970; Jones & Middlemiss, 1972). Stock solutions of catalase (in 10mM-KH₂PO₄-Na₂HPO₄ buffer, pH 7) were exhaustively dialysed against 0.1M-NaCl (AnalaR) to remove phosphate

buffering capacity. Peroxoacetic acid (Laporte Industries Ltd., Widnes, Lancs., U.K.) solutions were prepared, assayed and pretreated to remove residual H₂O₂ as described previously (Jones & Middlemiss, 1972). Stock solutions (approx. 5mM) were titrated to pH 7.2±0.1 with 0.1M-NaOH immediately before use. pH measurements were made with a Corning-Eel model 10 expanded-scale pH-meter linked to a Servoscribe recorder; the glass electrode was a Pye-Unicam Ingold Glass Combination Electrode. All experiments were performed at 20±1°C.

Results and discussion

Since the *pK* of acetic acid is about 4.7 and that of peroxoacetic acid is about 8.2, the optimum pH for carrying out the experiments is about 7. Unbuffered

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Fig. 1. Titration of catalase with acid

The changes in $[H^+]$ produced by successive additions of acetic acid (1 mM) to an unbuffered solution of ox liver catalase are plotted. The catalase concentration was $7.06 \mu M$ (in aq. 0.1 M-NaCl; initial pH = 7.20). The error bars correspond to ± 0.02 pH unit.

catalase solutions (in 0.1 M-NaCl at pH 7) possess the buffering capacity of the enzyme protein, so that measurements of pH change may not be used directly to obtain values for the concentration of acetic acid formed by reduction of peroxyacetic acid. Calibration curves were obtained by measuring the changes in pH on addition of known concentrations of acetic acid to catalase solutions. A typical result is shown in Fig. 1; the formal change in H^+ concentration calculated from the pH measurements is, to a good approximation, directly proportional to the concentration of acetic acid added in the pH range 7.2–6.5.

The formation of Compound I was studied as follows. A small quantity of peroxyacetic acid solution was added from a micro-pipette with stirring to 3 ml of unbuffered ox liver catalase solution (catalase concentrations 3.4 – $10.4 \mu M$, in 0.1 M-NaCl, initial pH in the range 7.17–6.95). The decrease in the pH of the solution was measured until a constant value had been reached (2–3 min). Final pH values were in the range 6.94–6.68. An internal calibration was then carried out by re-adjusting the pH as near to the starting value as possible by the addition of NaOH. At this point 0.1 ml of 1 mM-acetic acid was added and the resulting decrease in pH was recorded.

The results are presented in Fig. 2, in which the ratio [acetic acid released]:[catalase] is plotted against the ratio [peroxyacetic acid added]:[catalase]. The solid line represents a theoretical titration curve based on the assumptions that 1 mol of acetic acid is released on the oxidation of 1 mol of catalase ferrihaem to the Compound I state and that there are four ferrihaem moieties per molecule of catalase. The results are in good agreement with this model and

Fig. 2. Dependence of the release of acetic acid accompanying the formation of Compound I from ox liver catalase and peroxyacetic acid on the concentration of peroxyacetic acid

The solid line is based upon the assumptions that 1 mol of acetic acid is released on the oxidation of 1 mol of catalase ferrihaem to Compound I and that there are four ferrihaems per molecule of catalase.

hence are in accord with the formulation of peroxidase Compound I formation in eqn. (1). At higher ratios of [peroxyacetic acid]:[catalase] ($>10:1$) the curve departs markedly from the horizontal. Under these conditions significant irreversible oxidation of the catalase protein and enhanced rate of Compound II formation are observed (Middlemiss, 1973). The addition of peroxyacetic acid to unbuffered solutions of bacterial catalase also results in a decrease in pH, but measurements on this system are more difficult owing to the much lower rate of formation of Compound I (Jones & Middlemiss, 1972).

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