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The Rate of Formation of the Enzyme-Substrate Compound I between Hydroxymethylhydroperoxide and Horseradish Peroxidase

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Hydroxymethylhydroperoxide, $HOCH_2OOH$ (HMP) is a peroxide substrate and a rapid irreversible inhibitor of horseradish peroxidase.[1]* A direct determination of the rate constant (k_1) for the formation of the enzyme-substrate compound I[2] from peroxidase and HMP has been precluded by the obligate presence of H_2O_2 in HMP-preparations; an indirect method gave $k_1 \simeq 2 \times 10^5$ M^{-1} sec^{-1}. However, at a study of the effects of HMP on catalase it was found that the enzyme could remove H_2O_2 from aqueous HMP solutions.[4] The present paper reports a stopped-flow determination of the rate constant (k_1) for the formation of the enzyme-substrate compound I from HMP and peroxidase.

H_2O_2, HCHO, HMP, and bis(hydroxymethyl)peroxide, $HOCH_2OOCH_2OH$ (BHMP) form an equilibrium in water solution.[3] The equilibrium is catalyzed by H^+ and OH^-, predominantly by the latter at pH > 3.[3] At the conditions of the present experiments (pH 4.25, 25°C), HMP and BHMP are rather stable, the half-times of their hydrolyses being longer than 12 and 5 h, respectively.[3]

Results and discussion. Catalase was used to remove H_2O_2 from HMP solutions. The enzyme is partially transferred to the inactive[8] compound II by HMP,[4] but there is always some active catalase left to remove H_2O_2 from HMP solutions, as seen in Fig. 1.

The HMP solutions to be used in the stopped-flow experiments contained 5 times more catalase than was used in the experiment of Fig. 1. HMP was dissolved ($\simeq$ 0.45 mM) in 10 mM sodium acetate, pH 4.25, 0°, with 35 nM catalase ("stock solution"). After 20 min the H_2O_2 content was assumed to be low enough and a sample was diluted with 4, 9, or 19 volumes of the same buffer at 25°. These solutions were left to equilibrate for 10 min and then used in the stopped-flow apparatus. The content of HMP of the "stock solution" was repeatedly assayed by means of peroxidase and guaiacol.[1]

The stopped-flow runs (Fig. 2) gave an average k_1 of 5×10^5 M^{-1} sec^{-1} k_1 has previously been determined to 2×10^5 M^{-1} sec^{-1} by an indirect method in the presence of 6.7 mM guaiacol.[1] A part of this discrepancy may be explained by the blocking effect of hydrogen donor substrates on peroxidase (Ref. 9 and S. Marklund, unpublished experiments) at this rather high concentration. The previous investigation[1] was also performed with higher HMP-concentrations (50 – 90 μM) than the present (6.8 – 38.9 μM, Fig. 2) in which the observed k_1-values may show a tendency to decrease with increasing HMP concentration.

The higher k_1 of the present experiments cannot be due to interference from H_2O_2 still present in the catalase-treated HMP solutions. In the experiment with 6.8 μM HMP in Fig. 2, the presence of 0.23 μM H_2O_2 ($k_1 = 9 \times 10^6$ M^{-1} sec^{-1}, Ref. 2) would

* Horseradish peroxidase, Donor: Hydrogen peroxide oxidoreductase, E.C. 1.11.1.7; Catalase, Hydrogen peroxide : Hydrogen peroxide oxidoreductase, E.C. 1.11.1.6.

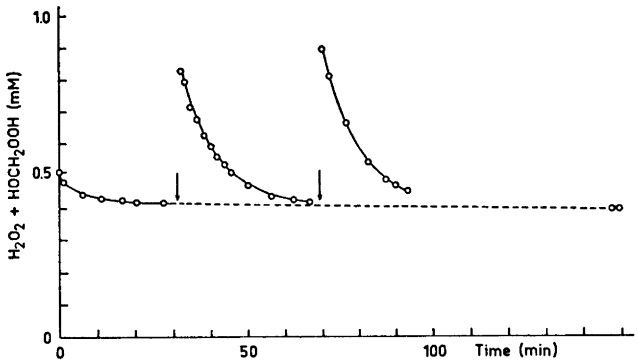

Fig. 1. Demonstration of the ability of catalase to remove H_2O_2 from an HMP-solution. Catalase (7 nM) was added to an HMP solution (0.42 mM HMP, 0.09 mM H_2O_2 and 0.16 mM BHMP) in 10 mM sodium acetate, pH 4.25, 0°C. The solution was repeatedly assayed for H_2O_2+HMP by means of horseradish peroxidase (1.5 μM) and guaiacol (8mM). (H_2O_2 and HMP are peroxide substrates of peroxidase [1].) After 31 min and 69 min H_2O_2 ($\cong$0.5 mM) was added.

cause an apparent increase in k_1 for HMP from 2×10^5 to 5×10^5 M^{-1} sec^{-1}. But as the concentration of peroxidase was 1.5 μM (Fig. 2), 0.2 μM H_2O_2 would soon be depleted and a bend in the line would be seen.

The rate constant (k_1) for the formation of the enzyme-substrate compound I from peroxidase and two alkyl-hydroperoxides has previously been determined;[2] methylhydroperoxide, $k_1 = 1.5 \times 10^6$ M^{-1} sec^{-1} and ethylhydroperoxide, $k_1 = 3.6 \times 10^6$ M^{-1} sec^{-1}. The α-hydroxyalkyl-hydroperoxide, HMP, reacts more slowly. This is probably not due to the size of the molecule which is almost equal to that of ethylhydroperoxide. The hematin group of peroxidase is also considered relatively easily accessible.[2]

The HMP molecule is probably more solvated than the alkylhydroperoxides and may also form an intramolecular hydrogen

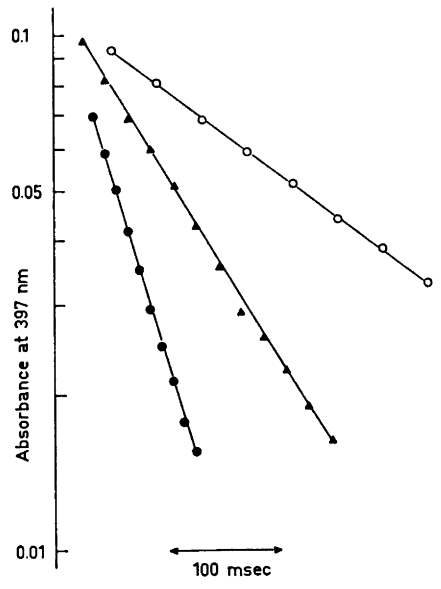

Fig. 2. The rate of formation of the enzyme substrate compound (compound I) between HMP and peroxidase. HMP solutions (their preparations are described in the text) were mixed with peroxidase (final concentration 1.5 μM) in a stopped-flow spectrophotometer and the absorbance at 397 nm recorded. Compound I and compound II of peroxidase are approximately isosbestic here.[11] The figure shows the decrease in absorbance of the peroxidase with that of compound I and compound II taken as zero. The symbols, ○, ▲, ●, represent the reactions in 6.8, 16.4, and 38.9 μM HMP (final concentrations), respectively, giving second-order rate constants of 5.5, 5.0, and 4.3×10^5 M^{-1} sec^{-1}. No compensations were made for the consumption of HMP during the reactions.

bond. Studies of the interactions of hydroxamic acids with peroxidase and the very high reactivity of the enzyme with peroxy acids containing aromatic side chains,[10] indicate the presence of a non-polar region near the sixth coordination position of the hematin iron, which should repel the polar HMP more than the alkyl-hydroperoxides.

Materials and methods. H_2O_2, *p.a.* Perhydrol, Merck, HMP was prepared according to Marklund.[3] The concentrations of the peroxides were determined with titanium (IV).[3] Horseradish peroxidase, fraction IIIb,[5] $\varepsilon_{mM\,403}=100$ cm^2 mol^{-1}. Catalase,* beef liver, Boehringer, Mannheim. $\varepsilon_{mM\,405}$ was taken as 297 cm^2 mol^{-1}.[6] H_2O was double distilled from quartz vessels.

For spectrophotometry a Beckman Acta III was used. A Durrum Gibson stopped-flow spectrophotometer,[7] thermostatted at $25\pm0.05°$, and equipped with a 2 cm cuvette, was used for the study of rapid reaction kinetics. Dead-time was <5 msec.

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Detection of General Acid-catalyzed Hydrolysis in Buffer Solutions in Mixed Solvents

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One of the main problems in studies of the acid-catalyzed hydrolysis of acetals and ortho esters has recently been the timing of the proton transfer relative to the cleavage of the carbon-oxygen bond of the substrate. In some particular cases a change in reaction mechanism with structure has been established.[1-5] Conclusions about reaction mechanism have usually been based on whether or not the reaction is subject to general acid catalysis.

In most of the earlier experiments to detect general acid catalysis, reactions have been run in buffer solutions in dioxane–water mixtures.[1,2,6-8] However, the conclusions about general acid catalysis were drawn without studying the possible influence of specific salt effects which are pronounced in this solvent as illustrated by data for the hydrolysis of triethyl orthobenzoate.[9] The appearance of "general acid catalysis" depended on the electrolyte used to maintain the ionic strength constant. The observed differences in the hydrolysis rates were quantitatively accounted for by specific salt effects on the hydronium ion catalysis.

It is sometimes necessary to study reactions in buffer solutions in mixed solvents because the substrates are sparingly soluble in pure water. Therefore it is important to find a way by which salt effects can be eliminated. The absence of general acid catalysis in the hydrolysis of triethyl orthoformate was verified by using a great excess of neutral electrolyte.[10] Unfortunately, the possibility of detecting a very weak general acid catalysis is lost at the same time. The reduction of the rate of the hydronium ion-catalyzed reaction by the organic solvent is compensated for by the accelerating effect of the relatively high ionic strength.

The aim of this study was to find mixed solvents suitable for preparing buffer solutions in which specific salt effects would be eliminated as far as possible.