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INTERACTIONS BETWEEN HYDROXYMETHYLHYDROPEROXIDE AND CATALASE

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SUMMARY

1. Hydroxymethylhydroperoxide reacts with catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) as a primary substrate with a rate constant of $3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

2. Hydroxymethylhydroperoxide induces the formation of catalase Compound II in a second-order reaction, $k = 273$ $M^{-1} \cdot s^{-1}$.

3. H_2O_2 accelerates the reactivation of the inactive catalase Compound II.

INTRODUCTION

Hydroxymethylhydroperoxide (HMP) is rapidly formed from hydrogen peroxide and formaldehyde in neutral aqueous solution[1]. It is a primary substrate and a rapid irreversible inhibitor of horseradish peroxidase[2] (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), but its effect on catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is unknown. The present paper describes HMP as a primary substrate of catalase and the ability of the peroxide to induce compound II formation. Further, a reactivating effect of H_2O_2 on the inactive catalase Compound II is demonstrated.

MATERIALS AND METHODS

BaO_2, Riedel-de Haen A. G. Ethylhydroperoxide, AB Ferrosan, Fack, S-201 10 Malmö, Sweden. Horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), Fraction IIIb (ref. 3) $\varepsilon_{403\ nm} = 1.0 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$. Catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6.), beef liver, Boehringer Mannheim Gmbh. $\varepsilon_{405\ nm}$ was taken as $2.97 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ (ref. 4) and the enzyme was assumed to contain 2 hematins per molecule[4]. $A_{405\ nm}/A_{280\ nm} = 0.91$. The catalase was not reducible with dithionite. Cacodylate buffer (British Drug Houses), which was used in many of the experiments, did not influence catalase activity more than the presumably innocuous

Abbreviations: HMP, hydroxymethylhydroperoxide $HOCH_2OOH$; BHMP, bis(hydroxymethyl)peroxide $HOCH_2OOCH_2OH$.

phosphate buffer (50 mM buffers, pH 6.4). Water was double-distilled from quartz vessels.

H_2O_2, p.a. Perhydrol, Merck. HMP and BHMP were prepared according to Marklund[1]. BHMP can be crystallized in the pure form, but HMP can only be obtained in equilibrium with BHMP, H_2O_2 and a negligible amount of formaldehyde. The concentrations of H_2O_2, HMP and BHMP were determined with titanium (IV)[1].

H_2O_2, formaldehyde, HMP and BHMP form an equilibrium in water solution[1]. The equilibrium is catalysed by H^+ and OH^-, mainly by the latter at pH >3. Under the conditions of the present experiments (pH 5, 25 °C) HMP and BHMP are rather stable, the half-times of their hydrolyses being about 115 min and 30 min, respectively[1].

For spectrophotometry a Beckman Acta III was used. All experiments were performed at 25 °C.

RESULTS AND DISCUSSION

HMP as a primary substrate of catalase

If catalase is added to an HMP solution, first a rapid and then a slower decrease in "hydroperoxide" (H_2O_2 + HMP) is recorded (Fig. 1). The rapid phase apparently corresponds to the catalatic disproportionation of the H_2O_2 in the solution, and the slower phase to a decrease in HMP. If a hydrogen-donor substrate of catalase *e.g.* formic acid, formaldehyde or ethanol (Fig. 1) is present, the reduction of HMP is markedly accelerated. (These compounds do not affect HMP in the absence of catalase.) This indicates that HMP, like methylhydroperoxyde and ethylhydroperoxyde[5], may serve as a peroxide substrate of catalase in its peroxidative reactions. (Guaiacol is also oxidized by HMP in the presence of catalase.)

As seen in Fig. 1, ethanol concentrations above 10–20 mM do not further accelerate the reduction of HMP, indicating that the reaction between the peroxide and catalase has become rate-limiting. The slope of the maximum decrease in HMP in the figure corresponds to a rate constant of $3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for the reaction. This value, which is calculated per mole of hematin, is a minimum because part of the catalase is transformed to the inactive[5] Compound II by HMP (*vide infra*).

The hydrogen-donor substrates protect against Compound II formation. Under the conditions described in the legend to Fig. 1, the increase in absorbance at 434 nm of the catalase ("free" catalase and Compound I isosbestic, ref. 6 and Fig. 2) in the presence of 17 mM ethanol is less than 20% of that found in the absence of hydrogen donor substrate.

The ability of HMP to serve as a primary substrate of catalase is also demonstrated by the formation of catalase Compound I upon the addition of HMP, Fig. 2 (*cf.* ref. 6). The transformation of catalase to Compound I is about as complete as that obtained with methylhydroperoxide[6] and much more extensive than that given by H_2O_2 (ref. 7), which excludes the possibility of the effect being due to H_2O_2 released from HMP by hydrolysis.

The rates of reaction of hydroperoxides with catalase decrease with increasing size of the peroxide molecule[5], and this is interpreted as being due to steric hindrance by the catalase protein to access to the hematins. HMP, the size of which is close to that of ethylhydroperoxide, accordingly reacts about as rapidly as the latter (k_1 =

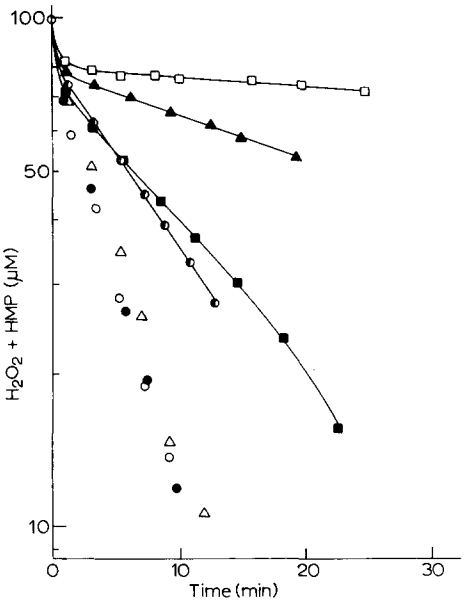

Fig. 1. HMP as peroxide substrate of catalase. Catalase (58 nM) was added to HMP solutions (82 μM HMP, 17 μM H_2O_2 and 43 μM BHMP) in 10 mM sodium cacodylate, pH 5.0, containing no or various amounts of hydrogen-donor substrate. The solutions were repeatedly analyzed for H_2O_2 *plus* HMP by means of peroxidase (1.5 μM) and guaiacol (8 mM). H_2O_2 and HMP are peroxide substrates of peroxidase[2]. □, denotes the reaction in the absence of hydrogen-donor substrate and ▲, ◑, ■, △, ● and ○ the reactions in 0.4 mM formaldehyde, 0.15 mM formic acid, 4, 12, 24 and 40 mM ethanol, respectively.

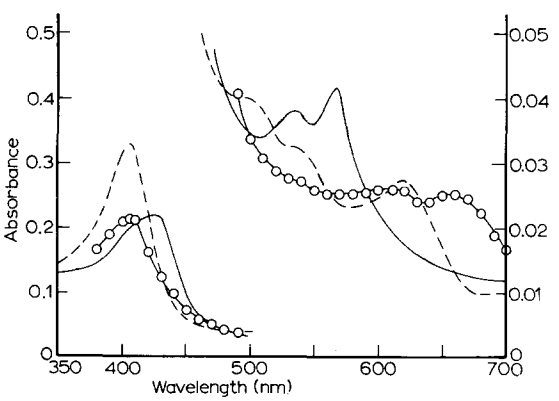

Fig. 2. The spectra of catalase, HMP–catalase Compound I and Compound II. Catalase (1.11 μM) was dissolved in 10 mM sodium cacodylate, pH 5.0 (— — —). HMP (0.35 mM) was added and the spectrum scanned within 1–4 min thereafter (———, Compound II). ○—○, shows the spectrum obtained 3 s after the addition of 56 μM HMP to the catalase (Compound I).

$2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$)[5] and more slowly than the smaller methylhydroperoxide ($k_1 = 8.5 \cdot 10^5$ $M^{-1} \cdot s^{-1}$)[5].

The formation of catalase Compound II by HMP

Catalase Compound II is red and probably has one oxidizing equivalent more than free catalase[8]. It may slowly, spontaneously form from H_2O_2 Compound I[8,9] and this reaction is accelerated by some anions[10]. It is also formed by the action of alkylhydroperoxides[5,11] and some "one electron donors"[12] on Compound I, and in some autooxidizing systems[8,13].

Like alkylhydroperoxides, HMP induces the formation of Compound II (Fig. 2). At a certain HMP concentration, Compound II formation follows first-order kinetics (determined at 434 nm, where catalase and Compound I are isosbestic, ref. 6 and Fig. 2) and the rate constants (s^{-1}) are proportional to the HMP concentration (Fig. 3). Compound II formation by HMP thus closely follows second-order kinetics. The slope of the line in the figure corresponds to a rate constant of 273 $M^{-1} \cdot s^{-1}$. The ability of HMP to cause Compound II formation is thus about as great as that of methylhydroperoxide[5] and much greater than that of ethylhydroperoxide[5]. The second-order rate constants for Compound II formation with methyl- and ethylhydroperoxide, however, especially the latter, decrease considerably with increasing peroxide concentration[5].

The straight line does not pass through the origin, which indicates that there is a spontaneous decay of HMP–catalase Compound I to Compound II with a rate constant of the order of $2 \cdot 10^{-3}$–$3 \cdot 10^{-3} s^{-1}$. Nicholls[8] obtained the value $2.8 \cdot 10^{-3} s^{-1}$ for H_2O_2 Compound I.

The spectrum of a catalase solution transformed to Compound II by HMP

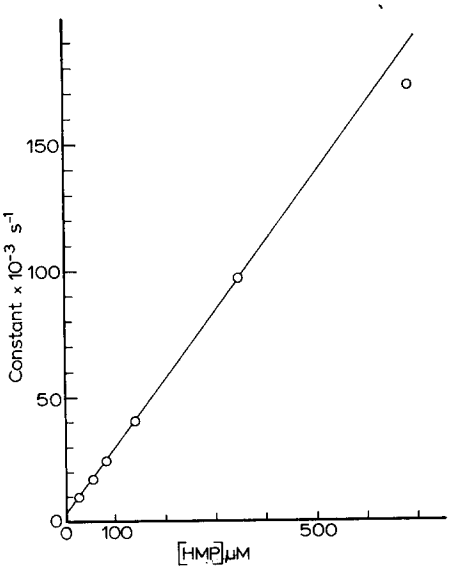

Fig. 3. The rate of formation of catalase Compound II as a function of HMP concentration. HMP was added to catalase solutions. The increase in absorbance at 434 nm (free catalase and Compound I isosbestic, *cf.* Fig. 2 and ref. 6) followed first-order kinetics up to a plateau. The rate constants were obtained from the straight lines in semilogarithmic plots.

HMP (μM)	28	56	84	139	345	684
Catalase (nM)	107	107	177	177	350	350
Increase in $A_{434\ nm}$ (%)	96	92	89	88	89	88

returns to an essentially unchanged free catalase spectrum when all HMP has been reduced. The related enzyme horseradish peroxidase, which like catalase may use HMP as a primary substrate[2], is, unlike catalase, rapidly and irreversibly inactivated by the peroxide due to an attack at a methene bridge in the hematin group. The inactivation which is accompanied by the formation of a peroxidase compound with a strong band at 670 nm[14], has a half-time of 1 min in 40 μM HMP. Even when exposed for hours to HMP, catalase forms no such band in the red, and thus seems resistant to this irreversible inhibitory effect of the peroxide.

Possibly the greater steric hindrance to access to the hematins by catalase[5] as compared with peroxidase prevents the approach of HMP in an orientation which allows it to attack a methene bridge.

Reactivation of catalase from Compound II by H_2O_2

Catalase Compound II is catalatically inactive[5]. It is stated to decay slowly ($t_{\frac{1}{2}}$ = 14 min[8]) and spontaneously to active catalase. If a sample from a HMP–catalase Compound II solution is assayed (Fig. 4, decrease in absorbance at 230 nm of 20 mM H_2O_2 in phosphate buffer, pH 7), the catalatic activity is initially low. (It is, however, not zero, indicating that not all catalase is in the form of Compound II). The activity gradually increases, and after 2 min it amounts to about 80% of the activity of a corresponding amount of "free" catalase. This rapid reactivation in H_2O_2 is not something specific for the Compound II formed by the action of HMP, as the same effect is found with that formed in ethylhydroperoxide. Incubation of a Compound II sample in the pH 7 phosphate buffer for 75 s before addition of H_2O_2, Fig. 4, leads in accord with the results of Nicholls[8] to very little increase in activity. The accelerated reactivation is thus due to the added H_2O_2 and not to some

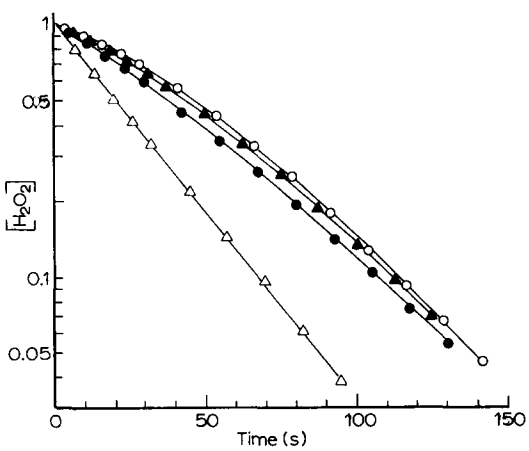

Fig. 4. Reactivation of catalase Compound II by H_2O_2. Catalase (0.47 μM) was incubated in 10 mM sodium cacodylate, pH 5.0, containing HMP or ethylhydroperoxide to give Compound II. Samples were then added (dilution 1/300) to 20 mM sodium phosphate (deaerated), pH 7.0, containing 20 mM H_2O_2 and the decrease in H_2O_2 followed at 230 nm. ○—○, reaction after 1 min incubation in 0.28 mM HMP; ●—●, reaction after 6 min in 3.3 mM ethylhydroperoxide; ▲—▲, reaction obtained when the catalase was incubated for 1 min in 0.28 mM HMP and the sample (1/300) then kept in the phosphate buffer for 75 s before H_2O_2 (20 mM) was added; △—△, blank reaction obtained after 1 min incubation of catalase in the absence of peroxide.

factor in the phosphate buffer. Identical results are obtained with H_2O_2 produced from BaO_2 and phosphoric acid, which excludes the possibility of the effect being due to impurities (stabilizers) in the commercial H_2O_2. This activating effect of H_2O_2 on catalase Compound II has apparently not been previously reported.

H_2O_2 has been stated not to influence catalase Compound II[9]. However, some conflicting results in the paper cited may be explained by a reactivating effect of H_2O_2 on Compound II, *e.g.* the finding that the conversion of catalase to Compound II is less complete in "bottle" H_2O_2 than in the low concentrations of H_2O_2 produced by the glucose oxidase system.

Catalase Compound II is transformed to Compound III (refs 6, 13) upon the addition of H_2O_2, with an apparent dissociation constant of 40 μM[6]. Thus, in 20 mM H_2O_2 (Fig. 4) "all" Compound II should be transformed to Compound III, suggesting that the reactivation of catalase proceeds by way of the latter compound. In accord with the dissociation constant[6], the reactivation in 3 mM H_2O_2 is as rapid as in 20 mM. Compound III has been stated to decay to Compound II (refs 12, 13), but the present results indicate that it also may decay to free catalase or Compound I.

CONCLUSIONS

HMP is rapidly formed from formaldehyde and H_2O_2 in neutral aqueous solution[1]. Other aldehydes also easily add to H_2O_2 and form α-hydroxyalkylhydroperoxides[15–17]. These peroxides are often much stronger inhibitors of biochemical systems than the parent compounds[2,18–30]. The extent to which they are formed in H_2O_2-producing biochemical systems is unknown. The present work shows that catalase can decompose at least the simplest homologue, HMP, by the peroxidatic reactions of the enzyme (Fig. 1), a type of reaction that has been shown to occur *in vivo*[31,32]. HMP probably may also be decomposed *via* reduction of HMP–catalase Compound I by H_2O_2, a reaction that is very rapid with alkylhydroperoxide Compound I (refs 9, 33).

The peroxide from two formaldehydes and one H_2O_2 (BHMP) apparently does not react with catalase. A tendency to formation of compound I may be seen, but it can fully be explained by the formation of HMP by BHMP hydrolysis[1].

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