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THE ACTIONS OF HYDROXYMETHYLHYDROPEROXIDE AND BIS(HYDROXYMETHYL)PEROXIDE ON FUMARATE HYDRATASE, LACTATE DEHYDROGENASE, ASPARTATE AMINOTRANSFERASE, GLUCOSE OXIDASE, AND ACID PHOSPHATASE

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SUMMARY

1. Many reports attribute biochemical actions to bis(hydroxymethyl)peroxide. Mixtures of hydrogen peroxide and formaldehyde are known to exert a greater effect on biochemical systems than either substance alone. In both cases the effects are probably caused by hydroxymethylhydroperoxide, formed from bis(hydroxymethyl)-peroxide by hydrolysis or from hydrogen peroxide and formaldehyde by a condensation.

2. Hydroxymethylhydroperoxide is a fairly potent inhibitor of fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2), lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), and aspartate aminotransferase (L-aspartate:2-oxo-glutarate aminotransferase, EC 2.6.1.1), whereas glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) and especially acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) are less affected.

3. 2-Mercaptoethanol and albumin protect against the inhibitory action of hydroxymethylhydroperoxide without reducing the amount of peroxide.

4. Fumarate hydratase and lactate dehydrogenase, exposed to hydroxymethyl-hydroperoxide, are not reactivated by 2-mercaptoethanol, whereas aspartate amino-transferase slowly partially regains its activity.

INTRODUCTION

Bis(hydroxymethyl)peroxide (BHMP) has been stated to be mutagenic[1,2] and to inhibit peroxidase[3], blood catalase[4], anaerobic glycolysis, cell respiration, aldolase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and Ehrlich-ascites-carcinoma cells *in vitro*[5,6], and *in vivo*[7]. The action of BHMP on cell division, glycolysis and respiration of synchronized yeast cells has been described[8].

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

Formaldehyde enhances the mutagenic effects of ultraviolet radiation[9,10]. Mixtures of formaldehyde and H_2O_2 are stronger mutagens than either substance alone[11], and they inhibit catalase[12].

BHMP and HMP form equilibria with H_2O_2, hydrated HCHO and H_2O (ref. 13).

$$H_2C(OH)_2 + H_2O_2 \rightleftharpoons HOCH_2OOH + H_2O \quad (1)$$
$$H_2C(OH)_2 + HOCH_2OOH \rightleftharpoons HOCH_2OOCH_2OH + H_2O \quad (2)$$

BHMP may be crystallized in the pure form, but HMP can only be obtained in equilibrium with BHMP and H_2O_2 and a negligible amount of HCHO. The attainment of equilibrium is catalyzed by OH^- and H^+, the effect of OH^- being predominant at pH > 3 (ref. 13). There is some effect of buffer acid and base. At pH 7 and 25° the reactions proceed rapidly, $t_{\frac{1}{2}}$ for the hydrolysis of BHMP to HMP being around 30 sec. A small amount of H_2O_2 added to 100 mM HCHO will have reacted to 50% within less than 10 sec (pH 8, 1 sec).

It was recently shown that the alleged inhibitory effect of BHMP on peroxidase actually was due to HMP formed from BHMP by hydrolysis[14]. Obviously other biochemical actions attributed to BHMP[1,2,4-8] and the effects of mixtures of H_2O_2 and HCHO[9-12] could have been produced by HMP. The present experiments were designed to investigate this possibility by an examination of the effects of H_2O_2, HMP, BHMP and HCHO on a number of enzymes. Some attempts to characterize the effects of HMP were also performed.

MATERIALS AND METHODS

All experiments were performed at 25°. H_2O_2 (30%, w/w)p.a., Perhydrol Merck. Hydroxymethylhydroperoxide and bis(hydroxymethyl)peroxide were prepared according to MARKLUND[13]. The concentrations of the three peroxides were determined with titanium(IV)[13]. 2-Mercaptoethanol, Eastman organic chemicals. Albumin, bovine, Fraction V; NADH, "Grade III"; Sigma Chemical Co. Horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7), Fraction IIIb (ref. 15), $\varepsilon_{mM}(403\ nm) = 100\ cm^2 \cdot mole^{-1}$. Water, double distilled in quartz vessels. Other reagents were of analytical grade.

The other enzymes were purchased from Boehringer Mannheim GmbH; glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4), *Aspergillus niger*, 140 units/mg; fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2), pig heart, 350 units/mg; lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), pig heart, 360 units/mg; aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), pig heart, 180 units/mg; acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), potato, 60 units/mg. The enzymes were thoroughly dialyzed, min. 3 days, against 0.1 M sodium phosphate, pH 7.0, at 4°, before use.

For spectrophotometry a thermostated Beckman DK2-A was used.

Assay of the enzyme activities

The activities were taken as the initial slopes of spectrophotometer tracings when the enzymes were added to the solutions given below. The methods were designed and checked not to be affected by the small amounts of inhibitors accompanying the enzymes. Glucose oxidase: $A_{470\ nm}$. 36 mM sodium acetate, pH 4.75, 100

mM glucose, 1.8 mM guaiacol and 1.4 μM peroxidase. Fumarate hydratase: $A_{250\ nm}$. 50 mM sodium phosphate, pH 7.30, 50 mM L-malate[16]. Lactate dehydrogenase: A_{340nm}. 50 mM sodium phosphate, pH 7.5. 0.3 mM pyruvate, 0.15 mM NADH. Aspartate aminotransferase: $A_{340\ nm}$. 100 mM sodium phosphate, pH 7.4, 8 mM 2-ketoglutarate, 0.15 mM NADH, 40 mM L-aspartate, 2 μg/ml malate dehydrogenase, and 2 μg/ml lactate dehydrogenase (enzymes from Boehringer). Acid phosphatase: The enzyme was added to 7 mM *p*-nitrophenylphosphate in 100 mM sodium citrate, pH 5.5. After 5 min, 2 vol. of 0.2 M NaOH were added and $A_{405\ nm}$ read against a blank.

Technique of the enzyme inactivation experiments

The protective agent, when present, the enzyme and the inhibitor, added in that order, were incubated aerobically in 50 mM sodium acetate, pH 5.20. The initial enzyme activity (= 1 in the figures) was determined immediately prior to the addition (time = 0) of the inhibitor. The enzyme inactivation was followed by the assay of samples from the incubation mixture after measured time intervals.

The pH of the incubation medium was chosen as a compromise between two effects: Lactate dehydrogenase and fumarate hydratase are inactivated at low pH and the hydrolyses of HMP and BHMP (Formulas 1, 2) are catalyzed by OH^- (ref. 13). After 10 min in 50 mM sodium acetate, pH 5.20, 27% of BHMP will have been hydrolyzed to HMP, and an "HMP mixture" of the representative percentual composition 1.89 mM BHMP, 3.83 mM HMP, 0.78 mM H_2O_2 and no formaldehyde will change to 1.36 mM BHMP, 4.0 mM HMP, 1.14 mM H_2O_2 and 0.89 mM released formaldehyde.

RESULTS AND DISCUSSION

Inhibiting peroxide

The actions of HMP, BHMP, H_2O_2, and HCHO on lactate dehydrogenase (Fig. 1), fumarate hydratase, and aspartate aminotransferase were investigated. Fumarate hydratase was about 5 times more sensitive to the peroxides than lactate dehydrogenase (Fig. 1) and aspartate aminotransferase one seventh, but the relative effects of the peroxides and formaldehyde were essentially the same. In the experiments with HMP as inhibitor, BHMP, H_2O_2, and HCHO were also present, since HMP cannot be prepared in the pure form[13]. H_2O_2 and HCHO, when added separately, were relatively weak inhibitors; BHMP appeared slightly stronger. The "HMP-mixture" was considerably more inhibitory and consequently its effect was caused mainly by HMP. After 10 min in the incubation medium, 27% of an added amount of BHMP will have hydrolyzed to HMP[13]. HMP thus released can account for all the enzyme inactivation apparently caused by BHMP. As mentioned, HMP is rapidly formed from HCHO and H_2O_2 at physiological pH (ref. 13). In the light of these findings, it seems reasonable to conclude that the alleged biochemical effects of BHMP[1-8] and mixtures of HCHO and H_2O_2 (ref. 9–12) are in fact caused by HMP.

Mechanism

Fumarate hydratase is reported to contain 12 thiol groups, 3 on each subunit, considered to be buried in the hydrophobic interior of the enzyme because of their slow reaction with *p*-mercuribenzoate and ethyleneimine (pH > 7)[17]. Phosphate and L-malate protect the enzyme against the above thiol reagents, possibly by stabilizing

the tetrameric form of the enzyme and not by covering the active site[17,18]. At lower pH (6.5) iodoacetate (and 4-bromocrotonate) inhibit the enzyme without much reaction with thiol groups. This inactivation is very efficiently prevented by 50 mM L-malate, and the sites of attack are shown to be methionyl and histidyl residues assumed to be near or at the active site[19].

Fumarate hydratase was the most HMP-sensitive enzyme of those tested. If L-malate (2–50 mM) was present during the treatment with HMP the rate of inactivation was reduced to one fifth. The enzyme was probably saturated with L-malate in these experiments, the K_m values with L-malate and fumarate being $< 10\ \mu M$ (ref. 20). The protection may be exerted by a stabilization of the enzyme and(or) a protection of the active site, and no conclusions on the site of attack of HMP may be drawn.

Lactate dehydrogenase contains about 16 cysteinyl residues. Four of them, one on each subunit, are reactive[21], and when they are blocked most of the enzyme activity is lost. NAD^+, NADH, and NAD^+ sulphite but not pyruvate and lactate[21–23] protect against sulfhydryl reagents. *N*-Ethylmaleimide-blocked enzyme still binds 4 NADH, though with decreased affinity[23], but the enzyme–NADH complex can not bind pyruvate[24]. There is evidence for the presence of an essential histidine residue at the substrate binding site which can be alkylated by bromopyruvate[25] or photooxidized[26]. The alkylation is accelerated by the presence of NAD^+.

Lactate dehydrogenase was easily inactivated by HMP (Fig. 1). The presence of 40 μM NADH protected the enzyme well (Fig. 2). Fumarate hydratase was also somewhat protected by NADH, probably unspecifically *via* the reducing ability of the coenzyme, *cf.* 2-mercaptoethanol below. It seems reasonable to suggest that HMP

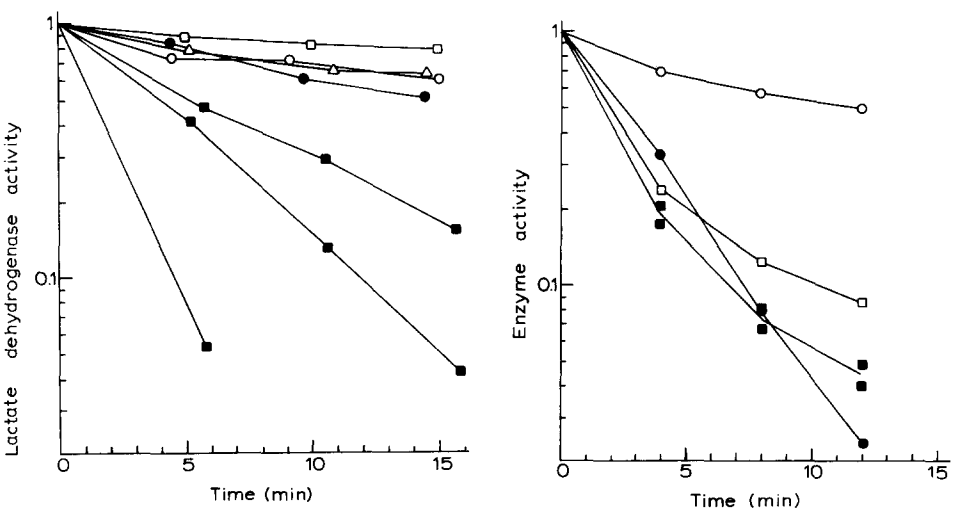

Fig. 1. The inactivation of lactate dehydrogenase by: ■, HMP (from above 0.22, 0.54, and 1.1 mM); ●, BHMP (1 mM); ○, H_2O_2 (1 mM); and △, HCHO (1 mM). □, denotes the spontaneous inactivation of the enzyme in the incubation medium. About 15 μg enzyme per ml.

Fig. 2. The protection of lactate dehydrogenase and fumarate hydratase against HMP by NADH. Lactate dehydrogenase (●) and fumarate hydratase (■) were incubated in 2.26 and 0.47 mM HMP, respectively. ○ and □ denotes the inactivation of lactate dehydrogenase and fumarate hydratase in the presence of the same (2.26 and 0.47 mM) amounts of HMP *plus* 40 μM NADH. About 15 μg and 20 μg, respectively, enzyme per ml incubation medium.

inactivates lactate dehydrogenase mainly by attacking the essential thiol groups, which can be protected by NADH.

Aspartate aminotransferase contains about 10 thiol groups per molecule (mol. wt. 90 000)[27,28], of which 5–7 are easily blocked by, *e.g.* iodoacetate and maleate with no loss of enzyme activity. 2 thiol groups are less accessible and when blocked (*p*-mercuribenzoate) the enzyme activity is decreased. The other thiols react sluggishly with concomitant changes in enzyme conformation[28]. The enzyme is more readily inactivated by thiol reagents (*p*-mercuribenzoate[29], 5,5'-dithiobis-2-nitrobenzoate[27]) in its amino form[30] or in the presence of L-glutamate[31] than in the presence of 2-ketoglutarate. Two of the thiol groups become more exposed when the enzyme is transferred to the amino form[27]. Experiments with photooxidation[28,31–34] and alkylation with 3-bromopropionyl chloride[35] suggest the presence of histidine at the active site of aspartate aminotransferase. Keto-[31] or aminosubstrate[28,31] did not alter the rate of inactivation. The commercial aspartate aminotransferase (crystal suspension in 3 M $(NH_4)_2SO_4$ with 2.5 mM 2-ketoglutarate and 50 mM maleate) employed in the present investigation probably had some of its thiol groups blocked with maleate[36]. Because of the presence of 2-ketoglutarate before dialysis the enzyme was likely to be in its aldimine form[37].

Aspartate aminotransferase is inhibited by HMP but 7 times more resistant than lactate dehydrogenase. The enzyme was twice as sensitive to HMP in the presence of aminosubstrate (1 mM L-aspartate) than in the presence of ketosubstrate (1 mM 2-oxoglutarate). This points at the two above-mentioned thiol groups as possible sites of attack of HMP.

Glucose oxidase contains no free thiol groups as determined by titration with *p*-mercuribenoate and *N*-ethylmaleimide[38] and the inhibition by Ag^+, Hg^{2+}, Cu^{2+}, and *p*-mercuribenzoate, has been attributed to interactions with the FAD groups of the enzyme[38]. Glucose oxidase is highly resistant to H_2O_2 in its oxidized form, but becomes sensitive when reduced[39]. Methionine sulfoxide is formed by the action of H_2O_2, more so when the enzyme is reduced[39].

Glucose oxidase appears in its oxidized form in 50 mM sodium acetate, pH 5.20, with free access to air and in its reduced form in an argon-covered O_2-free solution of 75 mM D-glucose in sodium acetate, pH 5.20. However, in 25 min 16 mM H_2O_2 caused a 40% loss of activity when the enzyme was kept in the reduced form whereas the oxidized form suffered no loss. HMP was a stronger inhibitor than H_2O_2 for oxidized glucose oxidase. Quite unexpectedly, however, reduced glucose oxidase was not more sensitive than the oxidized enzyme to HMP. Thus, when oxidized and reduced enzyme were kept in 7 mM HMP about 85 and 90% of the activity remained after 16 min. This points at some steric hindrance or different reaction mechanisms of the two peroxides.

Acid phosphatase from potato was the most resistant of the enzymes in this investigation, indicating that this enzyme has no readily oxidized essential groups. More than 80% of the activity remained after 15 min incubation in 17 mM HMP. Acid phosphatase from wheat germ is highly radioresistant (D_{37} = 290 krads)[40].

Protection by 2-mercaptoethanol and albumin

2-Mercaptoethanol and albumin protected fumarate hydratase (Fig. 3), lactate dehydrogenase and aspartate aminotransferase against HMP. This was demonstrated

not to be due to a reduction of the total amount of peroxide. 2-Mercaptoethanol (5 mM) was added to an HMP solution (1.9 mM BHMP, 3.8 mM HMP, 0.8 mM H_2O_2) in 50 mM sodium acetate, pH 5.20. After 10 min a sample (0.3 ml) was assayed for peroxides by means of the titanium(IV) reagent[13]. Within this time $\leq 4\%$ of the HMP disappeared. (The determination method was not appreciably affected by the accompanying 2-mercaptoethanol.) Similarly, HMP (0.62 mM BHMP, 1.23 mM

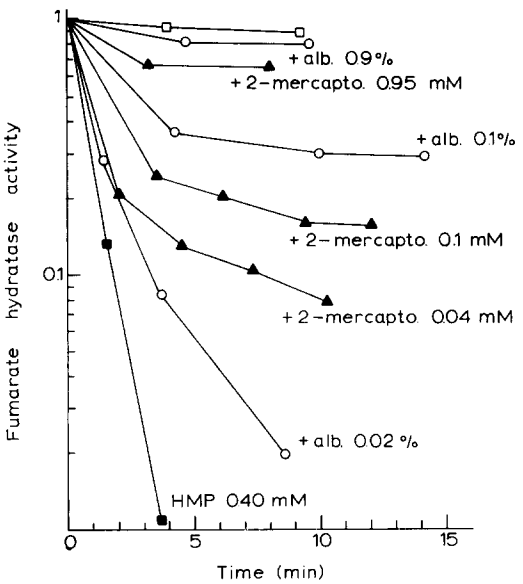

Fig. 3. The protection of fumarate hydratase by 2-mercaptoethanol and albumin. ■, the inactivation of fumarate hydratase in 0.40 mM HMP; ▲, the inactivation in 0.40 mM HMP + various amounts of 2-mercaptoethanol; and ○, the inactivation in 0.40 mM HMP + various amounts of albumin. □, denotes the spontaneous inactivation of the enzyme in the incubation medium. About 20 μg enzyme per ml in the incubation mixture.

HMP, 0.25 mM H_2O_2) was incubated in 50 mM sodium acetate, pH 5.20, in the presence and absence of 0.3% albumin. After 10 min, samples were brought to cuvettes with 6.7 mM guaiacol, 33 mM sodium acetate, pH 4.75, and 1.4 μM horseradish peroxidase, and $A_{470\ nm}$ was recorded. (H_2O_2 and HMP are peroxide substrates of peroxidase[14].) No decrease ($<1\%$) in total peroxide was noticed. The titanium method could not be used for the investigation of the effect of albumin because the protein precipitated.

Reactivation

Lactate dehydrogenase, fumarate hydratase, and aspartate aminotransferase were incubated with HMP and the enzyme activities followed by repeated assays of samples. After 5 min (residual enzyme activities about 20%), 2-mercaptoethanol (1.5 mM) or Tris buffer (final concn. 50 mM, pH 8) or both were added. Tris will rapidly remove BHMP and HMP (ref. 13). Further inactivation of lactate dehydrogenase and fumarate hydratase was stopped in all three media but no reactivation occurred. With aspartate aminotransferase a slow partial reactivation was caused by

2-mercaptoethanol: after 30 min about 20% of the lost activity had returned. The simultaneous addition of Tris hampered the reactivation.

Reaction models

The primary purpose of this investigation was to find out whether biochemical effects attributed to BHMP and mixtures of H_2O_2 and HCHO were caused by HMP. The experimental results also permit some discussion of the mode of action of HMP. The enzymes in this study reported to have essential thiol groups: fumarate hydratase, lactate dehydrogenase, and aspartate aminotransferase were readily inactivated by HMP. In the case of the latter two enzymes indirect evidence was produced that HMP actually attacked these essential thiols. 2-Mercaptoethanol and albumin protected the enzymes, but not by reducing the total amount of HMP. (Peroxidase is not protected by these compounds[41].) The inactivated enzymes could not be reactivated by 2-mercaptoethanol so this can also be excluded as a mechanism for the protection by the thiol, the partial reactivation of aspartate aminotransferase being too slow to account for the protection. Two models for the inactivation and protection may be discussed:

(1) Some activated species (HMP*) derived from HMP, reacts radically or ionically to inactivate the enzymes, 2-mercaptoethanol and albumin acting as scavengers. If this mechanism operates, less, probably much less, than 4% of HMP is transformed to HMP* in 10 min as this is the amount of HMP reduced by 5 mM 2-mercaptoethanol during the same time. Less than 1% disappeared in 0.3% albumin.

(2) HMP oxidizes some residue in the enzyme to a state which may either be reversed by 2-mercaptoethanol and albumin or proceed spontaneously or by further oxidation into an irreversible state. The nature of such a reversible state could be in the case of cysteinyl residues the labile sulfene; the sulfine and particularly the sulfone are not easily reduced and a disulfide is less probable both from steric and kinetic reasons. A methionine sulfoxide would possibly also fit in with the requirements.

Some combined effects could also be possible. Model 1 seems somewhat more probable. From steric reasons it seems difficult for the big albumin molecule to functio according to Model 2. However, albumin and 2-mercaptoethanol do not necessarily have the same modes of action. The inactivation of the enzymes by HMP, especially in the presence ot 2-mercaptoethanol and albumin, is most rapid initially which could be explained by accumulated HMP* in the HMP solution (aqueous, 0°) added to the incubation medium.

The oxidizing power of HMP is considerably greater than that of H_2O_2. It is very rapidly formed from H_2O_2 and the aldehyde at physiological pH (ref. 13) and one may speculate about the formation of this and similar compounds in H_2O_2-producing cells.

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