THE ISOLATION AND PROPERTIES OF AN ACTIVE PEROXIDASE FROM HEPATOCATALASE

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In a previous communication (Caravaca, May, Dimond, 1963) we reported that Caperase (lyophilized bovine hepatocatalase commercially available in Spain and France) lowers serum cholesterol levels in experimental animals and inhibits cholesterol biosynthesis from acetate and mevalonic acid in vitro. Further studies indicated that the inhibitory action of Caperase was not related to its catalatic activity. An investigation of various samples of hepatocatalase showed that, like Caperase, other preparations of commercial lyophilized hepatocatalase with much lower specific activity than crystalline hepatocatalase also inhibited cholesterol biosynthesis while the latter had little or no effect.

The lyophilized samples were found to contain a higher peroxidase activity thar crystalline hepatocatalase. In this connection it is of interest that Tanford and Lovrien (1961) have found that commercial lyophilized catalase is partially dissociated to half and quarter molecules. Inada et al (1961) have shown that alkali denaturation of catalase results in dissociation of the molecule to smaller su units with generation of high peroxidase activity.

The work described here was conducted in the course of our studies to determine whether a peroxidase subunit of catalase is indeed the inhibitory factor in

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cholesterol biosynthesis. This report deals with the isolation of an active mammalian peroxidase from hepatocatalase and some properties of the enzyme.

EXPERIMENTAL

Peroxidase was obtained from hepatocatalase (Caperase) in the form of a stable, lyophilized powder which can be stored for several months without losing enzymatic activity.

In a typical preparation, 500 mg. of enzyme protein, containing approximately 60,000 units of hepatocatalase activity* were dissolved in an alkaline medium (pH 11.1) consisting of 4:1:5 mixture of 0.5 M K_2HPO_4, 0.5 M KOH and water to give a final concentration of 3 mg./ml. The sample was hydrolyzed for 3 hours at 25° at which time generation of peroxidase activity reached its maximum. Hydrolysis over an extended period of time resulted in gradual loss of peroxidase activity with complete disappearance after 8 hours.** The hydrolyzed sample was lyophilized, the dry powder dissolved in 16 ml. of water and dialyzed against 0.005 M potassium phosphate buffer pH 8.5 over a period of 16 hours. The dialyzed sample was lyophilized and the dry powder stored at 4°. The preparation thus obtained is an active peroxidase containing less than 0.1% of the original catalase activity.

Peroxidase activity was assayed by measuring the rate of utilization of H_2O_2 to form a colored reaction product with guaiacol at 470 mμ. at 25°. In the measurements 0.2 ml. of a 0.06 M solution of H_2O_2 were added to a mixture containing 120 μmoles potassium phosphate buffer pH 7.4, 18 μmoles guaiacol and 150 μg. of enzyme in a final volume of 3 ml. A unit of peroxidase acti-

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*A unit of hepatocatalase activity is defined as that amount of protein which decomposes 10 mg. of H_2O_2/min. under the conditions of Beers and Sizer (1953).

**Under the same conditions, high peroxidase activity is also generated by crystalline hepatocatalase (Sigma C-100).

vity is defined as that amount of protein which causes a change in optical density of 0.001 sec. The lyophilized peroxidase contained 30 units/mg. of peroxidase activity and less than 0.05 unit/mg. of catalase activity.

Properties of hepatocatalase peroxidase: The lyophilized preparation is soluble in water at a concentration of 50 mg./ml. and the solution can be stored frozen over long periods of time. The oxidation of guaiacol by the H_2O_2-peroxidase system has an optimal pH range between 9.5 and 10. The activity fell rapidly as the pH was decreased below 9.5 and dropped to approximately one-third at neutral pH. The absorbtion spectra of hepatocatalase (Caperase) and hepatocatalase peroxidase are shown in Fig. 1. The 410/280 ratio of a 5% protein solution is 0.7 for Caperase and 0.48 for the peroxidase.

DPNH and TPNH oxidation: Hepatocatalase peroxidase catalyzes the oxidation of reduced pyridine nucleotides in the presence of H_2O_2 or molecular oxygen and low levels of Mn^{++}. The reaction is markedly stimulated by the addition of certain phenols including some naturally occurring phenolic hormones. The effects of thyroxine, estradiol-17β , estriol and the synthetic estrogen diethylstilbestrol on the peroxidase catalyzed oxidation of DPNH are shown in Fig. 2. Under the conditions described TPNH was oxidized at essentially the same rate. No effect of the hormones was observed in the absence of the peroxidase.

The peroxidase catalyzed aerobic oxidation of DPNH is markedly enhanced by Mn^{++} and proceeds quite readily in the presence of thyroxine. Under the particular conditions employed in Fig. 2 the rate of oxidation was constant until the reaction approached completion. Maximum stimulation was produced by thyroxine at pH 7.4. The activity fell rapidly as the pH was decreased below 7.4 and more slowly as the pH was increased above this value. Under the conditions employed for thyroxine, other simple phenols were less stimulatory.

LEGENDS FOR FIGURES

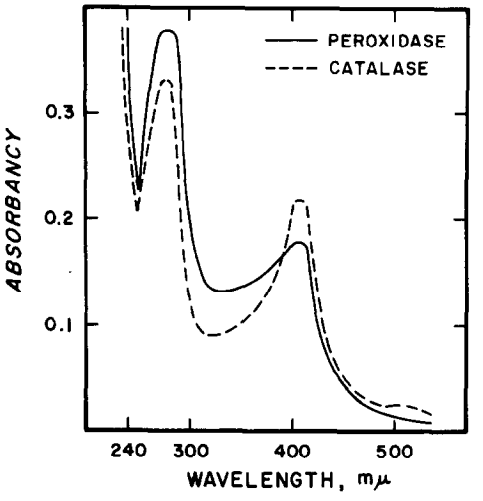

Fig. 1. Absorbtion spectra of hepatocatalase (Caperase) and hepatocatalase peroxidase at pH 8.5 (0.01 M potassium phosphate buffer).

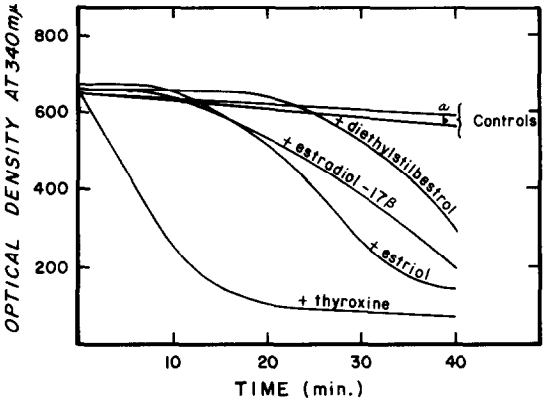

Fig. 2. Effect of thyroxine and estrogens on the oxidation of DPNH by the Mn^{++}-peroxidase-O_2 system. The reaction mixture contained 120 μmoles of phosphate buffer pH 7.4 (pH 8.5 in samples containing estrogens); 0.3 μmole of DPNH; 1.0 mg. of hepatocatalase peroxidase; 0.5 μmole of $MnCl_2$ and water to a final volume of 3.0 ml. Where indicated, sodium-l-thyroxine (0.03 μmole), diethylstilbestrol, estradiol-17β and estriol (0.08 μmole in 20 μl. of methanol) were added. Controls (complete system without hormones added) were run at pH 7.4 (a) and pH 8.5 (b). The latter contained 20 μl. of methanol. The reaction was started by the addition of the peroxidase. The blank contained no DPNH. The oxidation of DPNH was followed by measuring the decrease in light absorbtion at 340 mμ. in a Beckman model DU spectrophotometer at 25°.

Phenol and resorcinol had about 8 and 25% of the activity of thyroxine, respectively. Activation by diethylstilbestrol, estriol and estradiol-17β was optimal at a pH of about 8.5 and occurred following a definite lag period after which it proceeded at a rate which increased with time. The induction period may reflect a slow rate of oxidation of the hormones to a free radical form (Akazawa and Conn, 1958). Under the conditions described in Fig. 2 for the phenolic estrogens, androsterone and dehydroisoandrosterone were found to have no stimulatory effect on the peroxidase catalyzed oxidation of DPNH and TPNH.

The oxidation of reduced pyridine nucleotides by the Mn^{++}-peroxidase-O_2-thyroxine system was totally inhibited by uric acid, hydroxylamine, ascorbic acid and $CuSO_4$ at a concentration of 10^{-5} M. At the same concentration cyanide had no effect. Reduced glutathione, at a concentration of 10^{-4} M caused 90% inhibition. Oxidized glutathione was not inhibitory. The reaction was totally inhibited when 100 units of catalase were added to the system. Hepatocatalase peroxidase was found to inhibit cholesterol biosynthesis from acetate and mevalonic acid by rat liver homogenates (Table 1). The techniques for the preparation of liver homogenates and measurement of acetate and mevalonic acid incorporation into cholesterol were as previously described (Caravaca, May, Dimond, 1963).

DISCUSSION

The peroxidatic activity of catalase was first demonstrated by Keilin and Hartree (1945) who postulated that this function, rather than the ability to decompose H_2O_2 might be the primary biological role of this enzyme. However, in spite of the early recognition of its possible physiological significance, the study of the peroxidatic function of catalase has been complicated by the inter-

fering action of its high catalatic activity which imposed great limitations on the experimental approach.

TABLE 1

Effect of Hepatocatalase peroxidase on Cholesterol Biosynthesis from Acetate-1-2-C^{14} and Mevalonic Acid-2-C^{14}

Expt.	Substrate	Peroxidase added mg.	Cholesterol found (c.p.m./mg.)	% Inhibition
	Acetate	----	18,500	----
1	"	0.5	13,690	26
	"	1.0	8,417	54.5
	Mevalonic acid	----	16,250	----
	"	0.5	12,577	22.6
2	"	1.0	8,450	48

The complete system contained the following in a volume of 2.5 ml; homogenate supernatant fluid, 2 ml; potassium phosphate buffer, pH 7.4, 160 µmoles; $MgCl_2$, 10 µmoles; DPN, 2 µmoles; and substrate. In experiment 1, 10 µmoles of sodium acetate-1-2-C^{14} containing approximately 3 x 10^6 c.p.m. were added. In experiment 2, 4 µmoles of d-1-mevalonic acid-2-C^{14} containing approximately 5 x 10^5 c.p.m. were added. Incubations were carried out in a Dubnoff shaker at 37° for 1 hour under 100% oxygen.

The isolation of an active peroxidase from the catalase molecule in a stable form has made it possible to study the peroxidatic function of catalase without interference from its high catalatic activity. It is seen from these preliminary studies that hepatocatalase peroxidase shares many properties in common with the well characterized horseradish peroxidase (Akazawa and Conn, 1958; Klebanoff, 1959). The stimulatory effect of thyroxine and certain estrogens on the oxidation of DPNH and TPNH by the hepatocatalase peroxidase may be of physiological importance. It is possible that this might be related to the manner in which thyroxine stimulates oxygen consumption in vivo. It also points out a mechanism by which estrogens might participate in hydrogen or electron transport and thus alter the rates

of oxidation-reduction reactions. It is of interest that both thyroxine and estrogens have been shown to inhibit cholesterol biosynthesis (Scaife and Migicovsky 1957; Boyd and McGuire, 1956) which is largely dependent on the presence of reduced pyridine nucleotides.

These results when coupled with the results shown in this paper on the inhibitio of cholesterol biosynthesis by the peroxidase provide an interesting model for a homeostatic control mechanism of cholesterol biosynthesis mediated by an enzyme-hormone system. Further studies on the properties of the peroxidase an its in vitro and in vivo effects are now in progress.

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