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EVIDENCE FOR CATALYTIC SITE CYSTEINE AND HISTIDINE BY CHEMICAL MODIFICATION OF β-HYDROXY-β-METHYLGLUTARYL-COENZYME A REDUCTASE

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Summary: S-(4-Bromo-2,3-dioxobutyl)-coenzyme A inactivates both yeast and rat liver β-hydroxy-β-methylglutaryl-coenzyme A reductase. The inactivation is irreversible, complete in 15 s, and proportional to the concentration of the reagent. β-Hydroxy-β-methylglutaryl-CoA provides protection against inactivation, whereas NADPH does not. Inactivation is attributed to reaction with an essential cysteine at the β-hydroxy-β-methylglutaryl-CoA binding site. Experiments with other active site-directed reagents confirm the involvement of a cysteine and support the presence of an active-site histidine, but rule out the participation of arginine or serine.

Chemical modification of enzymes is a tool for identifying amino acids that participate in substrate binding and other aspects of catalysis (1). This technique has been applied to characterize active sites of key enzymes of a number of metabolic pathways. However, chemical modification to identify active-site amino acids has not been carried out previously on HMG-CoA reductase. This enzyme, the rate-limiting enzyme of cholesterol biosynthesis, exhibits a diurnal cycle, has a 2-h half-life, is hormonally regulated and subject to feedback repression, and has its activity profoundly affected by a number of stimulators and inhibitors (2). HMG-CoA reductase from Chinese hamster ovary has been sequenced by nucleotide sequencing (3). The enzyme is composed of a hydrophobic portion and a protease-released hydrophilic segment that contains the active sites required for catalysis. However, despite the

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Abbreviations used: HMG, β-hydroxy-β-methylglutaryl; BDB, S-(4-bromo-2,3-dioxobutyl).

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wealth of information about the structure and regulation of HMG-CoA reductase, the active-site amino acids participating in interactions required for catalysis have not been identified.

In the present study, we have used the solubilized, purified, catalytically active enzyme segments from yeast and rat liver for reaction with site-specific chemical probes. By this procedure we have demonstrated the effect of selected probes, which have been shown to react with specific amino acid functional groups, on the catalytic activity of HMG-CoA reductase. This is the first report on evidence by chemical modification for catalytic-site amino acids of HMG-CoA reductase.

MATERIALS AND METHODS

Chemicals. HMG-CoA, NADP, NADPH, diethylpyrocarbonate, O-phosphoryl-ethanolamine, phenylmethylsulfonyl fluoride, and diisopropylfluorophosphate were obtained from Sigma; 3-acetylpyridine, pyridinium hydrobromide perbromide, bromoacetyl bromide, iodoacetamide, phenylglyoxal, and 1,4-dibromo-2,3-butanedione from Aldrich; agarose-hexane-CoA affinity gel, type V, from P-L Biochemicals; dithiothreitol from Calbiochem; DEAE-cellulose from Schwarz-Mann, and Sephadex G-25 from Pharmacia. Fleischmann's active dry yeast for bakers was purchased locally. All other reagents were analytical grade. DEAE-Cellulose was acid-base washed and calcium phosphate gel was prepared as previously described (4).

Preparation of HMG-CoA Reductase. The HMG-CoA reductase from yeast was purified from Fleischmann's active dry bakers' yeast after solubilization of the enzyme during autolysis of the yeast. The purification procedure, including heat treatment, ammonium sulfate precipitation, calcium phosphate gel adsorption, DEAE-cellulose chromatography, and agarose-hexane-CoA affinity chromatography, has been described in detail (4). The 55,000 Da, catalytically active fragment of HMG-CoA reductase from rat liver was solubilized from the microsomes and purified as described (5, method 5).

Assay for HMG-CoA Reductase Activity. The assay was carried out spectrophotometrically by measuring the rate of oxidation of NADPH, as monitored by the decrease in absorbance at 340 nm (4). In addition to HMG-CoA reductase, 1-ml incubation mixtures contained potassium phosphate buffer pH 7.0, 100 µmol; dithiothreitol, 4 µmol; DL-HMG-CoA, 300 nmol; and NADPH, 150 nmol. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the oxidation of 1 nmol of NADPH per minute per milliliter of incubation mixture at 37°C.

Synthesis of BDB-CoA. BDB-CoA was synthesized by reacting CoA with freshly crystallized 1,4-dibromo-2,3-butanedione (6). Recrystallized BDB-CoA was stored under an atmosphere of nitrogen at -20°C. An aqueous solution of BDB-CoA was prepared immediately before use and the unused portion was discarded.

Synthesis of N-Bromoacetylethanolamine Phosphate and Bromoacetylpyridine. N-Bromoacetylethanolamine phosphate was synthesized from ethanolamine phosphate and bromoacetyl bromide (7). Synthesis of 3-bromoacetylpyridine hydrobromide was from pyridinium perbromide hydrobromide and 3-acetylpyridine (8).

Inactivation of HMG-CoA Reductase by BDB-CoA. Five milligrams of purified yeast or rat-liver HMG-CoA reductase was passed through a Sephadex G-25 column, 0.4 x 30 cm, which had been equilibrated with 0.1 M potassium phosphate buffer pH 7.0. This procedure removed dithiothreitol, which reacts with the alkylating reagent. The enzyme was then incubated at 37°C with the desired concentration of BDB-CoA in 0.1 M potassium phosphate buffer, pH 7.0. The reaction was stopped by adding dithiothreitol to a concentration of 4 mM, and residual HMG-CoA reductase activity was assayed.

RESULTS AND DISCUSSION

BDB-CoA inactivated yeast HMG-CoA reductase to a degree proportional to the amount of reagent (Fig. 1). The inactivation was fast and irreversible by dialysis in the presence of dithiothreitol, a sulfhydryl compound that protects HMG-CoA reductase from oxidation and from inactivation by BDB-CoA by reacting with that reagent. HMG-CoA reductase was very sensitive to inactivation with BDB-CoA; 0.03 nmol of the yeast enzyme [specific activity 20,000 units/mg, subunit M_r 60,000 (4)] was 50% inactivated by 0.35 nmol of BDB-CoA. The rat liver enzyme was even more sensitive, being almost completely inactivated at a 10:1 molecular ratio of reagent to enzyme. BDB-CoA inactivates

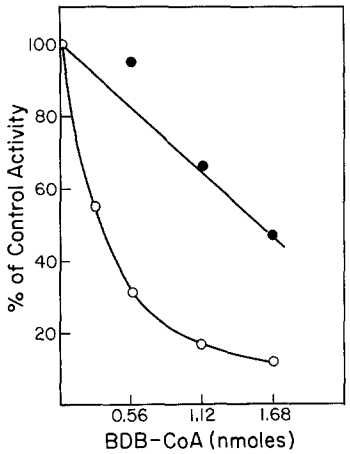

Fig. 1. Protection by HMG-CoA against BDB-CoA–inactivation of HMG-CoA reductase solubilized and purified from yeast. Samples containing 36 units of HMG-CoA reductase activity were incubated in a cuvette with the quantity of BDB-CoA indicated for 15 s at 37°C in 0.2 ml of 0.1 M potassium phosphate buffer, pH 7.0. The solution was diluted with 0.8 ml of the same buffer containing dithiothreitol, 4 μmol; NADPH, 150 nmol; and HMG-CoA, 300 nmol. The mixed solution was monitored spectrophotometrically at 340 nm. This procedure provided the data obtained for incubations in the absence of HMG-CoA, o—o. For the data obtained in the presence of HMG-CoA, •—•, this reagent was added just before BDB-CoA instead of after.

enzymes by alkylating the sulfhydryl group of essential cysteine (9-11). Because of this high sensitivity and some structural similarity between BDB and HMG, the alkylating reagent may show preference for covalent binding to a cysteine at the HMG-CoA binding site. BDB-CoA has been proposed as an affinity label designed for acyl-CoA sites (12).

HMG-CoA protects against short-term inactivation by BDB-CoA (Fig. 1), and the degree of protection is decreased by increasing the concentration of BDB-CoA. This is consistent with HMG-CoA blocking the binding-site cysteine from reaction with BDB-CoA. The measurements are complicated by the fact that HMG-CoA alone inactivates yeast HMG-CoA reductase (13). However, inactivation by HMG-CoA is much slower than by BDB-CoA, and by employing a 15-s incubation period before assay, HMG-CoA's protective effect against BDB-CoA inactivation is easily measurable (Fig. 1).

This protective effect was also observed with rat liver HMG-CoA reductase (Fig. 2). The top line shows that 85% inactivation occurred when BDB-CoA was

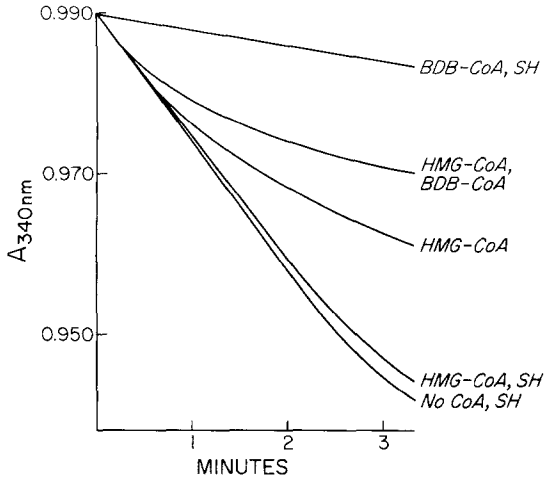

Fig. 2. Retardation by HMG-CoA of the rate of inactivation by BDB-CoA of HMG-CoA reductase solubilized and purified from rat liver. In a cuvette, 0.12 nmol of the catalytically active fragment of HMG-CoA reductase (estimated M_r 50,000) in 0.425 ml of 0.1 M potassium phosphate buffer, pH 7.0, was incubated for 30 s at 37°C with 1.2 nmol of BDB-CoA and/or 300 nmol of HMG-CoA, or without a CoA derivative, as indicated. To this solution was added with mixing 0.575 ml of the same buffer, which also contained 150 nmol of NADPH and 300 nmol of HMG-CoA (if not added before BDB-CoA), and in the determinations indicated by SH, 4 μmol of dithiothreitol. The change in absorbance with time was monitored.

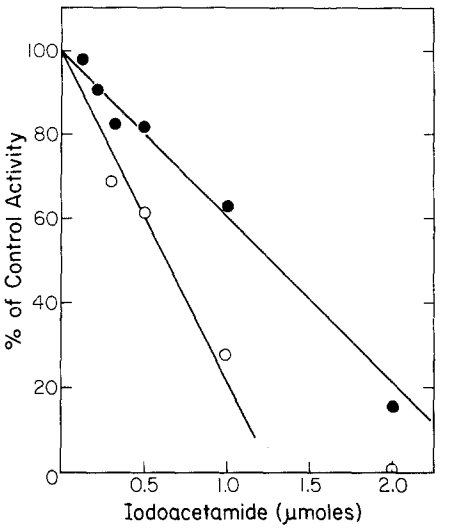

Fig. 3. Effect of temperature on the inactivation by iodoacetamide of HMG-CoA reductase solubilized and purified from yeast. Samples containing 22 units of HMG-CoA reductase activity and the amount of iodoacetamide indicated were incubated in 0.15 ml of 1.0 M potassium phosphate buffer, pH 7.0, for 1 min at 0°C, ●—●, or 37°C, o—o. Each reaction mixture, after the addition of 0.8 ml of the same buffer also containing 4 μmol of dithiothreitol, was incubated at 37°C for 5 min. Spectrophotometric monitoring of absorbance at 340 nm was carried out after addition of 0.05 ml of solution containing 150 nmol of NADPH and 300 nmol of HMG-CoA.

allowed to react with the reductase for 30 s before the unreacted reagent was destroyed with dithiothreitol. However, when BDB-CoA was in the presence of HMG-CoA, little inactivation occurred in 30 s, and when inactivation was allowed to proceed, 3 min was needed to approach 85% inactivation (as demonstrated by the next line in Fig. 2). The third line shows that much of the inactivation that occurred during the 3 min was due to the instability of the enzyme in the absence of dithiothreitol, rather than to inactivation by BDB-CoA. The next line shows that little inactivation of the enzyme occurred on a 30-s incubation with HMG-CoA before the addition of dithiothreitol. This series of time studies clearly shows that BDB-CoA inactivates HMG-CoA reductase rapidly in the absence of HMG-CoA and much more slowly in its presence.

NADPH was substituted for HMG-CoA to test the effect of the other substrate for HMG-CoA reductase on the rate of enzyme inactivation by BDB-CoA. The rate of inactivation by BDB-CoA was not affected by the presence of NADPH with either the yeast or the rat liver reductase.

Table 1. Effect of various chemical modifiers and active site-directed reagents on the activity of yeast HMG-CoA reductase

Reagent	Inhibition	Amount required for 50% inactivation of 36 units of enzyme activity (nmol)	Critical amino acid indicated
S-(4-bromo-2,3-dioxobutyl)-CoA	+++	0.35	cysteine
Bromoacetylpyridine	+	72	cysteine
Iodoacetamide	+	140	cysteine
Bromoacetylethanolamine phosphate	+	91	histidine
Diethylpyrocarbonate	+	125	histidine
Phenylglyoxal	-	-	-
Diisopropylfluorophosphate	-	-	-
Phenylmethylsulfonyl fluoride	-	-	-

Iodoacetamide, a reagent noted for reacting with cysteine sulfhydryls in proteins (14), inactivated HMG-CoA reductase at a rate dependent on the temperature and the concentration of reagent (Fig. 3). Another reagent that has been reported to react with active-site cysteine is bromoacetylpyridine (15). This reagent also inactivated HMG-CoA reductase.

Two reagents reported to react with active-site histidines, diethylpyrocarbonate (16) and bromoacetylethanolamine phosphate (17), inactivated HMG-CoA reductase. However, phenylglyoxal, an arginine probe (18), and serine probes diisopropylfluorophosphate (19) and phenylmethylsulfonyl fluoride, did not affect reductase activity. The effect of the various chemical modifiers is summarized in Table 1. BDB-CoA was by far the most potent for inactivation, probably because of greater selectivity for an active-site amino acid.

Work is in progress using radioactive BDB-CoA to confirm its specificity for one or more cysteinyl residues on HMG-CoA reductase and to determine the stoichiometry of binding.

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