Substrate-Competitive Inhibition of Bacterial Mevalonate:Nicotinamide–Adenine Dinucleotide Oxidoreductase (acylating CoA)*

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ABSTRACT: Soluble mevalonate:nicotinamide–adenine dinucleotide oxidoreductases from two bacterial species, a *Mycobacterium* and a *Pseudomonas*, have been partially purified and their inhibition by substrate analogs studied.

It is concluded that substrate binding to the enzyme requires a carboxyl, a 3-methyl, and a 3-hydroxy group. Any compound with this structure acts either as a substrate or as an inhibitor, competitive with mevalonate.

Soluble extracts of *Mycobacterium* S4 (Siddiqi, 1962; Siddiqi and Rodwell, 1962) or of *Pseudomonas* M1 (Fimognari, 1964; Rodwell *et al.*, 1965) catalyze both the mevalonate:NAD oxidoreductase (acylating CoA) E.C.1.1.1.x and the 3-hydroxy-3-methylglutaryl-CoA acetoacetate lyase E.C.4.1.3.4. reactions.

$$\text{mevalonate} + \text{CoA} + 2\ \text{NAD}^+ \longrightarrow 2\ \text{NADH} + 2\text{H}^+ + \text{HMG-CoA} \quad (1)$$

$$\text{HMG-CoA} \longrightarrow \text{acetoacetate} + \text{acetyl-CoA} \quad (2)$$

Reaction 1 is analogous to the mevalonate:NADP oxidoreductase (acylating CoA) E.C.1.1.1.34 or HMG-CoA[1] reductase reaction of mammalian liver (Knauss *et al.*, 1959) which is the principal site of "feedback" regulation of hepatic cholesterol synthesis (Siperstein and Fagan, 1964). The bacterial oxidoreductases differ from that of rat liver with respect to coenzyme specificity and, more significantly, in being soluble and far more active. Thus while assay of the liver oxidoreductase requires isotopically labeled substrates (Knauss *et al.*, 1959; Fimognari, 1964; Fimognari and Rodwell, 1965) the bacterial oxidoreductases may be assayed by conventional spectrophotometric techniques.

The observation (Siddiqi, 1962) that HMG inhibits mevalonate oxidation by cell-free extracts of *Mycobacterium* S4 prompted a study of a series of mevalonate analogs. Using soluble and partially purified oxidoreductase preparations from two bacterial sources we show that the minimum structural requirement for inhibition competitive with mevalonate to be that of a 3-methyl-3-hydroxycarboxylic acid. We further propose that mevalonate binding to the bacterial oxidoreductases involves only these same three groups.

Materials and Methods

Reagents. Compounds obtained commercially included: 3-hydroxy-3-methylbutyric acid, 2,2-dimethylpropionic acid, 3-methylbutanol-1, 1,3-butanediol, citric acid, and DEAE-cellulose (Distillation Products Ind.); DL-3-hydroxy-3-methylglutaric acid, DL-mevalonic acid δ-lactone, NAD, and CoA (Sigma Chemical Co.); glutaric acid and 3,3-dimethylacrylic acid (Calbiochem); 3-methylbutyric acid and sodium succinate (Fisher); sodium DL-3-hydroxybutyrate (British Drug House Mfg. Co.); and deoxycholate (Mann). DL-2-Phenylbutyric acid (Merck) was a gift of Dr. Lemuel D. Wright. Lithium acetoacetate was prepared according to Hall (1963).

Protein Determination. A spectrophotometric method (Waddell, 1956; Murphy and Kies, 1960) was employed using 0.9% NaCl as a diluent.

Determination of Enzyme Activity. The increase in absorbancy at 340 mμ due to NADH formation was measured at 30° using a Cary Model 14 spectrophotometer equipped with a thermostated cell compartment and a scale expander. For the *Pseudomonas* enzyme a 0.7-ml incubation mixture of the following composition was used: 100 mM in Tris-HCl, pH 7.1; 2.9 mM in NAD; 0.57 mM in CoA; and 3.57 mM in potassium DL-mevalonate, pH 7.1. The assay mixture for the *Mycobacterial* enzyme contained, in addition, 14 mM $MgCl_2$. One enzyme unit (mU) was defined as that amount of enzyme catalyzing the reduction of 1 mμmole of NAD/min.

* From the Department of Biochemistry, School of Medicine, University of California, San Francisco Medical Center, San Francisco. *Received May 27, 1965; revised July 6, 1965.* This work is from the Ph.D. thesis of Grace M. Fimognari. It was supported by a grant (GM-06468) from the U. S. Public Health Service. A preliminary report has appeared (Rodwell *et al.*, 1965).

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[1] Abbreviations used in this work: HMG, 3-hydroxy-3-methylglutarate; NAD^+, oxidized nicotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide.

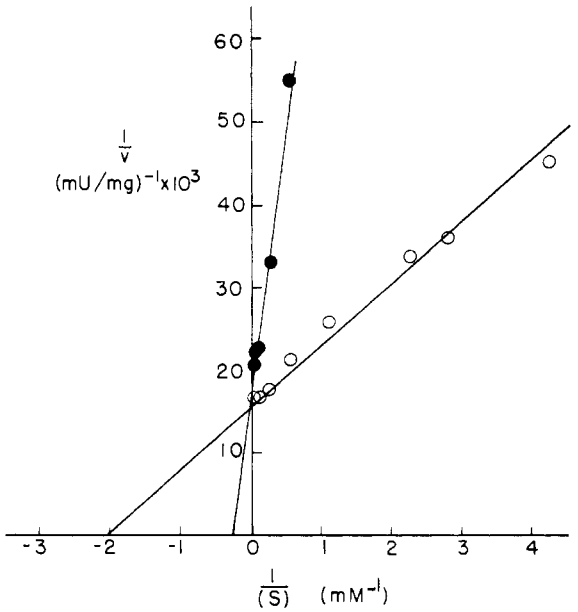


FIGURE 1: Competitive inhibition of the *Mycobacterium* oxidoreductase by DL-3-hydroxy-3-methylglutarate. Assay conditions were as follows: 100 mM Tris-HCl, pH 7.1; 14 mM $MgCl_2$; 2.9 mM NAD; 0.57 mM CoA; the indicated concentrations of DL-mevalonate, pH 7.1; either no inhibitor (open circles) or 1.4 mM DL-HMG, pH 7.1 (closed circles), and *Mycobacterium* oxidoreductase. The calculated K_i for DL-HMG was 0.02 mM.

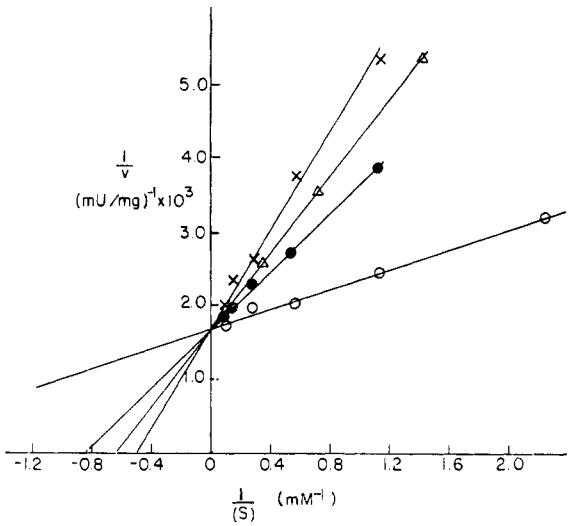


FIGURE 2: Competitive inhibition of the *Pseudomonas* oxidoreductase by various 3-hydroxy-3-methylcarboxylic acids. Buffer, NAD, $MgCl_2$, and CoA concentrations were as in Figure 1. Cuvets contained, in addition, the indicated concentrations of DL-mevalonate, pH 7.1; either no inhibitor (open circles); 1.4 mM DL-3-hydroxybutyrate, pH 7.1 (closed circles); 1.4 mM 3-hydroxy-3-methylbutyrate, pH 7.1 (×), or 1.4 mM DL-HMG (triangles) and *Pseudomonas* oxidoreductase.

Oxidoreductase from Pseudomonas M1. Cells were grown in shake culture at pH 7.0 and at 30° in a medium 25 mM in ammonium DL-mevalonate, 60 mM in potassium phosphate, 20 mM in sodium phosphate, 0.6 mM in $MgSO_4$, 0.02 mM in $FeSO_4$, and 0.002 mM in $MnSO_4$. Subsequent operations were at 0–5°. Cells were harvested by centrifugation (3000 × *g;* 30 min) at a bacterial density of about 720 mg of dry cells/l. as determined by turbidity measurement using a Klett photometer (Rao and Rodwell, 1962). The packed cells were washed once by resuspension in mevalonate-free medium and recentrifugation. Packed cells, which may be stored frozen for several months without appreciable loss of enzyme activity, were resuspended in 0.04 M potassium phosphate, pH 8.0, to give a 1% (dry wt/vol) suspension and ruptured by 30-min treatment at 100 v in a 9 kc Raytheon sonic oscillator. The resulting opalescent solution was centrifuged (3000 × *g;* 15 min) to remove unbroken cells and cell debris. The supernatant fraction or crude sonic extract was then centrifuged at 105,000 × *g* for 2 hr. The 105,000 × *g* supernatant fraction, which contains the activity, may be stored frozen. Supernatant fraction protein (50 mg of 105,000 × *g*) was applied to a 27 × 2.2 cm diethylaminoethylcellulose (DEAE-cellulose) column equilibrated with 0.04 M potassium phosphate, pH 8.0, and washed in with three column volumes (48 ml) of the same buffer. The enzyme was then eluted using a pH and salt gradient. The mixing chamber initially contained 200 ml of 0.04 M potassium phosphate, pH 8.0, and the reservoir 0.2 M potassium phosphate, pH 6.5. Fractions (9 ml) were collected at an elution rate of 1.5 ml/min. The active fractions were pooled, concentrated in dialysis tubing immersed in Carbowax 20 M, and stored frozen. The preparation used for the kinetic studies had a specific activity of 3260 mμmoles of NAD reduced or 1630 mμmoles of mevalonate oxidized min^{-1} per mg of protein.

Preparation of Oxidoreductase from Mycobacterium S4. Growth, harvesting, sonic rupture, and preparation of the 105,000 × *g* supernatant fraction were carried out as described for the *Pseudomonas* enzyme but substituting 0.08 M potassium phosphate, pH 7.0, for the 0.04 buffer, pH 8.0, prior to sonication. Supernatant fraction protein (50 mg of 105,000 × *g*) was applied to a 27 × 2.2 cm DEAE-cellulose column equilibrated with 0.08 M potassium phosphate, pH 7.0, and washed in with one column volume (16 ml) of the same buffer. The enzyme was then eluted by a salt gradient. The mixing chamber initially contained 35 ml of 0.08 M and the reservoir 0.48 M potassium phosphate, pH 7.0. Fractions (3 ml) were collected at an elution rate of 0.3 ml/min. The most active fractions were pooled, concentrated against Carbowax 20 M, and stored frozen. In contrast to the *Pseudomonas* enzyme, repeated freezing and thawing resulted in loss of activity. The preparation used for kinetic studies had a specific activity of 170 mμmoles of NAD reduced or 85 mμmoles of mevalonate oxidized min^{-1} per mg of protein.

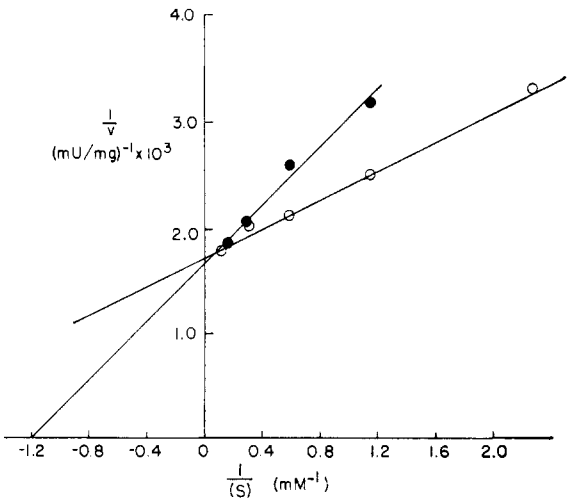


FIGURE 3: Competitive inhibition of the *Pseudomonas* oxidoreductase by deoxycholate. Buffer, NAD, $MgCl_2$, and CoA concentrations were as in Figure 1. Cuvets contained, in addition, the indicated concentrations of DL-mevalonate, pH 7.1; either no inhibitor (open circles) or 0.29 mM deoxycholate, pH 7.1 (closed circles); and *Pseudomonas* oxidoreductase.

TABLE I: Kinetic Constants for Mevalonate:NAD Oxidoreductase (acylating CoA) from *Pseudomonas* M1 and *Mycobacterium* S4.[a]

Source of Enzyme	K_m (mM) DL-Mevalonate	CoA	NAD
Pseudomonas M1	0.35	0.10	0.35
Mycobacterium S4	0.49	0.10	0.60

[a] Assays were carried out at the following concentrations of those substrates not under study: DL-mevalonate, 28.6 mM (85 × K_m); CoA, 0.57 mM (10 × K_m); NAD, 14.3 mM (40 × K_m); and $MgCl_2$, 14.3 mM. Data were evaluated graphically by plotting $1/v$ *vs.* $1/S$.

Both final preparations contained activity for HMG-CoA lyase (3-hydroxy-3-methylglutaryl-CoA acetoacetate lyase, E.C.4.1.3.4). The *Mycobacterium* enzyme requires added Mg^{2+} for full activity while the *Pseudomonas* enzyme showed no demonstrable requirement for a divalent metal.

Results

Michaelis Constants for Substrate, NAD, and CoA. The Michaelis constants for all three cosubstrates were evaluated in the conventional manner (Table I). These were quite similar for both the *Pseudomonas* and *Mycobacterium* enzymes and also all of a similar order of magnitude.

TABLE II: Compounds Tested as Inhibitors.[a]

Additions	*Pseudomonas* enzyme: Observed Activity (mU)	*Pseudomonas* enzyme: Fraction of Control (%)	*Mycobacterium* enzyme: Observed Activity (mU)	*Mycobacterium* enzyme: Fraction of Control (%)
None	5.7		6.4	
DL-3-Hydroxy-3-methylglutarate, 1.4 mM	3.4	59	3.4	53
DL-3-Hydroxybutyrate	2.7	47	0.90	14
3-Hydroxy-3-methylbutyrate	1.5	25	1.1	18
Citrate	5.8	102	5.9	93
Succinate	5.6	98	6.2	98
Glutarate	5.9	104	6.2	98
3,3-Dimethacrylate	5.5	96	5.9	93
2,2-Dimethylpropionate	5.4	94	6.7	105
Acetoacetate	5.6	98	6.6	104
Deoxycholate	0	0	0	0
Deoxycholate, 0.14 mM			4.7	72
None	3.5			
3-Methylbutanol-1	3.6	103		
DL-1,3-Butanediol	3.7	107		
3-Methylbutyrate	3.2	98		
None	1.5			
Decanedioate	1.6	108		
*None	3.1			
*DL-2-Phenylbutyrate, 5.1 mM	3.1	100		

[a] Enzyme assay conditions were as follows for all but the last two assays. Final volume, 0.7 ml; Tris-HCl, pH 7.1, 100 mM; DL-mevalonate, 3.6 mM; CoA, 0.57 mM; NAD, 14 mM; $MgCl_2$, 14 mM; neutralized solution of compound tested, 14 mM except where otherwise indicated and either the *Pseudomonas* or *Mycobacterium* oxidoreductase. Since DL-2-phenylbutyrate was relatively insoluble at pH 7.1, the starred (*) assays were conducted at pH 8.0 by substituting 200 mM potassium phosphate, pH 8.0, for the Tris buffer and adjusting the pH of all other solutions to 8.0.

Compounds Tested as Inhibitors. Several compounds structurally related to mevalonate were tested for their ability to inhibit mevalonate oxidation by either the *Pseudomonas* or *Mycobacterium* enzyme (Table II). Only 3-hydroxybutyrate, 3-hydroxy-3-methylbutyrate, 3-hydroxy-3-methylglutarate, and deoxycholate were inhibitory.

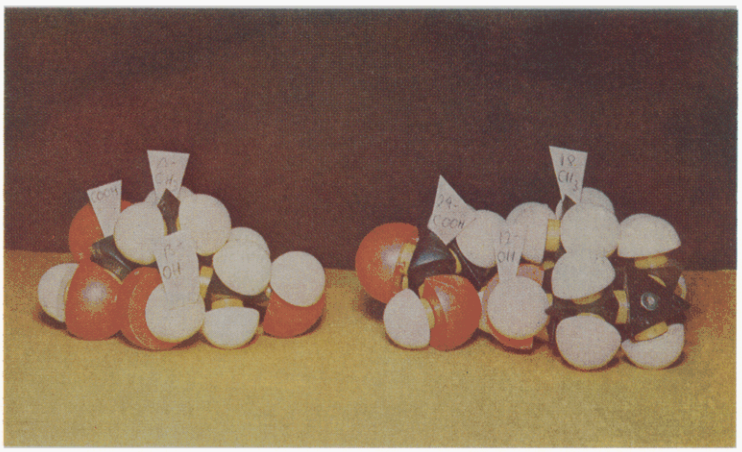


FIGURE 4: Molecular models of mevalonate and of the C and D rings of deoxycholate. Left, mevalonate; right, deoxycholate.

Competitive Inhibition by 3-Hydroxy-3-methylcarboxylic Acids. Each compound which inhibited mevalonate oxidation was further tested to establish the nature of the inhibition (Figures 1–3). In each case the inhibition was strictly competitive with mevalonate, and the K_i value (Table III) was of the same order of magnitude as the K_m for mevalonate.

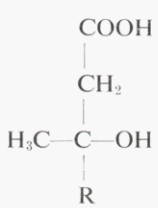
TABLE III: K_i Values for Competitive Inhibitors for the *Pseudomonas* Oxidoreductase.[a]

Inhibitor	K_i (mM)
3-Hydroxybutyrate	0.60
DL-3-Hydroxy-3-methylbutyrate	0.30
DL-3-Hydroxy-3-methylglutarate	0.40
Deoxycholate	0.26

[a] The data are from Figures 1 and 3.

Discussion

With the exception of deoxycholate, each competitive inhibitor is a 3-hydroxy-3-methylcarboxylic acid. That the minimum structural requirement for inhibition is indeed



is shown by the fact that all compounds with this structure were competitive inhibitors, and no compound lacking either the carboxyl, the 3-methyl, or the 3-hydroxyl showed significant inhibition. Neither 3-methylbutanol, which lacks both a carboxyl and a 3-hydroxy group, nor 1,3-butanediol, which lacks only the carboxyl group, was an inhibitor. 3,3-Dimethacrylate, acetoacetate, and 3-methylbutyrate are all 3-methylcarboxylic acids but were not inhibitors. The failure of acetoacetate to inhibit shows that the requirement for a 3-hydroxyl cannot be replaced by a 3-carbonyl function. Citrate also failed to inhibit, indicating that a 3-carboxyl group cannot replace a 3-methyl group. Finally, the failure of 2-hydroxy-2-methylpropionate to inhibit indicates that the hydroxyl and methyl groups must be β to the carboxyl group.

Deoxycholate was tested as an inhibitor since we had previously found that it inhibited mevalonate synthesis from acetate by rat liver "HMG-CoA reductase" (Fimognari and Rodwell, 1965). On the basis of the above studies it seemed clear that only 3-hydroxy-3-methylcarboxylic acids were competitive inhibitors, yet deoxycholate was an excellent competitive inhibitor with a K_i comparable to the K_m for mevalonate (Table III). The explanation is apparent when a molecular model of deoxycholate is compared with a model of mevalonate. As shown in Figure 4, the 24-carboxyl, the 18-methyl, and the 12-hydroxyl of deoxycholate can assume a configuration isosteric with mevalonate.

The close similarity of the K_m and K_i values together with the structural requirements for competitive inhibition suggest that binding of mevalonate to the enzyme takes place solely *via* the carboxyl, the 3-hydroxy, and the 3-methyl groups. The suggestions that enzyme-bound mevaldate is an intermediate between HMG-CoA and mevalonate (Ferguson *et al.*, 1959; Brodie and Porter, 1960; Siddiqi and Rodwell, 1962) and that enzyme-bound mevaldate hemimercaptal (Durr and Rudney, 1960; Siddiqi, 1962) and enzyme-bound HMG (Brodie *et al.*, 1964) also participate as intermediates have all been previously advanced. Both bacterial (Siddiqi, 1962) and yeast (Durr and Rudney, 1960)

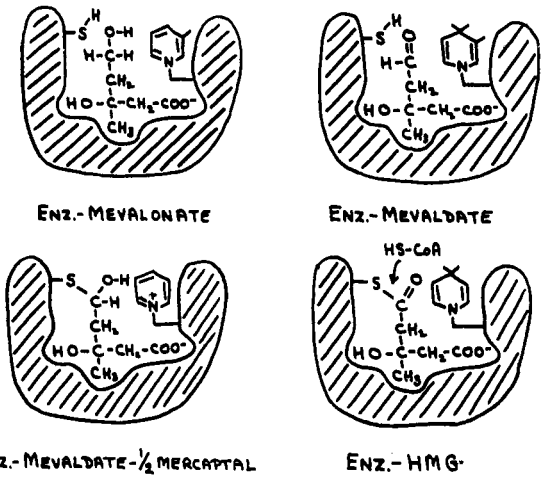


FIGURE 5: Hypothetical model of the active site of the *Pseudomonas* oxidoreductase.

enzymes are inhibited by thiol reagents, suggesting that an SH group is essential for activity. We therefore propose a model for the active site of the *Pseudomonas* oxidoreductase consistent with these observations (Figure 5). A similar model for the *Mycobacterium* enzyme would require Mg^{2+} in addition. Substrate binding is represented as involving only the indicated three groups. Only 1 mole of NAD is shown since the actual number bound is not known. Oxidation of I would yield enzyme-bound mevaldate, which might undergo nucleophilic attack on the carbonyl carbon by an SH of the enzyme yielding enzyme-bound mevaldate hemimercaptal. A second oxidation would yield enzyme-bound HMG which might then undergo an exchange reaction with free CoA, forming HMG-CoA, or might be cleaved to yield acetyl-CoA and acetoacetate (Siddiqi and Rodwell, 1962).

HMG and deoxycholate not only are competitive inhibitors for the bacterial oxidoreductases but also inhibit mevalonate synthesis from acetate by rat liver homogenates (Fimognari, 1964; Fimognari and Rodwell, 1965). Inhibition of the crude mammalian system by other 3-methyl-3-hydroxycarboxylic acids (*e.g.*, 3-hydroxy-3-methylbutyrate and 3-hydroxybutyrate) is not readily subject to analysis due to the presence of enzymes catalyzing their metabolism. The ease of preparation, solubility, and greater specific activity of the bacterial oxidoreductase preparations as compared to the rat liver preparation, together with demonstrated similarity in response to inhibitors, suggests that the bacterial enzymes may provide a useful model for the mammalian enzyme and be of use in the screening of pharmaceutical agents for treatment of hypercholesterolemia.

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