3-Hydroxy-3-methylglutaryl-CoA Lyase: Catalysis of Acetyl Coenzyme A Enolization†

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ABSTRACT: 3-Hydroxy-3-methylglutaryl-CoA lyase, which performs the cleavage of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to acetoacetate and acetyl-CoA by a Claisen-type reaction, also catalyzes enolization of acetyl-CoA. The rate of detritiation of methyl-labeled acetyl-CoA is proportional to enzyme concentration and is diminished by an antiserum that also inhibits the cleavage reaction. The tritium-exchange reaction requires both divalent cation and acetoacetate. An analogue of HMG-CoA, 3-hydroxyglutaryl-CoA, was prepared by reaction of acetonedicarboxylic anhydride with CoASH and reduction of the ketoacyl-CoA product with cyanohydridoborate. While 3-hydroxyglutaryl-CoA does not appear to be a substrate for HMG-CoA lyase, it competitively inhibits both the cleavage reaction (K_i = 50 μM) and the tritium exchange from acetyl-CoA (K_i = 95 μM). Agreement between the K_i values measured for cleavage and for tritium exchange supports the hypothesis that the slow tritium exchange is a lyase-dependent reaction. Initial attempts to demonstrate complete reversiblity of the cleavage reaction have not been successful. However, the data suggest that the cleavage of HMG-CoA is at least partially reversible and indicate that enolization of acetyl-CoA may be dependent upon a conformational change of HMG-CoA lyase, induced by binding of acetoacetate, in a manner analogous to the keto acid dependent tritium exchange catalyzed by malate synthase and citrate synthase [Eggerer, H., & Klette, A. (1967) *Eur. J. Biochem. 1*, 447; Srere, P. A. (1966) *Biochem. Biophys. Res. Commun. 26*, 609; Eggerer, H. (1965) *Biochem. Z. 343*, 111].

HMG-CoA[1] lyase (3-hydroxy-3-methylglutaryl-CoA acetoacetate-lyase, EC 4.1.3.4) catalyzes the cleavage of HMG-CoA to acetoacetate and acetyl-CoA in a Claisen retrocondensation reaction. The Claisen reaction is similar to the base-catalyzed aldol reaction, both involving C–C bond cleavage. Acetyl-CoA-utilizing Claisen enzymes are ubiquitous in nature and include many of the "citrate enzymes" including citrate synthase and several of the acyl-CoA ligases such as malate synthase. Both citrate synthase and malate synthase couple carbon–carbon bond formation to hydrolysis of a thio ester linkage with enolization of acetyl-CoA postulated as the rate-determining step (Klinman & Rose, 1971; Eggerer, 1965). In contrast to these enzymes, which have been studied in some detail (Srere, 1972, Higgins et al., 1972), the HMG-CoA cleavage enzyme has received relatively little attention, in part because stable purified preparations have only recently become available (Kramer & Miziorko, 1980). Both the bovine enzyme (Stegink & Coon, 1968) and the avian enzyme (Kramer & Miziorko, 1980) require divalent cation and sulfhydryl-containing reagents for catalytic activity. Studies on the partially purified enzyme have established the stereospecificity and stoichiometry of HMG-CoA cleavage (Stegink & Coon, 1968). Like all other acetyl-CoA-utilizing Claisen enzymes studied, the cleavage of HMG-CoA involves inversion about C2 of HMG-CoA (Messner et al., 1975). Stegink & Coon (1968) proposed a mechanism of HMG-CoA cleavage that involved abstraction of a proton from the 3-hydroxyl position followed by C–C cleavage and the formation of an enolate of acetyl-CoA. A reversal of this mechanism implies the enolization of acetyl-CoA, a situation directly analogous to that observed with citrate and malate synthase. The experiments described in this paper were designed to test whether HMG-CoA lyase does, in fact, support the postulated enolization of acetyl-CoA and to define the factors that support such a reaction. A preliminary account of this work has appeared (Kramer & Miziorko, 1982).

Experimental Procedures

Materials

Acyl-CoA thio esters were synthesized from the appropriate anhydrides (Simon & Shemin, 1953). HMG anhydride, both unlabeled and 3-^{14}C-labeled, was synthesized by the method described by Goldfarb & Pitot (1971). Because of limitation in quantities of [3-^{14}C]HMG acid employed, the acetone-soluble portion of the reaction mixture was removed with a pipet rather than by filtration. The solid dicyclohexylurea formed during anhydride production was washed with cold acetone in order to extract any residual [^{14}C]HMG anhydride. The acetone extract was evaporated under a stream of dry N_2, and the resulting residue was used directly in the preparation of the CoA ester.

Dithiothreitol was purchased from Boehringer. Hepes was obtained from Sigma Chemical Co., St. Louis, MO. Agarose–hexane–coenzyme A type I was purchased from P-L Biochemicals, Milwaukee, WI. Lithium acetoacetate was purchased from Sigma, and the rabbit antiserum prepared against purified HMG-CoA lyase was the same as described previously (Kramer & Miziorko, 1980). 3-Ketoglutaric acid and diethyl 3-hydroxyglutarate were obtained from Aldrich Chemical Co., Milwaukee, WI.

Methods

Assay of HMG-CoA lyase activity was measured as described previously (Kramer & Miziorko, 1980) or by the method of Clinkenbeard et al. (1975). Duck liver HMG-CoA lyase was prepared with a modification of the procedure of Kramer & Miziorko (1980). Following chromatography on hydroxylapatite, the partially purified enzyme was concen-

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPLC, high-pressure liquid chromatography; AcAc, acetoacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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trated in an Amicon ultrafiltration cell with a PM 30 membrane. Precipitation with ammonium sulfate was omitted. Affinity chromatography utilized agarose–hexane–coenzyme A (type I) instead of agarose–hexane–3′,5′-ADP due to considerable variability between lots of the latter resin in its ability to bind partially purified HMG-CoA lyase. Biospecific elution of the lyase enzyme was accomplished by using the competitive inhibitor 3-hydroxyglutaryl-CoA (100 μM) rather than coenzyme A.

^{3}H exchange from [2-^{3}H]acetyl-CoA into solvent water was measured by following the depletion of ^{3}H radioactivity from acetyl-CoA, determined after removal of water from aliquots of the incubation mixtures by lyophilization. An alternate method, which verified that substantial hydrolysis of acetyl-CoA does not occur under incubation conditions, involved using a mixture of ^{3}H- and ^{14}C-labeled acetyl-CoA. Aliquots of the incubation mixture were quenched at pH 1.6 prior to lyophilization to remove ^3H_2O. ^{3}H exchange was measured by the first-order rate of ^{3}H/^{14}C decrease. Any acetyl-CoA hydrolysis is detected by depletion of ^{14}C radioactivity. Nonenzymatic rates of ^{3}H exchange are less than 5% of the enzymatic rate measured in the presence of optimal Mg^{2+} and acetoacetate.

Synthesis of 3-Hydroxyglutaryl-CoA. The anhydride of acetonedicarboxylic acid was prepared according to the precedure of von Pechman & Neger (1893) using 3-ketoglutaric acid that has been recrystallized from ethyl acetate (Fieser & Fieser, 1967). The anhydride crystals were washed with benzene and stored in a desiccator. This material is not suitable for prolonged storage.

Reaction of the anhydride with CoA was performed as outlined by Simon & Shemin (1953). A total of 47 mg of the acetonedicarboxylic anhydride was added to an aqueous solution of coenzyme A (125 mg) that had been adjusted to pH 8.5 with $NaHCO_3$. The pH is maintained at 8.0 during the reaction while keeping the solution cold (on ice), stoppered, and under N_2 atmosphere while shaking vigorously for 5 min. The pH is then quickly brought to 3 by addition of 2 N HCl. Nitrogen is then bubbled through the mixture for 5 min.

The sample was then reduced to 3-hydroxyglutaryl-CoA with cyanohydridoborate since the mildly acidic conditions (pH 3) required for reduction of ketones (Borch et al., 1971) are ideal for stabilization of the carboxyl at C5 and maintenance of the thio ester linkage. Sodium cyanohydridoborate (31 mg) was added to the mixture. Because the reaction consumes acid with HCN evolution, it was necessary to maintain pH $\sim$3 by addition of HCl. After approximately 20 min, the pH appeared to remain constant, indicating that the reaction had reached completion (Borch et al., 1971). Complete conversion of the unreduced 3-ketoacyl-CoA intermediate to the 3-hydroxy derivative was verified by reverse-phase HPLC (Miziorko et al., 1982). The sample was then diluted in 5 mM LiCl containing 3 mM HCl and applied to a DE-52 column 1.5 × 30 cm that was eluted with a 20–400 mM LiCl gradient of 1000 mL, 3 mM in HCl. Fractions containing the thio ester product were examined by reverse-phase HPLC. The product exhibited a single absorbance peak (260 nm) with a retention time of 10.1 min at a flow rate of 1 mL/min in comparison to 11.2 min for the unreduced product. Pooled fractions from the DE-52 column were lyophilized, dissolved in methanol, and precipitated repeatedly from methanol/acetone (1:5) to remove LiCl. The purified product was dissolved in 3 mM HCl and stored at −20 °C.

An aliquot of the product was cleaved by alkaline hydrolysis in $LiOH/LiHCO_3$ (pH 11) for 2 h. The hydrolysate was acidified with HCl and the free acid extracted into ethyl acetate. The diethyl ester of 3-hydroxyglutaric acid was converted to the free acid by the same procedure and used as a standard for comparison. Comparison of the ^{1}H NMR spectra of the free acids confirmed the assignment of the reduced acyl-CoA as 3-hydroxyglutaryl-CoA.

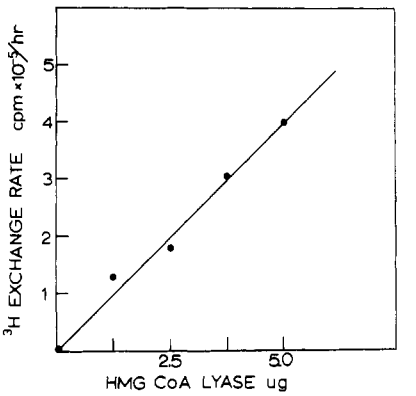

FIGURE 1: Tritium exchange as a function of HMG-CoA lyase concentration. Reaction mixtures contained 160 μmol of Hepes (pH 7.0), 40 μmol of lithium acetoacetate, 4 μmol of $MgCl_2$, 2 μmol of dithiothreitol, and HMG-CoA lyase, as indicated, in a total volume of 0.4 mL. The reaction was initiated by addition of 160 nmol of [^{3}H]acetyl-CoA (sp act. 5400 cpm/nmol). The experiment was performed at 30 °C. Reactions were quenched by freezing in a dry ice/acetone mixture. ^3H_2O was removed by lyophilization.

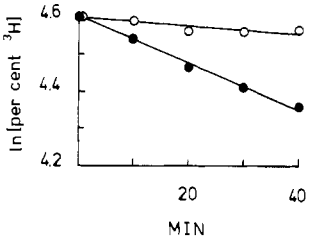

FIGURE 2: Acetoacetate requirement for ^{3}H exchange. 50-μL reaction mixtures contained 5 μmol of Hepes, pH 7.0, 0.5 μmol of $MgCl_2$, 0.5 μmol of dithiothreitol, 0.6 μg of HMG-CoA lyase (sp act. 350 units/mg), 70 nmol of acetyl-CoA, both 2-^{3}H and 1-^{14}C labeled (5680 and 1380 cpm/nmol, respectively), and 0 (○) or 100 mM (●) lithium acetoacetate. The temperature was maintained at 30 °C. Reactions were quenched by pipetting 10-μL aliquots into 100 μL of 1 M potassium phosphate, pH 1.6. ^3H_2O and any [^{3}H]- and [^{14}C]acetate formed by spontaneous hydrolysis of acetyl-CoA were removed by lyophilization. Rates of the first-order ^{3}H exchange are indicated by measuring the percentage of initial ^{3}H to ^{14}C ratio.

Results

Detritiation of [^{3}H]Acetyl-CoA. HMG-CoA lyase catalyzes tritium exchange from [^{3}H]acetyl-CoA. Upon incubation of acetyl-CoA tritiated in the methyl position with HMG-CoA lyase under appropriate conditions (Figure 1), loss of radioactivity from acetyl-CoA is observed. While the rate of ^{3}H exchange into the aqueous medium is 1000-fold slower than the rate of HMG-CoA cleavage catalyzed by the same quantity of enzyme under standard assay conditions, the rate was found to be directly proportional to the concentration of enzyme protein (Figure 1).

The tritium-exchange reaction is stimulated by acetoacetate, a coproduct of HMG-CoA cleavage (Figure 2). A plot of the initial tritium-exchange rate vs. acetoacetate concentration yields an hyperbolic saturation curve; a double-reciprocal plot of the data indicates an apparent K_m for acetoacetate equal to 36 mM (Figure 3). A control experiment (minus acetoacetate) was also performed at 5 mM Mg^{2+}. The detritiation

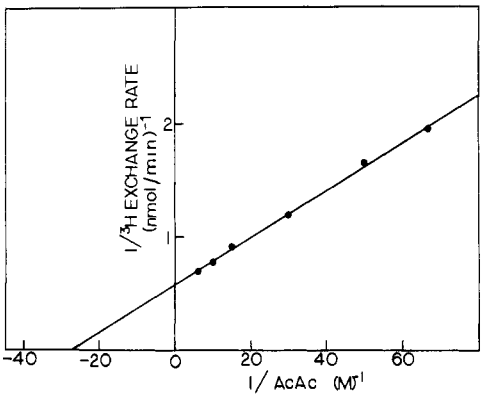

FIGURE 3: Dependence of ^{3}H-exchange rate on acetoacetate concentration. The detritiation rate was measured in a 0.4-mL reaction mixture at 30 °C containing 2.5 μg of HMG-CoA lyase (sp act. 350 units/mg), 160 nmol of [^{3}H]acetyl-CoA (5400 cpm/nmol), 4 μmol of $MgCl_2$, 2 μmol of dithiothreitol, 160 μmol of Hepes, pH 7.0, and 0–167 mM lithium acetoacetate, as indicated. Reactions were terminated by freezing in dry ice/acetone. ^3H_2O was removed by lyophilization.

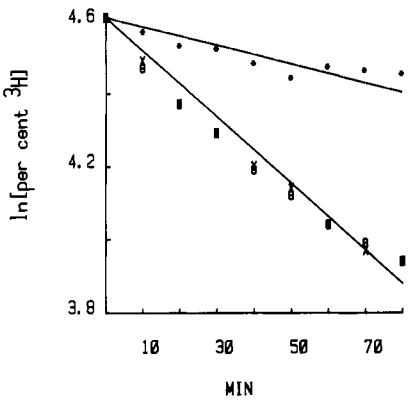

FIGURE 4: Stimulation of ^{3}H exchange by Mg^{2+}. Reaction mixtures contained either 0.1 μmol of EDTA and 0.5 μmol of $MgCl_2$ (×), 0.1 μmol of EDTA (*), or 0.5 μmol of $MgCl_2$ (⊙) in a 100-μL mixture containing 0.92 unit of HMG-CoA lyase. Conditions were otherwise as described in Figure 2. The ^{3}H-exchange rate was determined from measurement of the ratio of ^{3}H to ^{14}C.

rate was negligible under these conditions, as was the rate observed at 5 mM Mg^{2+} in the absence of enzyme.

The formation of HMG-CoA by a retrocondensation has not been detected. An HMG-CoA lyase catalyzed condensation of acetyl-CoA and [3-^{14}C]acetoacetate was attempted by incubation of 17 μg (6 units) of HMG-CoA lyase in a 100-μL volume containing 50 mM [^{14}C]acetoacetate (sp act. 10000 cpm/nmol) and 2 mM acetyl-CoA for 2 h at pH 7.5. Following precipitation of the protein by addition of methanol and the addition of unlabeled carrier HMG-CoA, the reaction mixture was analyzed by reverse-phase ion-pairing HPLC (Baker & Schooley, 1979; Miziorko et al., 1982). The HMG-CoA peak did not contain any ^{14}C radioactivity above base-line levels.

Stimulation of Tritium Exchange by Mg^{2+}. In view of the requirement for divalent cation in the cleavage reaction, the effect of divalent cation on acetyl-CoA enolization was investigated. The results presented in Figure 4 indicate that Mg^{2+} markedly stimulates the detritiation rate. The pseudo-first-order rate constant in the absence of Mg and with EDTA added was 3.4-fold smaller than that observed when an excess of Mg^{2+} (5 mM) is included in the reaction mixture. Since 5 mM Mg^{2+} supports an exchange rate identical with that measured in the presence of 5 mM Mg^{2+} plus 1 mM EDTA, the possibility that the failure to stimulate ^{3}H exchange

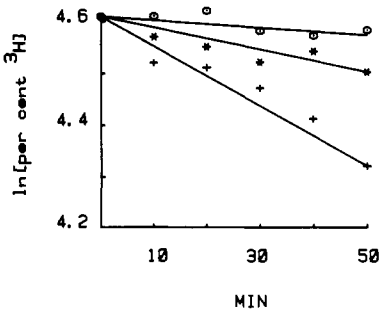

FIGURE 5: Inhibition of ^{3}H exchange by antiserum. Rabbit antiserum prepared against HMG-CoA lyase and rabbit antiserum prepared against bovine serum albumin were mixed with HMG-CoA lyase as follows: 20 μL of each antiserum was mixed with an equal volume of homogeneous HMG-CoA lyase (1.2 units) followed by addition of 20 μL of a 25 mM potassium phosphate solution containing 50% glycerol and 3 mM dithiothreitol (DTT). A third mixture was prepared that contained only 20 μL of anti-HMG-CoA lyase antiserum, 20 μL of the potassium phosphate–glycerol–DTT solution, and 20 μL of H_2O. Following an incubation of 2 h at 30 °C, 50-μL aliquots from each mixture were introduced into the assay mixture for measurement of ^{3}H exchange. The volume of the assay mix was 100 μL. The assay mixtures contained 30.0 μmol of Hepes, pH 7.0, 0.01 mg of bovine albumin, 0.45 μmol of $MgCl_2$, 0.45 μmol of dithiothreitol, 10 μmol of lithium acetoacetate, 1.0 unit of HMG-CoA lyase (activity determined prior to incubation with antiserum), and 0.10 μmol of acetyl-CoA containing both 2-[^{3}H]methyl and 1-^{14}C in the acyl group. The specific activities were 5600 and 280 cpm/nmol, respectively. The exchange was quenched by pipetting 15-μL aliquots into 100 μL of 1 M potassium phosphate, pH 1.6. ^3H_2O and any acetate produced by hydrolysis of the substrate were removed by lyophilization. The rates of tritium exchange are expressed as ln of the percentage of initial ratio of ^{3}H to ^{14}C for each reaction mix: antiserum against HMG-CoA lyase + HMG-CoA lyase enzyme (*); antiserum against bovine albumin + HMG-CoA lyase enzyme (+); antiserum against HMG-CoA lyase without added enzyme (⊙).

in the absence of Mg was the result of nonspecific inhibition by EDTA can be discounted. The magnitude of the stimulatory effect is similar to the 7-fold enhancement of HMG-CoA cleavage due to magnesium (Kramer & Miziorko, 1980).

The residual ^{3}H-exchange activity observed in the absence of added Mg was most likely the consequence of a tightly bound cation that could not be completely separated from the enzyme by brief exposure to EDTA in the reaction mixture. This interpretation is consistent with the observation[2] that slow passage of an HMG-CoA lyase sample over a column of Chelex 100 produces an enzyme sample that exhibits a 200-fold stimulation of cleavage activity by Mg in comparison to the rate measured in the absence of added Mg.

Inhibition of Tritium Exchange by Antiserum. In order to unequivocally demonstrate that the detritiation reaction is attributable to the action of HMG-CoA lyase, it was necessary to incubate enzyme with an antiserum prepared against homogeneous HMG-CoA lyase under conditions that produce specific inhibition of the cleavage reaction (Kramer & Miziorko, 1980). The antiserum-treated enzyme was evaluated for its ability to detritiate acetyl-CoA and was compared to enzyme that was treated with antiserum against bovine serum albumin. The results (Figure 5) indicate that antiserum specific for HMG-CoA lyase inhibits ^{3}H exchange as well as HMG-CoA cleavage. The inhibition is the result of specific antibody–antigen interaction, as incubation with a nonspecific serum is without effect. Control experiments show that antiserum alone does not stimulate ^{3}H exchange.

Interaction of HMG-CoA Lyase with 3-Hydroxyglutaryl-CoA. 3-Hydroxyglutaryl-CoA, an analogue of HMG-CoA

[2] P. R. Kramer, unpublished observations.

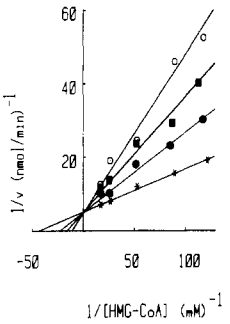

FIGURE 6: Inhibition of HMG-CoA lyase by 3-hydroxyglutaryl-CoA: kinetic determination of an inhibitor constant. Reaction mixtures contained Tris-Cl$^-$, pH 8.2, 40 μmol, $MgCl_2$, 2 μmol, dithiothreitol, 1 μmol, HMG-CoA lyase, 0.68 ng (sp act. 305 units/mg), bovine serum albumin, 20 μg, and [3-^{14}C]HMG-CoA (17.9×10^3 cpm/nmol) as indicated, in a total volume of 0.2 mL. Inhibitor levels were 0 (*), 200 μM (●), 400 μM (■), and 600 μM (⊙). Temperature was 30 °C. The complete reaction mixture, less substrate, was incubated for 10 min at 30 °C prior to initiation of the cleavage reaction by addition of substrate. Aliquots (40 μL) were withdrawn at 3-min intervals and pipetted into vials containing 0.1 mL of 2 N HCl. The acidified aliquots were taken to dryness at 90 °C. Reaction velocity is measured as the rate of depletion of acid-stable radioactivity (i.e., uncleaved [^{14}C]HMG-CoA).

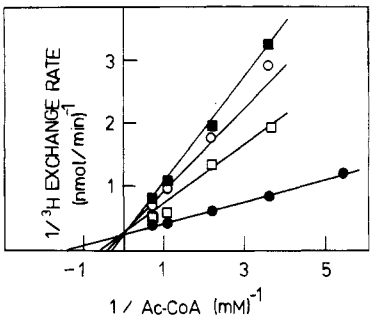

FIGURE 7: Inhibition of tritium exchange by 3-hydroxyglutaryl-CoA. Reaction mixtures containing 0.78 unit of homogeneous HMG-CoA lyase were assayed by the method described in Figure 5, except that the final concentration of Hepes, pH 7.0, was 0.1 M and the specific activity of isotopically labeled acetyl-CoA was 5100 and 1400 cpm/nmol in ^{3}H and ^{14}C, respectively. The concentration of 3-hydroxyglutaryl-CoA in each reaction mixture was 0 (●), 0.2 mM (□), 0.4 mM (○), and 0.6 mM (■).

lacking the methyl group at C3, proved to be a potent inhibitor of HMG-CoA lyase. This substrate analogue is a linear competitive inhibitor with respect to HMG-CoA in the cleavage reaction (Figure 6); secondary plots of the data (Segel, 1975) indicate a K_i = 50 μM for hydroxyglutaryl-CoA.

It was possible that binding of 3-hydroxyglutaryl-CoA to HMG-CoA lyase could lead to a slow turnover to form cleavage products acetyl-CoA and malonic semialdehyde. Therefore 1 unit of HMG-CoA lyase was incubated with 400 μM 3-hydroxyglutaryl-CoA in a 1-mL volume along with the components for the standard spectral assay for HMG-CoA cleavage, which detects formation of acetyl-CoA. No cleavage of 3-hydroxyglutaryl-CoA was observed.

An analogue of HMG-CoA might be expected to compete for binding at the active site with the products of the HMG-CoA cleavage reaction. The data of Figure 7 indicate that 3-hydroxyglutaryl-CoA competes effectively with acetyl-CoA as the varied substrate in the detritiation reaction. A replot of slope vs. inhibitor concentration (not shown) reveals a linear slope effect and a K_i of 95 μM, in reasonable agreement with the value obtained for inhibition of the cleavage reaction by this compound. The double-reciprocal plot indicates an apparent K_m for acetyl-CoA of 0.7 mM.

Discussion

The HMG-CoA lyase catalyzed cleavage of HMG-CoA to form acetoacetate and acetyl-CoA occurs via a Claisen mechanism. Scission of the bond between the α and β carbons of the 3-hydroxy-3-methylglutaryl moiety produces acetoacetate and the enolate of acetyl-CoA. Protonation of the latter completes the reaction sequence that has been proposed for the bovine enzyme (Stegink & Coon, 1968). This type of reaction is similar to a base-catalyzed aldol reaction except that the reactant in the Claisen reaction is an ester. Those acetyl-CoA-utilizing enzymes that function within pathways where the physiologically important direction is condensation couple carbon–carbon ligation to CoA thio ester hydrolysis. Notable examples include citrate synthase, malate synthase, and HMG-CoA synthase. Since the cleavage reaction is energetically favorable, enzymes found within pathways in which the physiologically important direction is cleavage preserve the thio ester bond. The HMG-CoA cleavage reaction is so highly favorable that, to date, no back-reaction has been reported. However, the chemistry of the Claisen and aldol reactions raises the possibility that HMG-CoA cleavage could be reversed. The first step in any potential condensation of acetyl-CoA with acetoacetate should be abstraction of a methyl proton from the acetyl moiety and formation of the enolate. The observation of detritiation of methyl-labeled acetyl-CoA implies enolization and demonstrates that at least a portion of the reaction can be reversed.

Since the detritiation is very slow in comparison to HMG-CoA cleavage, it is important to be certain that the exchange reaction is, in fact, attributable to the catalytic activity of HMG-CoA lyase. A comparison of the properties of the exchange vs. cleavage reactions supports this conclusion. Both HMG-CoA cleavage and detritiation of acetyl-CoA are stimulated by divalent cation. The magnitude of the stimulatory effect of Mg in the cleavage reaction is similar to the effect on enolization of acetyl-CoA. The dependence of acetyl-CoA detritiation on acetoacetate, a coproduct of HMG-CoA cleavage, also suggests that the detritiation reaction is catalyzed by HMG-CoA lyase. The K_m for acetoacetate agrees well with the K_i of acetoacetate observed in inhibition of HMG-CoA cleavage.[3] The competitive inhibitor 3-hydroxyglutaryl-CoA inhibits both exchange and cleavage reactions with similar efficacy, and the inhibition produced by an antiserum raised against HMG-CoA lyase in both the detritiation and HMG-CoA cleavage reactions is comparable. Taken together, the data indicate that there is little probability that the exchange reaction could be the result of a contaminant present in the enzyme preparation.

Stimulation of proton abstraction by Mg^{2+} may suggest the assignment of a catalytic role for divalent cation in coordination to the thio ester carbonyl. Studies of the effect of divalent metals in stimulation of HMG-CoA cleavage (Stegink & Coon, 1968; Kramer & Miziorko, 1980) have demonstrated a requirement for divalent cation in the cleavage reaction but did not suggest its function in the catalytic mechanism. The properties of the thio ester linkage are compatible with this interpretation. In comparison with oxygen esters, the thio ester carbonyl should be more polarizable as a consequence of the low electronegativity of sulfur. The enolate of the thio ester would be expected to be stabilized by both resonance and inductive effects (Bruice & Benkovic, 1965). Coordination of the carbonyl with divalent cation could further stabilize the

[3] P. R. Kramer, unpublished observations.

polarized carbonyl by functioning as an electron sink. A similar role for cation has been postulated for class II aldolases (Mildvan et al., 1971).

Magnesium-stimulated enolization of acetyl-CoA in the presence of a keto acid is directly analogous to the effect reported by Eggerer & Klette (1967) in their study of malate synthase in which pyruvate, an analogue of glyoxalate, stimulated exchange of tritium from ^3H_2O into acetyl-CoA. They had proposed that the cation acts as a Lewis acid acting cooperatively with base catalysis involving the carboxylate of pyruvate in analogy to the chemical model of the bifunctional catalyst (Swain & Brown, 1952). This hypothesis was consistent with the finding that malate synthase did not catalyze the condensation of acetyl-CoA and pyruvate to citramalate, seemingly due to steric hindrance from the methyl group of pyruvate, which prevented nucleophilic attack upon the ketocarbonyl. This interpretation was consistent with the observation that malate synthase did not attack citramalyl-CoA. However, it was subsequently shown that condensation reactions catalyzed by malate synthase and other acetyl-CoA-utilizing enzymes involved inversion about C2 of the acyl group, which tends to preclude the possibility that enolization of acetyl-CoA proceeds through participation of the carboxylate in base catalysis (Klinman & Rose, 1971). Induction of a catalytically active state by keto acid binding is currently a favored interpretation (Walsh, 1977). Similarly, binding of malate to citrate synthase (Eggerer, 1965) stimulates enolization of acetyl-CoA. However, malate is incapable of undergoing a condensation reaction since it lacks the ketone functionality of oxaloacetate. There is evidence to suggest that binding of oxaloacetate induces a change in the conformation of citrate synthase (Srere, 1966, 1967, 1972), which may activate the enzyme in the acetyl-CoA enolization process. However, gross conformational changes need not occur for transition from an inactive to a conformationally active state. Stimulation of detritiation of acetyl-CoA by acetoacetate in this study suggests, by analogy to the malate synthase and citrate synthase studies, the induction of an enzyme form that catalyzes enolization of acetyl-CoA upon acetoacetate binding to enzyme. It should be kept in mind that minute changes in conformation of the active site implied by an induced-fit mechanism may define a "conformationally active" state.

The apparent failure of 3-hydroxyglutaryl-CoA to undergo cleavage in the presence of HMG-CoA lyase despite effective binding at the active site (indicated by K_i values of 50 μM and 95 μM measured for the cleavage and exchange reactions, respectively) indicates that the methyl group at C3 of HMG-CoA is a necessary structural feature for the formation of an active enzyme–substrate complex. Comparison of K_m for HMG-CoA of 8 μM vs. K_i = 50 μM for 3-hydroxyglutaryl-CoA, which was determined under identical conditions, implies that the presence of the methyl group increases the observed binding affinity by at least a factor of 6. Recently, another analogue of HMG-CoA lyase, *S*-(4-carboxy-3-hydroxyisoamyl)-CoA was reported to efficiently inhibit HMG-CoA lyase competitively with K_i = 80 μM (Miziorko et al., 1982), suggesting the substitution of a thioether linkage in this analogue for the usual thio ester moiety perturbs binding by a factor comparable to that measured when hydroxyglutaryl-CoA is substituted for HMG-CoA. However, unlike hydroxyglutaryl-CoA, which fails to serve as a substrate for structural or steric reasons, the thioether analogue of HMG-CoA fails to turn over as a consequence of its inability to stabilize the negative charge that must develop at C2 during a cleavage event.

Acknowledgments

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Registry No. HMG-CoA, 1553-55-5; HMG-CoA lyase, 9030-83-5; acetyl-CoA, 72-89-9; 3-hydroxyglutaryl-CoA, 35192-10-0; CoA, 85-61-0; acetonedicarboxylic anhydride, 10521-08-1.

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