3-Hydroxyl-3-methylglutaryl-CoA synthase: Utility of acetyldithio-CoA in detecting the influence of active site residues on substrate enolization

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Running Title: HMG-CoA synthase catalysis of acetyldithio-CoA enolization

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SUMMARY

Hydroxymethylglutaryl-CoA synthase-catalyzed condensation of acetyl-CoA with acetoacetyl-CoA requires enolization/carbanion formation from the acetyl C2 methyl group prior to formation of a new carbon-carbon bond. Acetyldithio-CoA, a readily enolizable analog of acetyl-CoA, is an effective competitive inhibitor of avian HMG-CoA synthase ($K_i = 28 \mu M$). In the absence of cosubstrate, enzyme catalyzes the enolization/proton exchange from the C2 methyl group of acetyldithio-CoA. Mutant enzymes that exhibit impaired formation of the covalent acetyl-S-enzyme reaction intermediate exhibit diminished (D159A, D203A) or undetectable (C129S) rates of enolization of acetyldithio-CoA. The results suggest that covalent thioacetylation of protein, which has not previously been detected for other enzymes that enolize this analog, occurs with HMG-CoA synthase. Enzyme catalyzes the transfer of the thioacetyl group of this analog to 3'-dephosphoCoA suggesting the intermediacy of a covalent thioacetyl-S-enzyme species, which appears to be important for proton abstraction from C2 of the thioacetyl group. Avian enzyme glutamate-95 is crucial to substrate condensation to form a new carbon-carbon bond. Mutations of this invariant residue (avian enzyme E95A and E95Q; S. aureus enzyme E79Q) correlate with diminished ability to catalyze enolization of acetyldithio-CoA. Enolization by E95Q is not stimulated in the presence of acetoacetyl-CoA. These observations suggest either a direct (proton abstraction) or indirect (solvent polarization) role for this active site glutamate.
3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase catalyzes production of a key intermediate in ketogenic (1) and steroidogenic (2) pathways by a multi-step Claisen condensation reaction (3). The synthesis of a new C-C bond requires formation of a carbanion from the acetyl C2 methyl carbon of acetyl-CoA prior to attack on the C3 ketone of acetoacetyl-CoA.

Deprotonation of acetyl-CoA to form a carbanion that condenses with cosubstrate is a mechanistic step common to HMG-CoA synthase, citrate synthase (4), and malate synthase (5) reactions. In the absence of cosubstrate or a suitable analog, deprotonation of acetyl-CoA is not catalyzed by these enzymes at a substantial rate, possibly due to the high pKₐ value (18-20) for this proton. A more readily enolizable analog of acetyl-CoA, namely acetyldithio-CoA, is characterized by a lower pKₐ for the C2 protons (pKₐ = 12.5) and has proven to be a useful enolizable substrate analog in studies with citrate synthase (6, 7). Recent work has indicated that it also serves as an enolizable analog of the acetyl-CoA product of the HMG-CoA lyase reaction (8).
beta ketothiolase reaction, acetyldithio-CoA has been reported (9) to be unable to thioacetylate enzyme to form a covalent adduct. However, this reaction employs a second acyl-CoA molecule which, after enolization, condenses with the covalent acyl-enzyme species. Acetylated beta ketothiolase efficiently enolizes acetyldithio-CoA, bound noncovalently as an analog of the second substrate (9).

In order to investigate the enolization reaction catalyzed by HMG-CoA synthase, acetyldithio-CoA has been tested as a substrate analog. This report describes the results of these studies, which indicate that even in the absence of cosubstrate, enzyme does indeed enolize this low pKₐ analog. Moreover, the importance of formation of a covalent acyl-enzyme intermediate to the enolization process is also suggested by these studies.

EXPERIMENTAL PROCEDURES
Materials. Escherichia coli BL21 (DE3) and the expression vectors pET-3d and pET-23d were purchased from Novagen (Madison, WI). Deoxyoligonucleotides were purchased from Operon Technologies (Alameda, CA). QuickChange site-directed mutagenesis kits were obtained from Stratagene (LaJolla, CA). Qiagen (Chatsworth, CA) plasmid kits were used to isolate plasmid DNA from bacterial cultures. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Amersham Pharmacia Biotech, Inc (Piscataway, NJ). DNA sequencing was performed on an ABI 3100 Genetic Analyzer at the Protein/Nucleic Acid Facility of the Medical College of Wisconsin. Ampicillin and isopropylthiogalactoside were purchased from United States Biochemical
(Cleveland, OH). [1-14C] acetyl-CoA is a product of Moravek Biochemicals (Brea, CA). All other reagents were purchased from Sigma (St. Louis, OH), Aldrich (Milwaukee, WI), or Pharmacia Biotech, Inc. (Piscataway, NJ).

Construction of mutant HMG-CoA synthase. Mutagenesis to produce the E95Q form of avian enzyme and the E79Q form of the \textit{S. aureus} enzyme was performed using Stratagene’s “Quick Change” mutagenesis kit and pairs of appropriate complementary mutagenic primers. Mutant plasmids were used to transform competent XL1-Blue cells. Mutagenic plasmid DNA was isolated from selected transformants and analyzed by restriction mapping and DNA sequencing. Mutant clones that were determined to be free from any PCR artifacts and confirmed to contain the desired substitution were transformed into competent BL21(DE3) cells for expression and isolation as described below.

Isolation and assay of HMG-CoA synthase proteins. The procedure developed for purification of the avian wild-type enzyme (10) was followed for isolation of all mutant avian enzymes (C129S, D159A, D203A, E95A, E95Q) from IPTG induced bacterial cultures. Wild-type and E79Q \textit{S. aureus} HMG-CoA synthases have been engineered from genomic \textit{S. aureus} DNA to incorporate C-terminal histidine tags (procedure to be described elsewhere) and were isolated from a nickel affinity resin column (1.0 x 15 cm) which had been exhaustively washed with equilibration buffer (50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl, 20 mM imidazole, and 5 mM mercaptoethanol) prior to elution of homogeneous protein using an imidazole gradient.
(20 to 300 mM; 100ml volume). Protein content of the purified enzymes was estimated by the Bradford assay (11), using bovine serum albumin as the standard. All wild-type and mutant proteins are characterized by a high degree of homogeneity, as assessed by SDS-polyacrylamide gel electrophoresis.

Either the standard spectrophotometric assay (2) or the more sensitive radioisotopic assay (2) was used to measure activity. In the spectrophotometric assay, the reaction mix included 100 mM Tris-Cl, pH 8.2, 100 µM EDTA, appropriate amounts of HMG-CoA synthase (approximately 6 µg for wild-type enzyme), 50 µM acetoacetyl-CoA and 200 µM acetyl-CoA. The reaction was performed at 30 °C and acetyl-CoA dependent loss of acetoacetyl-CoA was measured as a decrease in 300 nm absorbance, using a millimolar extinction coefficient of 3.6. For improved sensitivity, the spectrophotometric assay can be performed in the presence of 40 mM MgCl₂; in such cases, the millimolar extinction coefficient of 20.0 is used for acetoacetyl-CoA.

For the radioisotopic assay, the reaction mixture included 100 mM Tris-HCl, pH 8.2, 100 µM EDTA, 50 µM acetoacetyl-CoA, 200 µM [¹⁴C]-acetyl-CoA (8,600-10,000 dpm/nmol), and appropriate amounts of wild-type or mutant HMG-CoA synthase. The reaction was initiated by addition of radiolabeled acetyl-CoA to the assay mixture containing the rest of the components at 30 °C. At specified time intervals, 40 µl aliquots were removed from the incubation mixture and acidified with 6 N HCl. The mixture was heated to dryness and acid-stable radioactivity due to [¹⁴C]-HMG-CoA was measured by liquid scintillation counting.
Assay of the acetyl-CoA hydrolysis partial reaction, performed as described by Misra et al. (10), involved measuring time-dependent depletion of $[^{14}C]$ acetyl-CoA. Unhydrolyzed substrate is measured as acid stable radioactivity after its conversion to citrate with excess citrate synthase and oxaloacetate.

Stoichiometry of covalent acetylation (i.e. acetyl-S-enzyme formation) was determined according to the procedure described by Miziorko et al. (12).

**Inhibition of HMG-CoA synthase by acetyldithio-CoA.** Initial velocity experiments were conducted in mixtures including acetoacetyl-CoA, Ac-CoA, and acetyldithio-CoA in 0.1 M Tris buffer, pH 8.2, containing 40 mM MgCl$_2$. Samples were incubated at 30 °C, then pre-incubated enzyme (6.5 µg) was added to start the reaction. The concentrations of Ac-CoA used were 133, 200, 300, 400, 600, and 1500 µM; the fixed concentrations of acetyldithio-CoA used were 0 (control), 25, 50 or 100 µM. This spectrophotometric assay measures the rate of AcAc-CoA depletion at 300 nm. Rate data were analyzed (Grafit) as $1/v$ versus $1/[AcCoA]$ at different concentrations of acetyldithio-CoA inhibitor.

**HMG-CoA synthase catalyzed exchange of acetyldithio-CoA C2 methyl protons.** Synthesis of acetyldithio-CoA from phenyl thioacetate (Aldrich) was performed as described by Wlassics et al. (6) with the following modifications. The exchange reaction between S-phenylthioacetate and CoASH was performed in 300 mM Li$_2$CO$_3$, pH=8.5. The reaction was terminated by acidification to pH=4 with 1N HCl followed by evaporation to reduce the volume to 3 mL. After extraction with 3 mL of ethyl acetate,
the aqueous phase was concentrated to 200 µL; 1.5 mL of cold methanol was added and acetyldithio-CoA precipitated as the lithium salt by addition of 10 mL of cold acetone. After a total of four precipitations from cold methanol/acetone, the product was dried under a stream of nitrogen. Acetyldithio-CoA eluted as a single peak on RP-HPLC using an isocratic elution, (70% 50mM sodium phosphate, pH 4.5: 30% methanol) indicating the absence of additional CoA species. Purity was also verified by $^1$H NMR and UV/Vis spectroscopy (ratio of $A_{260nm}/A_{306nm} = 1.3$).

The ability of HMG-CoA synthase to catalyze solvent deuterium exchange with methyl protons of acetyldithio-CoA was monitored by $^1$H NMR. HMG-CoA synthase proteins were subjected to three cycles of concentration in an Amicon ultra filtration device to 250 µL followed by resuspension in 3 mL of a D$_2$O solution of 20 mM potassium phosphate buffer, pD 7.8. Enzyme and acetyldithio-CoA solutions were both chelexed separately. Exchange reactions for HMG-CoA synthase proteins were measured using samples that included 9 mM acetyldithio-CoA, 0.2 mM EDTA in 50 mM potassium phosphate buffer, pD = 7.8 and various concentrations of enzyme. For selected enzymes, experiments using 5 mM acetyldithio-CoA were also performed; the exchange rate was unchanged at this substrate analog concentration (data not shown), suggesting that enzyme was saturated at 9 mM acetyldithio-CoA. The nonenzymatic exchange rates were measured by omitting HMG-CoA synthase from the reaction mixture. $^1$H NMR experiments were performed on a Bruker AC-300 instrument operating at 300 MHz for $^1$H. After adding enzyme to the reaction mixture, sample was loaded into a 5 mm NMR tube and spectra were recorded at 22 °C. $^1$H NMR spectra of
the reaction mixture were recorded every 5 minutes over a period of 3 hours. Each spectrum is comprised of a total of 64 acquisitions.

Proton exchange was estimated (7, 13) from the time dependent change of the ratio of the 2.8 ppm \( \alpha \)-hydrogen methyl resonance to the 0.75 ppm panthetheine methyl resonance (which is unaffected by the exchange process). \( k_{\text{obs}} \) was calculated from equation 1:

\[
    k_{\text{obs}} = k_{\text{enzyme}} - k_{\text{control}} \quad (1)
\]

where \( k_{\text{enzyme}} \) is calculated from the slope of the least squares fit of the data plotted as the log of the peak ratio versus time (SigmaPlot) for exchange reactions containing enzyme, while \( k_{\text{control}} \) is calculated from the slope measured in background controls performed without enzyme. The exchange rate, \( k_{\text{exch}} \), was calculated (14) using equation 2:

\[
    k_{\text{exch}} = \frac{3[A\text{cetyldithio-CoA}]k_{\text{obs}}}{[\text{Protein}]} \quad (2)
\]

HMG-CoA synthase-catalyzed transfer of a thioacetyl group from dithioacetyl-CoA to 3'-dephospho-CoA. Reaction mixtures (1.00 ml) containing wild-type avian HMG-CoA synthase, 125 \( \mu \)M dithioacetyl-CoA and 625 \( \mu \)M 3'-dephospho-CoA, were incubated in 0.1 M sodium phosphate buffer, pH 6.7 at ambient temperature (22°C). The final concentrations of HMG-CoA synthase used were 0 \( \mu \)M (as control), 15 \( \mu \)M, 30 \( \mu \)M and 60 \( \mu \)M. After incubating enzyme in buffer solution at ambient temperature for 15 min, dithioacetyl-CoA and 3'-dephospho-CoA were added. 150 \( \mu \)l aliquots were taken at 0.4, 2, 4, 6, 8 and 10 min. To each aliquot, 5 \( \mu \)l of acetic acid were added to quench the
exchange reaction and 45 μl of methanol (HPLC grade) were added to precipitate protein; the mixtures were then immediately frozen on dry ice. For further analysis, these mixtures (200 μL) were thawed and spin-filtered to remove denatured protein. 138 μl of the filtrates were injected for HPLC analysis. The HPLC column was a Sphereclone RP18 (250 x 4.6 mm; 5 μ) and the mobile phase was 50 mM NaPi buffer, pH 4.5/MeOH (77:23). The column was eluted at flow rate of 0.8 ml/min. The UV detection was at 260 nm (absorption of CoA nucleotide). Under these conditions, the retention time (Rt) of CoA was 2.8 min, 3’-dephospho-CoA was 4.1 min, dithioacetyl-CoA was 9.2 min. and dithioacetyl-dephospho-CoA was 16.6 min. The dithioacetyl-dephospho-CoA peak was further confirmed by its UV spectrum (A_{260nm}/A_{306nm} : 1.3).

A standard curve (integrated A_{260 nm} peak area upon HPLC isolation versus amount of CoA nucleotide in sample) was constructed by subjecting different amounts of acetyldithio-CoA standard to HPLC at the conditions indicated above. The amounts of acetyldithiocephospho-CoA produced in the transacylation reaction were calculated based on the standard curve.
RESULTS

Inhibition of HMG-CoA synthase by acetyldithio-CoA. The structural homology between acetyl-CoA and acetyldithio-CoA suggested that the latter compound might be an inhibitor of the HMG-CoA synthase reaction. Steady state kinetic experiments indicated that acetyldithio-CoA does not support substantial enzyme catalyzed condensation with acetoacetyl-CoA during the short time period (several minutes) required for standard spectrophotometric assays. Under such conditions, it is possible to straightforwardly demonstrate that this analog is a competitive inhibitor with respect to acetyl-CoA (Fig. 1). As a competitive inhibitor, acetyldithio-CoA exhibits relatively high affinity, with an estimated $K_i$ value of $28.0 \pm 2.6 \mu$M. Such a value is comparable to acetyl-CoA requirements (10-40 $\mu$M) for half saturation of wild-type and mutant enzymes in formation of binary complexes (15, 16) involved in enzyme acylation or acetyl-CoA hydrolysis partial reactions. These results prompted a test of whether enzyme-catalyzed exchange of the analog’s C2 methyl protons could be detected.

Enzyme catalyzed proton exchange from acetyldithio-CoA. Avian cytosolic HMG-CoA synthase has been observed to catalyze proton exchange from the C2 methyl group of acetyldithio-CoA (Fig. 2A). The 9 mM concentration used for NMR detection of proton exchange is large in comparison with the measured inhibitor constant ($K_i = 28 \pm 2 \mu$M). This concentration of analog is adequate to optimize binary enzyme-acetyldithio-CoA complex formation for wild type and mutant enzymes, based on previous observations.
(10, 15, 16) of Ac-CoA saturation of the mutants employed in these studies. This prediction has been tested by performing proton exchange experiments with wild-type and D203A mutant enzymes using either 5 mM or 9 mM levels of acetyldithio-CoA. The change in analog concentration has no influence on the measured exchange rate.

Under reaction conditions selected to maintain modest background levels of nonenzymatic exchange, it is possible to demonstrate that enzyme catalyzed exchange is dependent on protein concentration (Fig. 2B). In contrast with observations for wild-type enzyme, comparable levels of mutant C129S synthase do not stimulate proton exchange above background rates (Fig. 2A; Table I). Since cys-129 normally supports formation of the acetyl-S-enzyme reaction intermediate and is saturated by acetyl-CoA at levels comparable to wild-type synthase, it seemed possible that enzyme acylation might influence the enolization/proton exchange reaction. For this reason, two other HMG-CoA synthase mutants D159A and D203A, which do not exhibit substantially altered ability to form binary acyl-CoA complexes but which exhibit very slow kinetics of acylation by acetyl-CoA (15), were tested for their ability to enolize acetyldithio-CoA. Both mutant enzymes (Table I) exhibited diminished rates of proton exchange (6.5- and 4.4-fold, respectively), suggesting that the enolization process might involve an acyl-enzyme species rather than the original acyl-CoA derivatives.

**Acetyldithio-CoA dependent transacylation.** HMG-CoA synthase catalyzes the transfer (12) of the acyl group of acetyl-CoA to a variety of thiol containing acceptors (e.g. dephospho-CoA, cysteamine, etc.). The net transacylation reaction (illustrated below for
transfer to a dephospho-CoA acceptor) consists of two half-reactions and is mediated by formation of a covalent acyl-S-enzyme intermediate.

\[
\text{acyl-CoA + enzyme} \leftrightarrow \text{acyl-enzyme + CoA} \quad (1)
\]

\[
\text{acyl-enzyme + 3'-dephosphoCoA} \leftrightarrow \text{acyl-3'-dephosphoCoA + enzyme} \quad (2)
\]

When acetyldithio-CoA is tested as an acyl donor in the transacylation reaction, it is possible to measure the progress of the reaction at various time intervals by quenching, deproteinizing, and freezing aliquots of the reaction mixture. Subsequent estimate of acetyldithio-3'-dephosphoCoA formation is accomplished by HPLC separation and quantitation of the peak due to this product (A\text{260 nm} detection; retention time 16.6 min) which is well resolved from chromatogram peaks attributable to CoA, 3'-dephosphoCoA, and acetyldithio-CoA (retention times of 2.8, 4.1, and 9.2 minutes, respectively; reverse phase C\text{18} chromatography performed using 50 mM sodium phosphate (pH 4.5)/methanol (77:23) at a flow rate of 0.8 ml/min). Acetyldithio-3'-dephosphoCoA is characterized by an A\text{260 nm}/A\text{306 nm} ratio of 1.3, comparable to that measured for acetyldithio-CoA. The transacylation reaction supported by acetyldithio-CoA exhibits the expected linearity with time (Fig. 3A) and enzyme concentration (Fig. 3B). The observed transacylation, together with the contrasts between the abilities of wild-type enzyme and C129S, D159A, and D203A mutants to support enolization of acetyldithio-CoA, suggest that HMG-CoA synthase forms a covalent acetyldithio adduct to cys-129. These results for HMG-CoA synthase contrast with the reported inability (9) of beta ketothiolase to utilize acetyldithio-CoA to form an acyl-enzyme adduct.
Enolization of acetyldithio-CoA by glutamate-95 mutants. Previous work (16) has demonstrated that an E95A mutant form of HMG-CoA synthase is not substantially impaired in early stages of the reaction (e.g. Michaelis complex or covalent acetyl-enzyme formation) but fails to catalyze condensation with the second substrate, acetoacetyl-CoA, at detectable levels. The ability of E95A to support proton exchange from the C2 methyl group of acetyldithio-CoA was tested (fig 4). The exchange rate is diminished (2.9-fold; Table II) in comparison with wild-type enzyme. An E95Q mutant was also constructed to minimize steric differences between side chains. The E95Q protein has been purified and partially characterized (Table III). Even with the more conservative side chain substitution, lack of the active site carboxyl group in E95Q results in a diminution in specific activity for the overall condensation reaction of almost five orders of magnitude. As in the case of E95A, the E95Q mutant seems impaired in the latter chemical steps of the reaction, since Vm for the partial reaction involving hydrolysis of acetyl-CoA is reduced by only five-fold. Also, the KM for acetyl-CoA in this partial reaction is altered by only 2-fold (Table III), suggesting that interaction of E95Q with this substrate is not markedly different than observed for wild-type enzyme. While the combined stoichiometry of trappable Michaelis complex with acetyl-CoA and covalent acetyl-enzyme adduct is diminished by two fold, 13C NMR measurements of [1,2-13C]-acetyl-enzyme indicate the same distinctive chemical shifts that characterize wild-type enzyme (Table III). These observations do not suggest any perturbation of the local dielectric environment or thioester carbonyl polarization (17) for the E95Q mutant in comparison with wild-type enzyme. Thus, any differences in enolization/proton
exchange from acyl-enzyme species formed using E95Q are likely to primarily reflect the lack of a carboxyl side chain. E95Q exhibits a slightly larger diminution in exchange rate (4.1-fold; fig. 4; Table II) than observed for E95A. The observations for E95A and E95Q are also supported by preliminary results generated using a mutant of S. aureus HMG-CoA synthase in which the corresponding active site glutamate is replaced by glutamine (E79Q); a 6.9 fold diminution in exchange rate (versus wild-type S. aureus enzyme) is observed. While these rates are not reduced to the background levels measured for C129S synthase, the substantial effects implicate avian HMG-CoA synthase’s glutamate-95 in the enolization of the acyl-enzyme intermediate that precedes condensation with the second substrate, acetoacetyl-CoA.

Enolization of acetyl-CoA by several Claisen condensation enzymes (e.g., malate synthase, citrate synthase) is enhanced in the presence of an analog of the cosubstrate (4, 5). The inability of glutamate-95 mutants of HMG-CoA synthase to catalyze the condensation reaction with the second substrate, acetoacetyl-CoA, allowed a test of the influence of second substrate site occupancy on the efficiency of enolization. Interestingly, inclusion of a 2-fold excess of acetoacetyl-CoA, a high affinity substrate (Km ≈ 1 µM), with E95Q enzyme did not stimulate but instead diminished the rate of proton exchange from acetyldithio-CoA (fig. 4). This observation for HMG-CoA synthase, which appears to enolize the covalent acyl-enzyme adduct, contrasts with the substrate analog stimulation of proton exchange by malate synthase (5) and citrate synthase (4). However, those enzymes do not form covalent enzyme adducts from acyl-CoA substrates.
DISCUSSION

C-C bond forming enzymes such as malate synthase, citrate synthase, and beta ketothiolase share with HMG-CoA synthase the requirement to deprotonate/enolize their common substrate, acetyl-CoA, prior to condensation with their respective cosubstrate. While proton exchange from the acyl-CoA substrate is not facile for these enzymes, such exchange is accelerated for malate synthase and citrate synthase when analogs of cosubstrate (e.g. pyruvate, S-malate, respectively) are present. In the case of HMG-CoA synthase some contrasts are apparent. When acetyldithio-CoA is employed as a substrate analog, substantial proton exchange/enolization is catalyzed in a time- and enzyme concentration-dependent reaction. No cosubstrate is required to support the observed exchange reaction. The availability of a mutant HMG-CoA synthase (avian E95Q) that does not catalyze the overall C-C bond forming reaction at any substantial rate allows use of the actual cosubstrate, acetoacetyl-CoA to test the impact on acetyldithio-CoA enolization. In contrast to results for malate synthase or citrate synthase, no stimulation of HMG-CoA synthase E95Q-catalyzed exchange by acetoacetyl-CoA is observed. Instead a slight decrease in proton exchange rate is measured. While such a decrease could be attributable to subtle changes in the E95Q active site, they may simply reflect hindered solvent access (D. H. T. Harrison and H. M. Miziorko, unpublished structural observations).
Another significant difference between C-C bond forming enzymes that can utilize acetyl-CoA and/or acetyldithio-CoA is illustrated by the inability of beta ketothiolase to form a thioacetyl adduct to enzyme (9). HMG-CoA synthase utilizes such an intermediate in catalyzing exchange of the acetyldithio moiety between CoA and 3'-dephosphoCoA groups. In other respects, these enzymes catalyze more homologous chemical events. Thiolase enolizes noncovalently bound acetyldithio-CoA prior to condensation with an acetyl-S-thiolase intermediate. HMG-CoA synthase enolizes covalent thioacetyl-S-enzyme even in the absence of the cosubstrate that would be attacked by the C2 carbanion of thioacetyl-S-enzyme. This observation raises a question over whether acetyldithio-CoA is actually a slow alternate substrate, rather than merely a substrate analog, for HMG-CoA synthase. HPLC monitoring of acyl-CoA derivatives produced from a mixture containing HMG-CoA synthase, acetyldithio-CoA, and acetoacetyl-CoA suggests that there is indeed a slow formation of a condensation product. However, after desalting of the putative product HPLC peak (C₁₈ SepPak), a methanol solution of the CoA derivative was subjected to MALDI analysis which indicated a molecular mass equivalent to HMG-CoA. Thus, using this mass spectroscopy approach, it was not possible to detect significant sulfur substitution in the C5 carboxyl of HMG-CoA. Model organic studies (18, 19) suggest that nonenzymatic sulfur/oxygen exchange of similar compounds occurs and may account for lack of detection of a sulfur-substituted C5 carboxyl in HMG-CoA. Alternatively, reversible enzyme-catalyzed addition of solvent water across a dithioester linkage in either a thioacetyl-S-enzyme or an enzyme-dithioHMG-CoA adduct is quite possible. Collapse of the tetrahedral intermediate formed after solvent attack will reform the covalent adducts
but may result in loss of sulfur since this would be a good leaving group (20). Such sulfur/oxygen exchange from an enzyme-dithioHMG-CoA adduct, followed by cleavage of the covalent linkage to enzyme, would produce unsubstituted HMG-CoA. Another possibility involves sulfur/oxygen exchange of the thioacetyl-S-enzyme adduct. This seems unlikely since the $A_{260\text{ nm}}/A_{306\text{ nm}}$ ratio does not change and NMR measurements do not detect significant formation of acetyl-SCoA during proton exchange from acetylthio-CoA. However, it may not be possible to exclude the slow formation of a low concentration of the normal acetyl-S-enzyme reaction intermediate that rapidly condenses with acetoacetyl-CoA to produce authentic HMG-CoA. Thus, it remains unclear whether dithioacetyl-CoA can support the condensation reaction with acetoacetyl-CoA that would qualify this sulfur-substituted analog as an alternate substrate.

In contrast with the difficulty involved in detecting a sulfur-substituted C5 carboxyl of HMG-CoA, the existence of a stable C1-substituted HMGdithio-CoA has been well documented (21). This compound, prepared by HMG-CoA synthase catalyzed condensation of acetoacetylthio-CoA with acetyl-CoA, is a substrate for HMG-CoA lyase (22) and a potent inhibitor of HMG-CoA reductase (21).

In the context of HMG-CoA synthase catalyzed proton exchange from acetylthio-CoA, diminished rates are exhibited not only by mutant enzymes that are impaired in acetyl-enzyme or thioacetyl-enzyme formation, but also by glutamate-95 mutants. E95A has been previously demonstrated (16) to be deficient in condensation of acetyl-enzyme
with acetoacetyl-CoA. Enolization of acetyl-enzyme is a necessary step in the condensation reaction. The diminished rates of enolization of acetyldithio-CoA measured using active site glutamate mutants (E95A, E95Q, bacterial enzyme E79Q) confirm a defect in condensation and are compatible with assignment of a direct (general base catalyst) or indirect (solvent polarization) role for the glutamate C5 carboxyl in deprotonation of acetyl-enzyme.

ACKNOWLEDGEMENTS
Dr. Kelly Chun provided the plasmid encoding the E95A mutant HMG-CoA synthase mutant used in some of these experiments.

FOOTNOTES

1Abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Ac-CoA, acetyl-Coa; AcAc-CoA, acetoacetyl-CoA; HPLC, high pressure liquid chromatography.
REFERENCES


Table I. Comparison of enzyme catalyzed proton exchange from acetyldithio-CoA by HMG-CoA synthase and acylation deficient mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{exch}$ (min$^{-1}$)$^b$</th>
<th>$k_{exch-wt}/k_{exch-mutant}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG-CoA Synthase$^a$</td>
<td>4.5 ± 0.23</td>
<td>−</td>
</tr>
<tr>
<td>(wild-type)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C129S</td>
<td>Not detectable</td>
<td>−</td>
</tr>
<tr>
<td>D159A</td>
<td>0.69 ± 0.01</td>
<td>6.5</td>
</tr>
<tr>
<td>D203A</td>
<td>1.03 ± 0.13</td>
<td>4.4</td>
</tr>
</tbody>
</table>

$^a$ Enzyme-catalyzed proton exchange of acetyldithio-CoA with HMG-CoA synthase proteins was conducted using 9 mM acetyldithio-CoA in 50 mM potassium phosphate buffer, pD 7.8 containing 0.2 mM EDTA.

$^b$ $k_{exch}$ was calculated from the equation: 3 [Acetyldithio-CoA] $k_{obs}$ / [Enzyme]. $k_{obs}$ was calculated using the equation: $k_{enzyme} - k_{control}$, where k is the first order rate constant, determined from the slope of the least squares fit of the log of the ratio of the $\alpha$-hydrogen resonance at 2.8 ppm to the pantetheine methyl resonance at 0.75 ppm versus time. The control sample was identical to enzyme samples, except enzyme was excluded from the reaction mixture.
Table II. Comparison of enzyme catalyzed proton exchange from acetyldithio CoA by wild-type HMG-CoA synthase and active site glutamate mutants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{\text{exch}}$ (min$^{-1}$)$^b$</th>
<th>$K_{\text{exch-wt}}$/$K_{\text{exch-mutant}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG-CoA Synthase$^a$ (wild type)</td>
<td>4.5 ± 0.23</td>
<td>–</td>
</tr>
<tr>
<td>E95A</td>
<td>1.55 ± 0.19</td>
<td>2.9</td>
</tr>
<tr>
<td>E95Q</td>
<td>1.10 ± 0.03</td>
<td>4.1</td>
</tr>
<tr>
<td>E95Q + acetoacetyl-CoA</td>
<td>0.33 ± 0.07</td>
<td>13.6</td>
</tr>
</tbody>
</table>

$^a$ Enzyme-catalyzed proton exchange of acetyldithio-CoA with HMG-CoA synthase proteins was conducted using 9 mM acetyldithio-CoA in 50 mM potassium phosphate buffer, pH 7.8 containing 0.2 mM EDTA. When included in the exchange reaction sample, acetoacetyl-CoA concentration was 0.4 mM.

$^b$ $k_{\text{exch}}$ was calculated from the equation: $3 \frac{[\text{Acddithio-CoA}]}{k_{\text{obs}}}$ / [Enzyme]. $k_{\text{obs}}$ was calculated using the following equation: $k_{\text{enzyme}} - k_{\text{control}}$, where $k$ is the first order rate constant, determined from the slope of the least-squares fit of the log of the ratio of the $\alpha$-hydrogen resonance at 2.8 ppm relative to the pantetheine methyl resonance at 0.75 ppm versus time. The control sample was identical to enzyme samples, except enzyme was excluded from the reaction mixture.
Table III. Characterization of E95Q HMG-CoA synthase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E95Q HMG-CoA synthase</th>
<th>wild-type HMG-CoA synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (overall reaction; U/mg)</td>
<td>1.4 ± 0.2 (x 10^{-5})</td>
<td>1.0 ± 0.1(^a)</td>
</tr>
<tr>
<td>Vm (acetyl-CoA hydrolysis partial reaction; U/mg)</td>
<td>0.0032 ± 0.0001</td>
<td>0.016 ± 0.003(^b)</td>
</tr>
<tr>
<td>(K_M) (acetyl-CoA hydrolysis partial reaction; (\mu)M)</td>
<td>20 ± 2</td>
<td>11 ± 2(^b)</td>
</tr>
<tr>
<td>Ac-CoA binding stoichiometry(^c)</td>
<td>0.51 ± 0.01</td>
<td>0.99 ± 0.05</td>
</tr>
</tbody>
</table>

\(^{13}\)C chemical shifts for [1,2-\(^{13}\)C] acetyl-S-enzyme

<table>
<thead>
<tr>
<th></th>
<th>C-1</th>
<th>C-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>184 ppm</td>
<td>26 ppm</td>
</tr>
</tbody>
</table>

\(^a\)Data reported by Misra et al. (10).

\(^b\)Data reported by Chun et al. (16).

\(^c\)Estimated by the centrifugal gel filtration method described by Vollmer et al. (23). Stoichiometry estimate, based on 57.6 kDa subunit, includes both Michaelis complex and covalent acetyl-S-enzyme adduct.
FIGURE LEGENDS

Figure 1. Inhibition of recombinant avian HMG-CoA synthase by acetyldithio-CoA. A double reciprocal plot indicating the dependence of reaction rate on acetyl-CoA concentration at various inhibitor levels is displayed. Inhibition experiments were conducted in mixtures containing 0.1 M Tris buffer, pH 8.2, 20 µM acetoacetyl-CoA, 40 mM MgCl₂, 133-1500 µM acetyl-CoA, and HMG-CoA synthase (6.5 µg). Enzyme activity was estimated at 30 °C using a spectrophotometric assay that measures AcAc-CoA disappearance at 300 nm. The final concentrations of acetyldithio-CoA inhibitor are as follows: (○) 0 µM, (●) 25 µM, (□) 50 µM, and (■) 100 µM. Analysis of the data (Grafit) indicates that acetyldithio-CoA is a competitive inhibitor with respect to acetyl-CoA; the Ki value of acetyldithio-CoA is 28.0 ± 2.6 µM.

Figure 2. HMG-CoA synthase catalyzed exchange of the α-hydrogens of acetyldithio-CoA. Panel A depicts the time dependence of the exchange reaction measured using 9 mM acetyldithio-CoA in 50 mM potassium phosphate buffer, pD 7.8, containing 0.2 mM EDTA. Data are plotted as the log of the ratio (R) of the dithioacetyl methyl proton resonance (2.8ppm) to the pantetheine methyl proton resonance (0.75ppm) versus time. A control experiment (♦) was performed using conditions outlined above but no enzyme was included. In measurements on enzyme-catalyzed exchange, the identities and concentrations of HMG-CoA synthase enzymes are: (■) wild-type, 0.1 mM, (●) wild-type, 0.2 mM, (▲) C129S, 0.2 mM, and (▼) C129S, 0.4 mM. In panel B, kₜₐₙ (calculated by a least-squares fit of the rate data with a correction for the nonenzymatic control
exchange rate) is plotted as a function of concentration of wild-type avian HMG-CoA synthase used in the exchange reaction.

Figure 3. HMG-CoA synthase catalyzed transfer of a thioacetyl group from acetyldithio-CoA to 3'-dephospho-CoA. Enzyme catalyzed thioacetyl transfer was conducted in 1.0 ml reaction mixtures containing 125 µM acetyldithio-CoA, 625 µM 3'-dephospho-CoA and 0.1 M sodium phosphate buffer, pH 6.7 at ambient temperature (22 °C). Acetyldithio-3'-dephospho-CoA levels in reaction aliquots withdrawn at the indicated time points were estimated from the integrated area of the corresponding HPLC peak (260 nm detection; refer to experimental procedures for HPLC details). Panel A indicates the enzyme dependence of the transacylation reaction. Concentrations of avian HMG-CoA synthase used were: (●) 15 µM, and (□) 30 µM. A buffer control reaction (○) was performed under the same conditions indicated above, except enzyme was excluded. In panel B, the transacylation rate (nmol/min) for production of acetyldithio-3'-dephospho-CoA as a function of enzyme concentration is displayed.

Figure 4. HMG-CoA synthase glutamate-95 mutant catalysis of proton exchange from acetyldithio-CoA. The time dependence of the exchange reaction was measured in samples containing 9 mM acetyldithio-CoA in 50 mM potassium phosphate buffer, pD 7.8, containing 0.2 mM EDTA. Data are plotted as the log of the ratio (R) of the dithioacetyl methyl proton resonance (2.8ppm) to the pantetheine methyl proton resonance (0.75ppm) versus time. A control experiment (●) was performed using conditions outlined above and no enzyme was included. In measurements on
glutamate-95 mutant enzyme catalyzed exchange, the following enzymes were employed: (▼) E95A, 0.2 mM; (■) E95Q, 0.2 mM; (▲) E95Q, 0.2 mM in the presence of 0.4 mM acetoacetyl-CoA.
Fig 1.
Fig 2A
Fig. 2B

![Graph showing a linear relationship between enzyme concentration and $k_{\text{obs}} \times 10^{-3}$ (min$^{-1}$). The x-axis represents [Enzyme] (mM) ranging from 0.0 to 0.4, and the y-axis represents $k_{\text{obs}} \times 10^{-3}$ (min$^{-1}$) ranging from 0 to 60. The graph includes data points at [Enzyme] = 0.0, 0.1, 0.2, 0.3, and 0.4 mM, with corresponding $k_{\text{obs}} \times 10^{-3}$ values at 0, 10, 20, 30, and 50 (min$^{-1}$) respectively. A linear trend line is drawn through the data points.]


Fig. 3A
Fig. 3B

[Graph showing the relationship between Acetyldithio-3'-dephosphoCoA (nmol/min) and [Enzyme] (µM).]

- Y-axis: Acetyldithio-3'-dephosphoCoA (nmol/min)
- X-axis: [Enzyme] (µM)

The graph shows a linear increase in Acetyldithio-3'-dephosphoCoA production as the concentration of enzyme increases.
Figure 4
3-hydroxy-3-methylglutaryl-CoA synthase: Utility of acetyldithio-CoA in detecting the influence of active site residues on substrate enolization
Chang-Zeng Wang, Ila Misra and Henry M. Miziorko

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