3-Hydroxy-3-methylglutaryl-CoA Synthase

PARTICIPATION OF ACETYL-S-ENZYME AND ENZYME-S-HYDROXYMETHYLGLUTARYL-SCoA INTERMEDIATES IN THE REACTION*

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Acetyl-CoA reacts stoichiometrically with a cysteinyl sulfhydryl group of avian liver 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to yield acetyl-S-enzyme (Miziorko H. M., Clinkenbeard, K. D., Reed, W. D., and Lane, M. D. (1975) J. Biol. Chem. 250, 5768-5773). Evidence that acetyl-S-enzyme condenses with the second substrate, acetoacetyl-CoA, to form enzyme-S-HMG-SCoA has been obtained by trapping and characterizing this putative intermediate. [14C]Acetyl-S-enzyme was incubated briefly at -25° with acetoacetyl-CoA, precipitated with trichloroacetic acid, and the labeled acylated enzyme species were isolated. Performic acid oxidation of the precipitated [14C]acetyl-S-enzyme intermediates produced volatile [14C]acetic acid from unreacted [14C]acetyl-S-enzyme and nonvolatile [14C]3-hydroxy-3-methyl glutaric acid from enzyme-S-[14C]HMG-SCoA. Condensation of unlabeled acetyl-S-enzyme with [14C]acetoacetyl-CoA or acetoacetyl-[14C]CoA also produced labeled enzyme-S-[14C]HMG-SCoA. Thus, the acetyl moiety from acetyl-CoA and the acetoacetyl and CoA moieties from acetoacetyl-CoA all are incorporated into the HMG-CoA which is covalently-linked to the enzyme. Enzyme-S-[14C]HMG-SCoA was subjected to proteolytic digestion under conditions favorable for intramolecular S to N acyl transfer in the predicted cysteine-S-[14C]HMG-SCoA fragment. Performic acid oxidation of the protease-digested material yields N-[14C]HMG-cysteic acid indicating that HMG-CoA had been covalently bound to the enzyme via the -SH of an active site cysteine.

An isotope trapping technique was employed to test the kinetic competence of acetyl-S-enzyme as an intermediate in the HMG-CoA synthase-catalyzed reaction. Evidence is presented which indicates that the rate of condensation of acetoacetyl-CoA with acetyl-S-enzyme to form enzyme-S-HMG-SCoA is more rapid than either the acetylation of the synthase by acetyl-CoA or the overall forward reaction leading to HMG-CoA. These observations, together with indirect evidence that hydrolysis of enzyme-S-HMG-SCoA is extremely rapid, suggest that acylation of synthase is the rate-limiting step in HMG-CoA synthesis.

While the participation of an enzyme-S-HMG-SCoA intermediate in the reaction had been suggested earlier (2, 4), attempts to demonstrate its existence were unsuccessful. In view of more recent findings (3), including those reported in this paper, it appears that earlier failures to detect this intermediate can be attributed to the slow rate of its formation (Reaction 3) relative to hydrolysis (Reaction 4). In the present

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3-Hydroxy-3-methylglutaryl coenzyme A synthase (EC 4.1.3.5) catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA (Reaction 1). Investigations in this

Acetyl-CoA + acetoacetyl-CoA + H₂O → HMG-CoA + CoA (1)

laboratory (1–3) have shown that homogeneous avian liver HMG-CoA synthase is acetylated by acetyl-CoA at a cysteinyl-SH giving rise to acetyl-S-enzyme. This observation and other evidence (4–7) support the proposal that acetyl-S-enzyme is an intermediate in HMG-CoA synthase from acetyl-CoA and acetoacetyl-CoA. Based on the premise that acetylation of the enzyme by acetyl-CoA occurs first in the reaction sequence, it follows that condensation with acetoacetyl-CoA, the second substrate in the reaction, would generate the novel intermediate, enzyme-S-HMG-SCoA. A final hydrolytic step would then be required to release HMG-CoA from its thioester linkage to the enzyme. The three step reaction sequence outlined below (Reactions 2 to 4) accounts for all pertinent experimental facts in the literature bearing on the mechanism of action of HMG-CoA synthase.

CH₃C-SCoA + HS-Enz → CH₃C-S-Enz + CoASH (2)

acetyl-CoA

acetyl-enzyme

CH₃C-SCoA + CH₂=C-SCoA + CH₂=C-S-Enz → CH₃C-C-SCoA + CH₂=C-SCoA + Enz-SH (4)

acetoacetyl-CoA

HMG-CoA-enzyme

HMG-CoA

While the participation of an enzyme-S-HMG-SCoA intermediate in the reaction had been suggested earlier (2, 4), attempts to demonstrate its existence were unsuccessful. In view of more recent findings (3), including those reported in this paper, it appears that earlier failures to detect this intermediate can be attributed to the slow rate of its formation (Reaction 3) relative to hydrolysis (Reaction 4). In the present

1 The abbreviation used is: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
investigation, conditions were employed which made it possible to trap and, therefore, to characterize the elusive enzyme-S-HMG-SCoA intermediate. Furthermore, it was established that the rate of condensation of acetyl-S-enzyme with acetoacetyl-CoA to form this intermediate is more rapid than the overall forward reaction of HMG-CoA synthesis (Reaction 1). This observation indicates that acetyl-S-enzyme is kinetically competent to participate as an intermediate in the HMG CoA synthase-catalyzed reaction. A preliminary account of this work has appeared (8).

**EXPERIMENTAL PROCEDURES**

**Materials**

Coenzyme A was obtained from P-L biochemicals: HMG from Schwarz/Mann; diketone from Sigma; [3H]coenzyme A, [1-14C]acetic anhydride, and ethyl [3-14C]acetacetate from New England Nuclear; and morin dihydride (3,4,5,7-pentahydroxyflavone) from Aldrich. Pronase, a Streptococcus griseus protease, was purchased from Calbiochem. Glass fiber filters (2.5 cm) were obtained from H. Roeox Angol and DRAE-cellulose and PEI-cellulose thin layer chromatographic sheets from Baker.

**Methods**

Homogeneous chicken liver mitochondrial HMG-CoA synthase was prepared and assayed as previously described by Reed et al. (9). The enzyme was stored at -60° in 25 mm potassium phosphate, pH 7.0, containing 50% v/v glycerol, 0.1 m dithiothreitol, and 0.1 m EDTA. Protein was determined by the method of Lowry et al. (10). For the mitochondrial synthase, conversion to refractometrically determined protein can be made by multiplying protein determined by the Lowry method by 0.775 (9).

For the mitochondrial synthase, conversion to refractometrically determined protein can be made by multiplying protein determined by the Lowry method by 0.775 (9). Unlabeled and [1-14C]acetyl-CoA were prepared from the anhydride by the method of Simon and Shemin (11); [3-14C]acetacetate was chromatothegraphically purified as previously described (12). Unlabeled acetoacetyl-CoA and acetoacetyl-[14C]CoA were prepared similarly using diketene instead of the anhydride. [3-14C]Acetacetate CoA was prepared by a modification of the method of Horch and Jencks (13). [3-14C]Acetic acid was generated by hydrolyzing 7.2 μmol of ethyl [3-14C]acetacetate (22.4 x 10⁶ cpm/μmol) with 20 μmol of LiOH in a final volume of 1 ml. After allowing hydrolysis to proceed for 7 h at 25°, 20 μmol of Tris/sulfate were added, the pH was brought to 2.5 μmol of unlabeled acetoacetate, 10 μmol of L-arginine, 10 μmol of EDTA, and 0.6 unit of kidney succinyl-CoA-acetacetate-CoA transferase were added bringing the total volume to 2 ml. Exchange of [3-14C]acetacetate into acetoacetate-CoA was allowed to proceed for 30 min at 25°. The reaction mixture was then cooled to 4° and brought to pH 2 with HCl. After being diluted 100-fold, the [1-14C]acetyl-CoA was purified by DEAE-cellulose chromatography (1.8 x 20 cm column), elution being accomplished with a LiCl gradient (10 m to 200 m containing 3 mm HCl). The eluate was monitored for absorbance at 280 and 300 nm and for 14C. Fractions containing [3-14C]acetacetate-CoA were lyophilized and then dissolved in a minimal volume of H2O. Addition of 8 volumes of cold methanolic acetone (1:3) resulted in the precipitation of 5 μmol of [3-14C]acetacetate-CoA (lium salt; 5.5 x 10⁶ cpm/μmol).

N-(3-Hydroxy-3-methylglutaroyl) cysteic acid (N-HMG-cysteic acid) was prepared by mixing cysteine at pH 8 with a 1.1-fold molar excess of HMG anhydride prepared as described by Goldfarb and Pitot (14). Acdylation was allowed to proceed at 25° for 30 min with constant stirring. The reaction mixture was stored at room temperature overnight to allow completion of the S to N intramolecular rearrangement which occurs spontaneously at neutral pH (15, 16). The sample was then taken to dryness and was oxidized with performic acid (17). After completion of the oxidation, a large excess of water was added and the sample was flash-evaporated to remove formic acid. The overall yield of N-HMG-cysteic acid was 85%. Thin layer chromatography of the concentrated sample, employing a variety of systems (cf. Fig. 1), produced a single spot, detectable with either bromocresol purple or morin (18). For further characterization by mass spectrometry, a portion of the product was chromatographed on a Dowex 50 (H+ form) column for conversion to the free acid which was then methylated with excess diazomethane. Mass spectroscopic analysis showed that the methylated product was indeed the trimethyl ester of N-HMG-cysteic acid. The spectrum obtained under electron impact contains a molecular ion peak at m/e 355, accompanied by M-15, M-31, and M-59 peaks. Fragmentation characteristic of the amide linkage leads to ions of mass 159, comprising the hydroxycarboxylic acid moiety. The spectrum obtained using isobutane reagent gas contains an intense M + H peak at m/e 356, accompanied by a small M + H - H2O ion.

Preparation of [3-14C]- and Unlabeled Acetyl-S-enzyme Used for Production of Enzyme-S-HMG-SCoA—Sixty nanomoles of acetyl-CoA or 50 nmol of [1-14C]acetyl-CoA (6000 cpm/nmol) were added to 1.5 nmol of HMG-CoA synthase in a solution (total volume, 0.2 ml) containing 2 μmol of potassium phosphate, pH 7.5, and glycerol (24%, v/v). After incubation at 25° for 5 min to ensure stoichiometric acetylation of enzyme (3), the reaction mixture was quickly cooled to 0° and being frozen at -60° overnight. Each sample was subsequently inserted in a -25° bath and 40 μl of 1 m acetate buffer, pH 5.3, was added, followed by 80 μl of cold ethanol bringing the mix (final volume, 0.32 ml) to 25% (v/v) in ethanol and permitting thawing at the reduced temperature. Reaction with acetoacetyl-CoA was initiated as soon as complete mixing of the sample was achieved. No attempt was made to separate acetyl-S-enzyme from unreacted acetyl-CoA prior to carrying out the condensation reaction, since the enzyme is neither acetylated nor reacetylated by the excess acetyl-CoA under the low temperature conditions of these experiments.

**RESULTS**

**Formation of Enzyme-S-HMG-SCoA from Acetyl-S-enzyme and Acetoctyl-CoA—** It is possible to trap an intermediate having the properties of the condensation product of [1-14C]acetyl-S-enzyme and acetoacetyl-CoA by controlling conditions (3) that affect the relative rates of the partial reactions of HMG-CoA synthesis (Reactions 2 to 4). [1-14C]Acetyl-S-enzyme is prepared by incubating [1-14C]acetyl-CoA with HMG-CoA synthase for 5 min at 25° and then freezing the mixture at -60°. The acetylated form of the synthesis is precipitated with trichloroacetic acid and contains approximately one acetyl group per molecule of enzyme (Table IA). Previous investigations (2) showed that the acetyl group of acetyl-enzyme is covalently attached to the enzyme in thioester linkage to a cysteinyl-SH. This acetyl group is cleaved and volatilized upon exposure of acetyl-S-enzyme to performic acid vapor (Table IA). When incubated with acetoacetyl-CoA for 10 min at -35° (in the presence of 25% ethanol and 15% glycerol), [1-14C]acetyl-S-enzyme is converted in 16 to 20% yield to a trichloroacetic acid
Acetyl-enzyme was generated by incubating 80 nmol of [1-\(^{14}\)C]acetate-S-enzyme, acetocetyl-S-[\(^{14}\)H]CoA, or [3-\(^{14}\)C]acetocetyl-S-CoA in Experiments B and C for 5 min at 25\(^\circ\) in 0.2 ml containing 1.5 nmol of HMG-CoA synthase, 2 \(\mu\)mol of potassium phosphate, and 24\%, v/v, glycerol. Reaction mixtures were cooled to 0\(^\circ\) and frozen at \(-60^\circ\) overnight. Prior to initiating the condensation reaction, the frozen samples were placed in a \(-25^\circ\) bath and 40 \(\mu\)l of cold 1 M acetic buffer, pH 5.0, were added followed by 80 \(\mu\)l of cold ethanol, bringing the mix (final volume, 0.32 ml) to 25\%, v/v, in ethanol and permitting thawing at reduced temperature. Condensation was initiated by addition of 20 nmol of unlabeled acetoacetyl-CoA (Experiment A), 14 nmol of acetocetyl-S-[\(^{14}\)H]CoA, 50,000 cpm/mmol (Experiment B), or 20 nmol of [3-\(^{14}\)C]acetocetyl-CoA, 5500 cpm/mmol (Experiment C). After 10 s, the reactions were terminated, precipitated with trichloroacetic acid, filtered, and oxidized with performic acid as described under "Experimental Procedures." AcAc refers to acetoacetyl-

| Additions | Exposure to performic acid vapor | Radioactivity in protein precipitate |
|-----------|---------------------------------|-----------------------------------|
| Experiment A | [\(^{14}\)C]Acetyl-S-Enz | No | 9600 (<50)\(^a\) | 1.00 |
| [\(^{14}\)C]Acetyl-S-Enz + AcA-CoA | No | 7300 (<50)\(^a\) | 0.76 |
| [\(^{14}\)C]Acetyl-S-Enz | Yes | 200 (0)\(^a\) | 0.00 |
| [\(^{14}\)C]Acetyl-S-Enz + AcA-CoA | Yes | 1800 (1500)\(^a\) | 0.16 |
| Experiment B | Enz + AcAc-[\(^{14}\)H]CoA | No | 210 | |
| Acetyl-S-Enz + AcA-[\(^{14}\)H]CoA | No | 5550 | 0.07 |
| Experiment C | Enz + [3-\(^{14}\)C]Acet-CoA | | 80 | |
| Acetyl-S-Enz + [3-\(^{14}\)C]Acet-CoA | No | 1390 | 0.16 |

\(^a\) \(^{14}\)C activity as free HMG extractable with ethanol after performic acid oxidation.

The identity of the \(^{14}\)C-labeled compound in the ethanol extract was unequivocally established as 3-hydroxy-3-methylglutaric acid by co-crystallization of this material to constant melting point (19). Subsequent crystallizations were performed in a similar fashion. At each crystallization step, the melting point was determined and a portion of the product was weighed and counted for \(^{14}\)C activity.

\(^a\) Represents 200 mg of carrier HMG added.

\(^{14}\)C in a stoichiometry equal to that obtained by reacting [\(^{14}\)C]acetate-S-enzyme with unlabeled acetocetyl-CoA under identical conditions (Table IA). As expected, the formation of \(^{14}\)C-labeled "enzyme-S-HMG-CoA" from [\(^{14}\)C]acetocetyl-CoA requires the presence of the acetylated form of the synthase. Similar experiments conducted with acetocetyl-[\(^{14}\)H]CoA (Table IB) reveal that the \([^{14}]\)HCoA moiety is incorporated into enzyme-S-HMG-CoA, supporting our previous assertion that the trapped intermediate contains CoA (3). As observed in the analogous [\(^{14}\)C]acetocetyl-CoA labeling experiments, the incorporation of [\(^{14}\)H]CoA is absolutely dependent upon the presence of the acetylated form of enzyme. In this experiment, however, the incorporation of [\(^{14}\)H]CoA is lower than that expected on the basis of the [\(^{14}\)C]-labeling experiments (Table I, A and C). This discrepancy in stoichiometry could be due to partial hydrolysis of the thioester bond to CoA catalyzed by the synthase or promoted by the work-up before filtration. It should be emphasized that the ability to detect enzyme-S-HMG-CoA depends entirely upon the ratio of the rates of condensation (Reaction 3) and hydrolysis (Reaction 4). At room temperature, practically no condensation product is trapped on the enzyme by the techniques described above, presumably because the ratio of condensation rate to hydrolysis rate is lower under these conditions.

Finally, it should be noted that in the experiments with [3-\(^{14}\)C]acetocetyl-CoA, which employed the same procedure used to generate and detect [\(^{14}\)C]acetate-S-enzyme, no evidence was obtained for the covalent linkage of an acetocetyl group to the synthase. This observation is of interest in view of the earlier reports by Brodie et al. (20, 21) of the participation of an
acetoacetyl-enzyme in the biosynthesis of mevalonic acid. While it is possible that such an adduct forms, but is very labile, our negative findings are in agreement with those of Middleton and Tubbs (7), who, using an indirect acylation assay with the yeast synthase, also failed to detect such an acetoacetyl-enzyme adduct.

Isolation and Characterization of the Active Site Fragment Containing the 3-Hydroxy-3-methylglutaryl Condensation Product – It has been established (1, 2) that the site of acetylation of HMG-CoA synthase by acetyl-CoA is a cysteinyl-SH group. If, as we propose (2, 3), acetyl-enzyme reacts with acetoacetyl-CoA in a second step (Reaction 3), the immediate condensation product, i.e. HMG-CoA, should remain covalently bound to the same cysteinyl-SH group of the synthase. This postulate is consistent with the finding that performic acid, the enzyme-S-[14C]HMG-SCoA condensation product was mechanically disrupted in 2 ml of 20 mM imidazole chloride buffer, pH 6.8, containing 1 mg of pronase and 5% ethanol. Proteolysis was allowed to proceed for 60 h at 37° after which the digest was centrifuged and the pellet washed with imidazole buffer. The pooled supernatant solution and wash, containing essentially all of the 14C activity initially trapped, was lyophilized, dissolved in concentrated formic acid containing 1.5% hydrogen peroxide, and incubated overnight at 4°. The sample was then taken to dryness, the residue dissolved in HCl, pH 1.3, and loaded on a Dowex 50W-X4 column (12 × 0.9 cm) previously equilibrated with an HCl solution at pH 1.5. The 14C activity was eluted as a single peak upon washing the column with HCl, pH 1.5. The 14C-containing fractions were taken to dryness and subjected to thin layer chromatographic characterization (Fig. 1). AcAc refers to acetoacetate- and TCA to trichloroacetic acid.

1. [1-14C]Acetyl-Enz
Before performic acid 96,000 100
After performic acid 2,000 2
2. Total [14C]Acetyl-Enz after AcAc-CoA
TCA precipitate 73,000 76
TCA precipitate after performic acid 18,000 19
3. Pronase digest of [14C]Acyl-Enz
Before performic acid 73,000 76
After performic acid 73,000 75
4. [14C]-labeled acylaminos acids eluted from Dowex 50
N-[14C]Acetyl-cysteic acid 63,000 66a
N-[14C]HMG-cysteic acid 46a

The washed [14C]acyl-enzyme precipitate was hydrolyzed with pronase, a proteolytic enzyme preparation, at neutral pH to liberate the labeled acylated amino acid fragments. Under these conditions, the S-[14C]acyclolysteine derivatives should spontaneously rearrange to their corresponding N-[14C]acyclolysteine analogues. Strong chemical precedents exist for S to N intramolecular acyl transfers of this type (15), e.g. the spontaneous rearrangement of S-acetylcytoeamine to form acetyl- and acetoacetyl-, respectively.

Homogenous HMG-CoA synthase (15 nmol) was incubated for 5 min at 25° in triplicate with [1-14C]acetyl-CoA (750 nmol, 6500 cpm/ nmol) in 8 mM potassium phosphate buffer, pH 7.5, containing 24% glycerol. Reaction mixtures (total volume, 2.0 ml) were quickly cooled to 0° and then frozen overnight at -60°. The samples were thawed rapidly at -25° after addition of cold acetate buffer, pH 5.0, (final concentration 125 mM) and then addition of cold ethanol (final concentration 25%, v/v). Final sample volume after these additions was 3.2 ml. The protein in two samples was precipitated by addition of cold 10% trichloroacetic acid. In the third sample, the condensation reaction was initiated rapidly by the addition of 200 nmol of acetoacetyl-CoA and was allowed to proceed for 10 s before termination by the addition of cold 10% trichloroacetic acid. Precipitated protein was trapped on glass fiber filters and washed as described under "Experimental Procedures." One of the two filters containing [1-14C]acyl-enzyme was counted directly and the other was used to determine performic acid-stable 14C activity as described under "Experimental Procedures." The filter containing the 14C-labeled condensation product was mechanically disrupted in 2 ml of 20 mM imidazole chloride buffer, pH 6.8, containing 1 mg of pronase and 5% ethanol. Proteolysis was allowed to proceed for 69 h at 37° after which the digest was centrifuged and the pellet washed with imidazole buffer. The pooled supernatant solution and wash, containing essentially all of the 14C activity initially trapped, was lyophilized, dissolved in concentrated formic acid containing 1.5% hydrogen peroxide, and incubated overnight at 4°. The sample was then taken to dryness, the residue dissolved in HCl, pH 1.3, and loaded on a Dowex 50W-X4 column (12 × 0.9 cm) previously equilibrated with an HCl solution at pH 1.5. The 14C activity was eluted as a single peak upon washing the column with HCl, pH 1.5. The 14C-containing fractions were taken to dryness and subjected to thin layer chromatographic characterization (Fig. 1). AcAc refers to acetoacetate- and TCA to trichloroacetic acid.

Acyl-enzyme Intermediates in HMG-CoA Synthesis

| Step | 14C activity recovered |
|------|-----------------------|
| 1. [1-14C]Acetyl-Enz | 96,000 100 |
| 2. Total [14C]Acetyl-Enz after AcAc-CoA | 73,000 76 |
| 3. Pronase digest of [14C]Acyl-Enz | 73,000 76 |
| 4. [14C]-labeled acylaminos acids eluted from Dowex 50 | 63,000 66a |

a Constitutes a recovery of 87% of the acyl-enzyme intermediates trapped at Step 2.

b Averaged results of three thin layer chromatographic systems (Fig. 1).
N-acetylcysteic acid, respectively.

Acyl-enzyme Intermediates in HMG-CoA Synthesis

A comparison of the specific activities of the $[^{14}C]$HMG-CoA formed (including enzyme-specific activity) with that of the $[^{14}C]$acetyl-S-enzyme initially present provides a measure of the extent of isotope dilution of $[^{14}C]$acetyl-S-enzyme by unlabeled acetyl-CoA prior to condensation. The extent of isotope dilution is a measure of the relative rate of acetyl transfer relative to condensation. If the extent of isotope dilution is small, then the rate of acetyl transfer is slow compared to the rate of condensation. If the extent of isotope dilution is large, then the rate of acetyl transfer is fast compared to the rate of condensation.

Relative Rates of Acetyl Transfer and Condensation Reactions of Acetyl-S-enzyme—The reactions in which acetyl-S-enzyme may participate in the presence of CoA and acetoacetyl-CoA are indicated in Scheme 2. Knowledge of the rate of condensation of acetyl-S-enzyme with acetoacetyl-CoA, relative to the rate of acetyl transfer from acetyl-S-enzyme to CoA, would provide an independent means of evaluating the role of acetyl-S-enzyme in HMG-CoA synthesis (Reaction 1). It has already been established (2) that the rate of acetyl transfer (k$_1$, Scheme 2) is at least as fast as the rate of HMG-CoA formation from acetyl-CoA and acetoacetyl-CoA. Thus, if condensation (k$_2$, Scheme 2) were as rapid as acetyl transfer, acetyl-S-enzyme would meet the kinetic requirements of a participant in the overall process. The relative rates of the competing condensation and acetyl transfer reactions were estimated by using a variation of the isotope trapping technique developed in Meister’s laboratory (22, 23). To monitor the relative rate of the alternative reactions of the proposed intermediate, $[^{14}C]$acetyl-S-enzyme is mixed simultaneously with CoA, a substoichiometric amount of acetoacetyl-CoA, and a large excess of unlabeled acetyl-CoA. Upon mixing, any $[^{14}C]$acetyl group transferred to CoA will undergo extensive isotope dilution with the unlabeled acetyl-CoA pool. Hence, acetyl-S-enzyme formed by reacetylation will have a greatly reduced specific activity. Alternatively, $[^{14}C]$acetyl-S-enzyme may initially condense with acetoacetyl-CoA to form a labeled product with the same specific activity as the starting $[^{14}C]$acetyl-S-enzyme. By maintaining acetoacetyl-CoA at levels that are substoichiometric with respect to that of $[^{14}C]$acetyl-S-enzyme, multiple productive enzyme turnovers cannot occur since the condensation reaction is virtually irreversible (4, 24). Hence, there is no need to rapidly quench the reaction to prevent multiple turnovers which would complicate interpretation of the results.

A comparison of the specific activity of $[^{14}C]$HMG-CoA formed (including enzyme-specific activity) with that of the $[^{14}C]$acetyl-S-enzyme initially present provides a measure of the extent of isotope dilution of $[^{14}C]$acetyl-S-enzyme by unlabeled acetyl-CoA prior to condensation. The extent of isotope dilution is a measure of the relative rate of acetyl transfer relative to condensation. If the condensation process is very rapid, the specific activity of the filtered acyl-enzyme precipitates before and after exposure to performic acid vapor. In a control experiment (results not shown) $[^{14}C]$HMG-CoA subjected to the same procedure, i.e. pronase digestion, performic acid oxidation, Dowex 50 chromatography, and thin layer chromatography, was recovered quantitatively in the form of $[^{14}C]$HMG, as expected. Hence, any $[^{14}C]$HMG-CoA bound noncovalently to the enzyme and not removed by extensive washing of the $[^{14}C]$acetyl-enzyme precipitates would have been readily distinguished by thin layer chromatography. All data generated in this series of experiments are consistent with the proposal that the condensation product of the HMG-CoA synthase-catalyzed reaction is attached to the enzyme at an active site cysteine.
product, $[^{14}C]HMG-CoA$, should approach that of the $[^{14}C]acetyl-S$-enzyme added initially. If acetyl transfer to CoA is much more rapid than condensation, $[^{14}C]HMG-CoA$ will have a specific activity comparable to that of the isotope-diluted $[^{14}C]acetyl-CoA$ pool.

Fig. 2 illustrates the kinetics and CoA concentration dependence of acetyl transfer from acetyl-S-enzyme to CoA in the absence of acetoacetyl-CoA. It is evident that when the CoA concentration is greater than 1.0 mM, acetyl transfer reaches completion in less than 10 s. In all subsequent isotope trapping experiments, the CoA concentration was maintained at 1.6 mM to ensure quantitative acetyl group transfer from $[^{14}C]acetyl-S$-enzyme to CoA within the 10-s time frame of the comparative rate studies. Under similar conditions, $[^{14}C]acetyl-S$-enzyme was mixed with solutions containing substoichiometric amounts of acetoacetyl-CoA and also CoA and unlabeled acetyl-CoA, each maintained at final concentrations of either 0 or 1.6 mM. The results of these experiments are summarized in Fig. 3. The slopes of Lines A, B, and C are measures of the actual and expected specific activities of $[^{14}C]HMG-CoA$ formed under different conditions. Line A represents the theoretical production of $[^{14}C]HMG-CoA$ of a specific activity equal to that of the $[^{14}C]acetyl-S$-enzyme added, i.e., without isotope dilution by the unlabeled acetyl-CoA pool. Data generated in an experiment with substoichiometric levels of acetoacetyl-CoA, but without unlabeled acetyl-CoA present, fit the theoretical line (Line A, Fig. 3) quite well. Line C (Fig. 3) would be expected if the label in $[^{14}C]acetyl-S$-enzyme completely equilibrated with the unlabeled acetyl-CoA pool before condensation (i.e., were $k_1 >> k_2$ in Scheme 2).

The points on Line B (Fig. 3) were obtained in an experiment in which both the condensation and acetyl transfer routes were available to the added $[^{14}C]acetyl-S$-enzyme, i.e., both acetoacetyl-CoA and unlabeled acetyl-CoA were present. While the ratio of any pair of points on Lines A and B obtained at the same concentration of acetoacetyl-CoA may be used to determine the relative rates of condensation and acetyl transfer ($k_2$ and $k_1$, respectively, in Scheme 2), the averaging achieved by comparing the slopes of lines fit to these points provides a better measure of the relative rates. The slope of Line B is two-thirds that of Line A, indicating that, under the conditions of this experiment, condensation ($k_2$, Scheme 2) is twice as rapid as acetyl transfer ($k_1$, Scheme 2). In view of our earlier finding (2, 6) that $k_2$, (Scheme 2) is as rapid as the overall process, i.e., the formation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA, it follows that condensation ($k_2$) is at least as rapid as the overall reaction. It can be concluded, therefore, that acetyl-S-enzyme meets the kinetic criteria for participation as an intermediate in HMG-CoA synthesis.

**DISCUSSION**

The finding that HMG-CoA synthase is rapidly acetylated by acetyl-CoA at a cysteinyl-SH (1, 2) implicated acetyl-S-enzyme as a probable intermediate in the synthesis of HMG-CoA. Assuming that this is the first step in the reaction sequence, it follows (2–4) that subsequent condensation of this intermediate would generate an enzyme-S-HMG-SCoA intermediate. Hydrolysis of the acyl-S-enzyme thioester bond would then liberate HMG-CoA. This sequence for the condensation-acetyl transfer reaction is consistent with the observed specific activities of HMG-CoA formed under different conditions. The theoretical line (Line A, Fig. 3) is quite well fitted by the data generated in experiments with substoichiometric levels of acetyl-CoA and acetoacetyl-CoA. Data generated in an experiment with substoichiometric levels of acetoacetyl-CoA, but without unlabeled acetyl-CoA present, fit the theoretical line (Line A, Fig. 3) quite well. Line C (Fig. 3) would be expected if the label in $[^{14}C]acetyl-S$-enzyme completely equilibrated with the unlabeled acetyl-CoA pool before condensation (i.e., were $k_1 >> k_2$ in Scheme 2).

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Acyl-enzyme Intermediates in HMG-CoA Synthesis

sation and hydrolysis steps has a sound chemical basis. Prior hydrolysis of the acetyl-enzyme thioester linkage would cause premature loss of the carbanionic character of the acetyl methyl group necessary for condensation. Moreover, it would lead to the loss of the high acyl transfer potential needed to pull the thermodynamically unfavorable condensation.

The failure of earlier attempts (4) to detect enzyme-S-HMGSCoA suggests a rate of hydrolysis (Reaction 1) which is rapid relative to the rate of condensation (Reaction 3). While investigating the rates of the partial reactions, it became evident (3) that the avian liver HMG-CoA synthase undergoes a reversible temperature-dependent conformational change which affects the rate of first partial reaction, i.e. acetylation of the enzyme (Reaction 2). It was established that a conformational change in the enzyme affected both its capacity for rapid acetylation by acetyl-CoA and ability to catalyze the synthesis of HMG-CoA. The possibility was considered that the relative rates of the other partial reactions, i.e. condensation and hydrolysis, might also be affected by altering the thermal history of the enzyme and the temperature at which the condensation reaction is performed. Preliminary studies (3, 8) verified the feasibility of this approach to the trapping of the postulated enzyme-S-HMG-SCoA intermediate. Therefore, in the present investigation, the condensation of acetyl-S-enzyme with acetocetyl-CoA was performed under conditions which block the first partial reaction (Reaction 2) and increase the ratio of the rates of condensation (Reaction 3) to hydrolysis (Reaction 4). From the results of these experiments (Table III), it can be calculated that condensation exceeded hydrolysis by a factor of nearly 2. Of the 94,000 cpm of [4C]acetyl-S-enzyme present initially, 21,000 cpm (i.e. 94,000 - 73,000 cpm) were apparently lost via hydrolysis of enzyme-S-[4C]HMG-SCoA (Reaction 4) while 18,000 cpm were recovered as enzyme-S-[4C]HMG-SCoA in the performic acid-treated enzyme precipitate. Therefore, the total amount of enzyme-S-[4C]HMGSCoA formed during condensation was 39,000 cpm (i.e. 18,000 + 21,000 cpm). Hence, condensation must have occurred at a rate almost twice that of hydrolysis. These results, together with those previously reported (3), support the hypothesis that the condensation/hydrolysis ratio can be increased through alterations in the conditions under which the condensation reaction is performed.

Additional information concerning the relative rates of the partial reactions was obtained from isotope trapping experiments (Fig. 3) which indicate that the condensation of acetyl-S-enzyme with acetocetyl-CoA is more rapid than acetyl transfer from acetyl-S-enzyme to CoA (Scheme 2). If previous estimates of $K_{eq} = 1$ for the acetylation reaction are correct (2, 7), it may be inferred that condensation occurs more rapidly than acetylation. While direct measurements of the rate of hydrolysis of enzyme-S-HMG-SCoA have not been made, the difficulties encountered in trapping appreciable amounts of this species suggest that hydrolysis is extremely rapid, particularly at 25°. Thus, it is likely that formation of acetyl-S-enzyme (Reaction 2) is the rate-limiting step in HMG-CoA synthesis. A conformational change may well be involved in converting the synthase to the "active," i.e. acetylatable, form required to initiate a catalytic cycle. Several lines of evidence are consistent with this possibility. First, acetylation of the enzyme (Reaction 2) appears to be the slow step in the overall process. Secondly it has been established (3) that the synthase can be thermally perturbed, i.e. inactivated, by lowering the temperature and that the return to a catalytically active state (or conformation) at a temperature <25° is exactly correlated with the return of the capacity of the enzyme to undergo acetylation by acetyl-CoA. Direct demonstration that a conformational change occurs prior to acetylation of enzyme and proof that the kinetics of such a change determines the rate of acetylation (and, therefore, the rate of the overall reaction) will require additional experimentation. Fluorescence or spin-labeling approaches could prove valuable in monitoring changes in protein conformation over a wide range of temperature. Attempts are currently being made to develop an appropriate probe for such studies.

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