TRAPPING OF A NOVEL COENZYME A CONTAINING INTERMEDIATE OF 3-HYDROXY-3-METHYLGLUTARYL-CoA SYNTHASE

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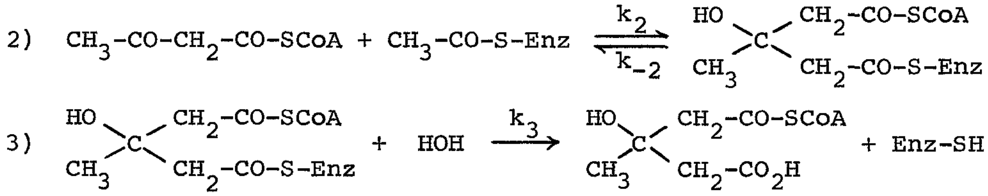
SUMMARY:

By controlling conditions which affect the relative rates of the partial reactions in HMG-CoA synthesis, it has been possible to trap and partially characterize HMG(-SCoA)-S-enzyme, the postulated central intermediate in catalysis. At -25° in 25% ethanol, the breakdown of HMG(-SCoA)-S-enzyme to HMG-SCoA and HS-enzyme is slowed relative to its rate of formation from acetoacetyl-SCoA and acetyl-S-enzyme. Under these conditions label from acetoacetyl-S[^{3}H]CoA or [^{14}C]acetyl-S-enzyme can be trapped in the accumulated intermediate with [^{14}C] activity appearing in the HMG moiety.

3-Hydroxy-3-methylglutaryl(HMG)CoA synthase is rapidly acetylated by acetyl-CoA at a cysteinyl -SH to yield acetyl-S-enzyme (1). These and other findings (2,3), led to the proposal that acetyl-enzyme is an intermediate in HMG-CoA synthesis. Assuming that the formation of acetyl-S-enzyme is the initial step in the sequence (Reaction 1), it follows that subsequent condensation of acetoacetyl-CoA with the enzyme-bound acetyl group would generate the unusual CoA-containing intermediate, HMG(-SCoA)-S-enzyme (Reaction 2). Hydrolysis of the acyl-enzyme thioester linkage in the presumed intermediate would then liberate HMG-CoA (Reaction 3), the product of the overall reaction sequence.

$$1)\quad \mathrm{CH_3\text{-}CO\text{-}SCoA + HS\text{-}Enz} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{CH_3\text{-}CO\text{-}S\text{-}Enz + CoASH}$$



$$2)\quad \mathrm{CH_3\text{-}CO\text{-}CH_2\text{-}CO\text{-}SCoA + CH_3\text{-}CO\text{-}S\text{-}Enz} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \begin{array}{c}\mathrm{HO}\diagdown\quad\diagup\mathrm{CH_2\text{-}CO\text{-}SCoA}\\ \mathrm{C}\\ \mathrm{CH_3}\diagup\quad\diagdown\mathrm{CH_2\text{-}CO\text{-}S\text{-}Enz}\end{array}$$

$$3)\quad \begin{array}{c}\mathrm{HO}\diagdown\quad\diagup\mathrm{CH_2\text{-}CO\text{-}SCoA}\\ \mathrm{C}\\ \mathrm{CH_3}\diagup\quad\diagdown\mathrm{CH_2\text{-}CO\text{-}S\text{-}Enz}\end{array} + \mathrm{HOH} \xrightarrow{k_3} \begin{array}{c}\mathrm{HO}\diagdown\quad\diagup\mathrm{CH_2\text{-}CO\text{-}SCoA}\\ \mathrm{C}\\ \mathrm{CH_3}\diagup\quad\diagdown\mathrm{CH_2\text{-}CO_2H}\end{array} + \mathrm{Enz\text{-}SH}$$

Although the participation of an HMG(-SCoA)-S-enzyme intermediate had been postulated (4), attempts to detect it were unsuccessful initially, apparently because the rate of thioester hydrolysis (k_3) greatly exceeds the rate of condensation (k_2). Recently, however, it was recognized that the synthase (from avian liver mitochondria) undergoes a reversible temperature ependent conformational change which appears to alter the relative rates of he partial reactions. By controlling these conditions it has been possible o trap and partially characterize the putative HMG(-SCoA)-S-enzyme interediate.

ETHODS

HMG-CoA synthase was prepared from chicken liver mitochondria and ssayed by methods described earlier (5). Acetyl-CoA was prepared from he anhydride by the method of Simon and Shemin (6). Labeled and unabeled acetoacetyl-CoA was synthesized by the same procedure, with dikeene replacing the anhydride. $[^{3}H]$CoA was obtained from New England Nulear. The formation of $[^{14}C]$acetyl-S-enzyme was measured by precipitaing the enzyme and carrier serum albumin from the incubation mixture ith cold trichloroacetic acid as previously described (1); following entrifugation, the resuspended protein precipitates were loaded onto lass fiber filters (2.5 cm diameter, H. Reeve Angel), washed extensively ith trichloroacetic acid, pyrophosphate in HCl, and ethanol after which he filters were counted in toluene-Triton scintillator (1). Radioactivty incorporated into $[^{14}C]$HMG(-SCoA)-S-enzyme was determined similarly xcept that filters loaded with protein were exposed to performic acid apor for 20 hrs after which $[^{14}C]$acetic acid (from $[^{14}C]$acetyl-S-enzyme) as removed *in vacuo* over NaOH. Radioactivity remaining on the filters as determined as above.

SULTS AND DISCUSSION

Earlier we reported (1), that HMG-CoA synthase thawed from storage -60° is rapidly, though not stoichiometrically, acetylated (at 0°) by etyl-CoA. More recently, it became apparent that the fraction of enzyme apable of being rapidly acetylated is increased by prior exposure to emperatures above 0°. As illustrated in Fig. 1, enzyme thawed at 0° and hen preincubated for 15 min at 0°, 10°, or 25° undergoes rapid acetylaion (i.e., within 2 min) to extents 30%, 65% and 100% of maximal. Neither igher temperature nor a longer preliminary incubation at 25° leads to a reater extent of acetylation. Importantly the extent to which synthase an be rapidly acetylated is not determined by the temperature at which e reaction *per se* is performed, but rather by the thermal history of e enzyme. Thus, prior exposure to 25° followed by a brief incubation d acetylation at 0° produces the same extent of acetylation as prior cubation and acetylation at 25° (Fig. 1). Although a significant burst f acetylation occurs both at 0° and 10°, 6-8 hours are required for acetlation to approach an endpoint which corresponds to 1 acetyl group per nthase dimer (105,000 daltons (5)). These findings suggest that the nthase undergoes a temperature-dependent conformational change from an nactive to an active form which can be acetylated by acetyl-CoA. The apid (or burst) phase of acetylation is presumably a measure of the mount of enzyme already present in the "active" conformation. The slow

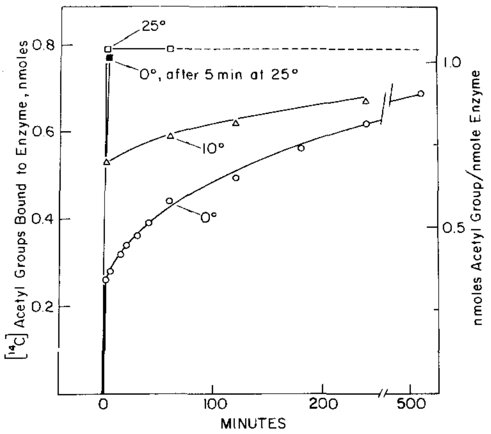


Fig. 1. Effect of temperature and prior thermal treatment on the kinetics of [^{14}C]acetyl-S-enzyme formation. Reaction mixtures contained 0.76 nmole of HMG-CoA synthase, 10 μmoles of potassium phosphate, pH 7.5, and 55 nmole of [1-^{14}C]acetyl-CoA (7250 cpm per nmole) in a total volume of 0.1 ml. Froz stock HMG-CoA synthase solutions (see legend of Table I for composition) we transferred from -60° to 0° and thawed rapidly; enzyme solutions were mixed with buffer and allowed to incubate at the indicated temperature for 15 min at which time acetylation was initiated by addition of [^{14}C]acetyl-CoA. At the times indicated, the reaction was stopped by addition of 1 ml of cold 10% trichloroacetic acid. [1-^{14}C]Acetyl-S-enzyme formation was assessed as described in Methods.

phase (0° and 10° in Fig. 1) probably represents a slow conformational change to the active state, the rate of which limits acetylation.

To determine whether this temperature-induced change is accompanied by an increase in catalytic activity, enzyme preparations with differing capacities for rapid acetylation were generated as described for Fig. 1 and immediately assayed for HMG-CoA synthase activity. Activity measurements were performed at 0° to minimize temperature-induced changes in the enzymes' potential for acetylation during the assay. As illustrated by Fig. 2, there is an excellent correlation between temperature-induced change in catalytic activity and capacity for rapid acetylation by acetyl-CoA. This suggests that a common conformational change affects both and is consistent with acetylation of the enzyme by acetyl-CoA as a step in the mechanism of HMG-CoA formation (Reaction 1).

Since the thermal history of HMG-CoA synthase determines both its capacity to undergo acetylation by acetyl-CoA (Reaction 1) and its ability to catalyze HMG-CoA formation (Reactions 1-3), the possibility was considered that the relative rates of condensation (k_2, Reaction 2) and hydrolysis (k_3, Reaction 3) might also be affected. Moreover, it appears that previous failures to detect the proposed HMG(-SCoA)-S-enzyme inter-

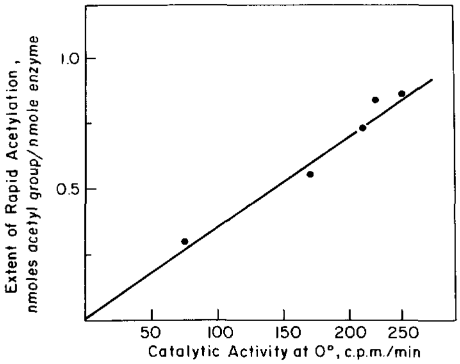


Fig. 2. Correlation of catalytic activity and capacity for rapid acetylation of HMG-CoA synthase at 0°. Reaction mixtures contained the same ratio of components described in Fig. 1. in a final volume of 0.11 ml. Enzyme samples varied in time and temperature (0° or 25°) of preincubation. 10 µl samples were withdrawn at the time acetylation was stopped and these samples were assayed for HMG-CoA synthase activity as previously described (5) except that assays were performed at 0°. The remaining 100 µl were used to assess the capacity of the synthase for rapid (within 2 min) acetylation as described in Fig. 1 and Methods.

mediate might be attributable to a slow rate of condensation, k_2, and a fast rate of hydrolysis, k_3. By reducing the temperature during the condensation reaction and through appropriate modification of other conditions, it was possible to trap and partially characterize the intermediate. A sensitive procedure was developed to detect the putative HMG(-SCoA)-S-enzyme intermediate in the presence of acetyl-S-enzyme. Initially, [1-^{14}C]acetyl-S-enzyme is incubated briefly either in the presence or absence of acetoacetyl-CoA. All labeled acyl-enzyme forms are then denatured and precipitated with cold trichloroacetic acid, filtered and washed on a glass fiber filter, and subjected to oxidation by performic acid vapor as previously described (1). After cleavage of thioester linkages, [^{14}C]acetic acid originating from [^{14}C]acetyl-S-enzyme is removed *in vacuo* over NaOH. This procedure eliminates [^{14}C] activity arising from [^{14}C]acetyl-S-enzyme, but does not volatilize [^{14}C]HMG produced from [^{14}C]HMG(-SCoA)-S-enzyme. Hence, [^{14}C]HMG remaining on the filter can be counted directly.

As illustrated in Table I brief acetylation of synthase with [^{14}C]-acetyl-CoA gives rise to 9000-9600 c.p.m. as acetyl-S-enzyme (0.92-0.99 nmole [^{14}C]acetyl-/nmole of enzyme). When [^{14}C]acetyl-S-enzyme is permitted to undergo condensation with acetoacetyl-CoA at 0° for 15 sec (Table IA) only a small percentage, ~2%, of the [^{14}C] activity present initially as [^{14}C]acetyl-enzyme is converted to a protein-bound form

TABLE I. Trapping of HMG(-SCoA)-S-Enzyme

Stock enzyme solutions containing 1mg of HMG-CoA synthase per ml, 20mM potassium phosphate, pH 7.0, 0.1mM dithiothreitol, 0.1mM EDTA and 30% glycerol were stored at -60°. For A), reaction mixtures containing 1.5 nmole HMG-CoA synthase, 1.6 μmole potassium phosphate, pH 7.5, and 80 nmole [1-^{14}C]AcCoA (6500 cpm per nmole) in a total volume of 0.2 ml were incubated at 25° for 5 min and then cooled to 0° or frozen at -60° overnight before rapid thawing. Prior to initiating the condensation reaction 40 μmoles of acetate buffer, pH 5.0, were added to each sample. In the -25° experiment, cold ethanol was rapidly added to 25% (v/v) to permit thawing at the reduced temperature. The condensation reaction was initiated by the addition of 20 nmoles of acetoacetyl-CoA. After 15 sec the reaction was terminated by addition of 1.5ml of cold 10% trichloroacetic acid. Fifty micrograms of carrier bovine serum albumin were added, the precipitated protein washed, and [^{14}C]activity bound to synthase determined as described in Methods. For B), reaction mixtures were the same as above, except that 60 nmoles of unlabeled acetyl-CoA were used to acetylate the enzyme and 14 nmoles of acetoacetyl-[^{3}H]CoA (51,000 cpm per nmole) were added to initiate the condensation reaction. The incubation procedure and technique for precipitating the [^{3}H] labeled synthase was identical to that for A).

Additions	Conditions	Radioactivity in precipitated enzyme	
		Before	After
		Performic acid vapor treatment	
		cpm	cpm
A) <u>[1-^{14}C]Acetyl-enzyme and unlabeled AcAc-CoA</u>			
[^{14}C]Ac-Enz	0°	9100	180
[^{14}C]Ac-Enz + AcAc-CoA		-	400
[^{14}C]Ac-Enz	[^{14}C]Ac-Enz (-60°) thawed	9600	230
[^{14}C]Ac-Enz + AcAc-CoA	rapidly, then ± AcAc-CoA at 0°	-	730
[^{14}C]Ac-Enz	[^{14}C]Ac-Enz (60°) thawed	9000 (<25)^1	220 (<25)^1
[^{14}C]Ac-Enz + AcAc-CoA	rapidly, then ± AcAc-CoA at -25°	-	1800 (1530)^1
B) <u>AcAc[^{3}H]CoA and unlabeled acetyl-enzyme</u>:			
AcAc-[^{3}H]CoA + Enz	0°	380	-
AcAc-[^{3}H]CoA + Ac-Enz		650	-
AcAc-[^{3}H]CoA + Enz	Enz or Ac-Enz (-60°) thawed	380	-
AcAc-[^{3}H]CoA + Ac-Enz	rapidly, then AcAc-[^{3}H]CoA at -25°	2920	-

^{1}[^{14}C] activity extractable with cold ethanol.

Abbreviations: Ac, acetyl and AcAc, acetoacetyl

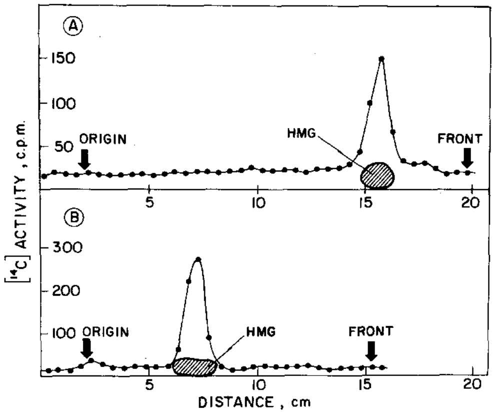


Fig. 3. Thin layer chromatographic characterization of the $[^{14}C]$ labeled fragment released from HMG-CoA synthase by performic acid oxidation. A sample containing $[^{14}C]$ labeled "acyl"-S-enzyme was prepared by incubating acetoacetyl-CoA at -25° and $[1\text{-}^{14}C]$acetyl-S-enzyme at -25° as in Table IA and trapped by precipitation with trichloroacetic acid. The precipitate was loaded onto a glass fiber filter, washed as indicated in Methods and subjected to oxidation by performic acid vapor. After volatilization of $[^{14}C]$acetic acid from $[^{14}C]$acetyl-S-enzyme *in vacuo* over alkali, the filter was extracted with 25 ml of cold ethanol. The extract was concentrated and chromatographed using the systems listed below. In A) the ethanol extract was applied with authentic 3-hydroxy-3-methylglutaric acid (HMG) to a silica gel sheet and developed for 14 hrs in a butanol/acetic acid/water (4:1:1) solvent system. The HMG spot and $[^{14}C]$ activity have an R_f of 0.76. In B) the ethanol extract and authentic HMG were cochromatographed on a silica gel sheet for 4 hr. in an n-amyl formate/water/formic acid (7:1:2) solvent system. HMG and $[^{14}C]$ activity have an R_f of 0.39. HMG was detected on chromatograms with a bromocresol purple spray.

having the characteristics of HMG(-SCoA)-S-enzyme, i.e. precipitable with trichloroacetic acid and not volatilized by performic acid. Allowing longer for condensation to occur does not improve the yield of enzyme-bound $[^{14}C]$HMG. However, if $[^{14}C]$ acetylated enzyme is first frozen overnight at -60°, then thawed rapidly at 0°, and condensation with acetoacetyl-CoA initiated immediately, the yield of $[^{14}C]$ activity as enzyme-bound HMG is more than doubled (Table IA). Moreover, the yield of "HMG(-SCoA)-S-enzyme" trapped can be increased even further to 15-20% of theoretical by rapidly thawing $[^{14}C]$acetyl-S-enzyme frozen at -60° and performing the condensation at -25° in the presence of 25% ethanol. Under these conditions, i.e. at -25° in the presence of 25% ethanol, the synthase cannot be reacetylated by $[^{14}C]$acetyl-CoA.

Proof that the condensation product is an HMG-enzyme derivative lies in the following facts. Prior to performic acid oxidation none of the $[^{14}C]$ activity precipitated by trichloroacetic acid can be extracted with ethanol (Table IA). However, after performic acid oxidation and volatilization of [^{14}C]acetic acid arising from [^{14}C]acetyl-enzyme, virtually all of the residual [^{14}C] activity on the filter, presumed to arise from [^{14}C]HMG(-SCoA)-S-enzyme, is extractable with ethanol. Thin layer chromatography of the ethanol extract gives rise to a single radioactive peak which comigrates with authentic carrier HMG using two solvent systems (Fig. 3). Therefore, it is evident that the intermediate contains HMG linked to the enzyme in thioester linkage presumably to a cysteinyl -SH like its precursor, acetyl-S-enzyme (1).

Finally, evidence has been obtained that the HMG-enzyme derivative formed during condensation (Reaction 2) contains covalently-bound CoA derived from acetoacetyl-CoA. As shown in Table IB, acetoacetyl-S-[^{3}H]CoA, incubated briefly with unlabeled acetyl-S-enzyme, gives rise to substantial [^{3}H]activity precipitable with trichloroacetic acid. Dependence upon the acetylated form of the synthase is indicated by the fact that substituting unacetylated enzyme leads to a considerably lower level of precipitable radioactivity that is constant, being unaffected by reaction conditions or the thermal history of the enzyme. Like the corresponding experiments with [^{14}C]acetyl-CoA (Table IA), the yield of [^{3}H]-labeled "HMG(-SCoA)-S-enzyme" trapped depends upon the temperature during condensation and the thermal history of the synthase.

Together with our earlier work (1), the present communication provides compelling support for the occurrence of the partial reactions depicted above (Reactions 1-3). Thus, both postulated covalently-bound enzyme intermediates, acetyl-S-enzyme and HMG(-SCoA)-S-enzyme, have now been isolated or trapped and partially characterized.

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