3-Hydroxy-3-methylglutaryl-coenzyme A Synthase Reaction Intermediates: Detection of a Covalent Tetrahedral Adduct by Differential Isotope Shift ^{13}C Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Binding of [1,2-^{13}C]acetyl-CoA to wild-type 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase is characterized by large upfield shifts for C1 (184 ppm, $\Delta\delta = 20$ ppm) and C2 (26 ppm, $\Delta\delta = 7$ ppm) resonances that are attributable to formation of the covalent [1,2 -^{13}C]acetyl-S-enzyme reaction intermediate. NMR spectra of [1,2-^{13}C]acetyl-S-enzyme prepared in $H_2{}^{16}O$ versus $H_2{}^{18}O$ indicate a 0.055 ppm upfield shift of the C1 resonance in the presence of the heavier isotope. The magnitude of this ^{18}O-induced ^{13}C shift suggests that the 184 ppm resonance is attributable to a reaction intermediate in which C1 exhibits substantial carbonyl character. No significant shift of the C2 resonance occurs. These observations suggest that, in the absence of second substrate (acetoacetyl-CoA), enzymatic addition of $H_2{}^{18}O$ to the C1 carbonyl of acetyl-S-enzyme occurs to transiently produce a tetrahedral species. This tetrahedral adduct exchanges oxygen upon backward collapse to re-form the sp^2-hybridized thioester carbonyl. In contrast with HMG-CoA synthase, C378G *Zoogloea ramigera* β-ketothiolase, which also forms a ^{13}C NMR-observable covalent acetyl-enzyme species, exhibits no ^{18}O-induced shift. Formation of the [^{13}C]acetyl-S-enzyme reaction intermediate of HMG-CoA synthase in D_2O versus H_2O is characterized by a time-dependent isotope-induced upfield shift of the C1 resonance (maximal shift = 0.185 ppm) in the presence of the heavier isotope. A more modest upfield shift (0.080 ppm) is observed for C378G *Z. ramigera* β-ketothiolase in similar experiments. The slow kinetics for the development of the deuterium-induced ^{13}C shift in the HMG-CoA synthase experiments suggest a specific interaction (hydrogen bond) with a slowly exchangeable proton (deuteron) of a side chain/backbone of an amino acid residue at the active site.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)[1] synthase catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to produce a key intermediate in steroidogenic and ketogenic metabolic pathways. The importance of this enzyme to metabolism is underscored by the observed regulation of the various isozymes (*1*) as well as by the recent documentation of a metabolic disease attributable to a deficiency in this enzyme (*2*). Mechanistic and protein chemistry studies indicate that a reasonably stable covalent acetyl-S-enzyme reaction intermediate forms as a prelude to the condensation with acetoacetyl-CoA that accounts for HMG-CoA production (*3*).

Recently Vinarov et al. (*4*) have used ^{13}C NMR to successfully detect the covalent acetyl-S-enzyme reaction intermediate, which exhibits large upfield shifts compared to those observed for free acetyl-CoA in buffered solutions (184 ppm, $\Delta\delta = 20$ ppm for C1 and 26 ppm, $\Delta\delta = 7$ ppm for C2). These observations have been also extended by an investigation of β-ketothiolase C378G, which accumulates an acetyl-S-enzyme intermediate that displays ^{13}C NMR shifts similar to those observed with HMG-CoA synthase (*4*). One possible explanation for the large effects on ^{13}C chemical shifts is that acetyl-S-enzyme formation or dissipation involves transient production of a tetrahedral species in which C1 is transformed into an sp^3-hybridized carbon, which would be expected to resonate around 100 ppm (*5, 6*). Rapid exchange between such species (low in steady-state concentration) and a dominant acetyl-S-enzyme species (with an sp^2-hybridized C1 carbonyl) could account for a component of the upfield shift ($\Delta\delta = 20$ ppm for C1 and $\Delta\delta = 7$ ppm for C2) in the observed signals for each enzyme. However, at this time the hypothesis of the tetrahedral intermediate formation in the process of enzyme acetylation is based largely on solid bioorganic precedent (*7*) rather than direct experimental observations.

The substitution of heavy stable isotopes (e.g., D for H, ^{18}O for ^{16}O) in metabolites produces small upfield shifts of the NMR signal of the carbon to which they are covalently linked (*8, 9*). When deuterium is substituted for hydrogen, the observed effects are largest when the deuteron is directly bonded to the ^{13}C atom (α-effect) and are rapidly attenuated if the deuteron is separated by two (β-effect) or three (γ-effect) bonds (*8, 10*). In the case of ^{18}O-induced isotope effects, the magnitude of the shift is a function of the bond

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[1] Abbreviations: DIS, differential isotope shift; NMR, nuclear magnetic resonance; acetyl-CoA, acetyl-coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-thiogalactopyranoside; TMS, tetramethylsilane.

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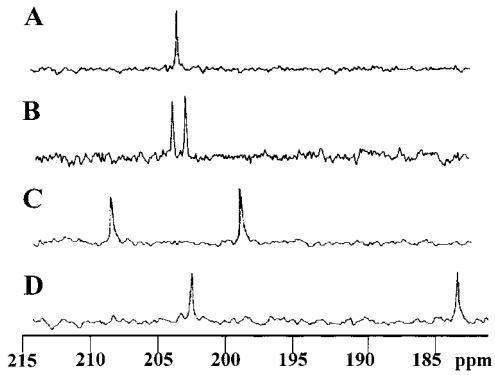

FIGURE 1: NMR spectra of ^{13}C-labeled acyl-CoA derivatives. Proton-decoupled ^{13}C NMR spectra (180−220 ppm, referenced to TMS) were measured at 21 °C on a Bruker-AC300 spectrometer operating at 75.469 MHz for ^{13}C. Samples, buffered with 10 mM potassium phosphate (pH 7.0, 20% D_2O for internal lock), contained (A) 2.0 mM [1-^{13}C]acetyl-CoA, (B) 2.0 mM [1,2-^{13}C]acetyl-CoA, (C) 2.0 mM [1,3-^{13}C]acetoacetyl-CoA, or (D) 2.0 mM [1,3,5-^{13}C]HMG-CoA. Spectra were zero-filled to 64K points and processed with 5 Hz line broadening to improve signal-to-noise ratio. Spectra A−D were obtained with 2000 transients each, which required approximately 1.5 h of acquisition time.

order (e.g., C−^{18}O produces half of the effect observed with C═^{18}O) (*9*). Application of the differential isotope shift technique has been used to demonstrate formation of tetrahedral adducts between inhibitors and proteases (*11*). As this report will demonstrate, the unusual stability and the well shifted peaks of HMG-CoA synthase's and β-ketothiolase's [^{13}C]acetyl-S-enzyme species afford a unique opportunity to extend the isotope shift approach to study actual intermediates in C−C bond formation/cleavage reactions. The approach provides direct experimental evidence for the formation of tetrahedral adducts in these reactions.

EXPERIMENTAL PROCEDURES

Synthesis of Unlabeled and ^{13}C-Enriched Metabolites. Unlabeled acetyl-CoA was synthesized from acetic anhydride by the procedure of Simon and Shemin (*12*).

[1-^{13}C] and [1,2-^{13}C]Acetyl-CoA. [1,1-^{13}C] and [1,1,2,2-^{13}C] acetic anhydride (99% enrichment) were purchased from Isotec. Synthesis employed the anhydride method of Simon and Shemin (*12*). Briefly, a solution of 100 μmol of CoA in 1 mL of nitrogen-purged water was adjusted to pH 8.0 with LiOH prior to addition of 200 μmol of [1,1-^{13}C] or [1,1,2,2-^{13}C] acetic anhydride. The mixture was periodically vortexed over 5 min, after which the reaction was complete, as demonstrated by a negative nitroprusside test (*13*). The pH was readjusted to 3.0 with HCl. [1-^{13}C]acetyl-CoA or [1,2-^{13}C]acetyl-CoA was precipitated with chilled MeOH−acetone (1:3) three times and recovered with a yield of 95%. The purity of the product was evident from a proton-decoupled ^{13}C NMR spectrum, which showed a resonance (a doublet in the case of [1,2-^{13}C]acetyl-CoA) at 204 ppm, corresponding to C1 of acetyl-CoA (Figure 1A,B), and a doublet in the case of [1,2-^{13}C]acetyl-CoA at 33 ppm corresponding to C2 of acetyl-CoA (not shown).

[1,3-^{13}C]Acetoacetyl-CoA. [1,3-^{13}C]Acetoacetyl-CoA was prepared following the method of Hersh and Jencks (*14*) as modified by Miziorko and Lane (*15*). Ethyl [1,3-^{13}C]-acetoacetate (99% enrichment, Isotec) was hydrolyzed with LiOH (1:2 ratio, micromoles) in a final volume of 1 mL at 30 °C for 5 h. After the hydrolysis, pH was adjusted to 7.2. Unlabeled acetoacetyl-CoA (Li^+ salt), EDTA, and 1 unit of porcine heart succinyl-CoA transferase (Sigma) were added to the hydrolyzed product in a final volume of 2 mL. The exchange was allowed to proceed for 30 min at 30 °C. The reaction mixture was cooled on ice and brought to pH 2 with HCl. The mixture was extracted with a 3-fold excess of ether three times to remove free acetoacetate. The aqueous layer was taken to near dryness and cooled. The sample was dissolved in chilled MeOH prior to addition of 3 volumes of cold acetone to precipitate [1,3-^{13}C]acetoacetyl-CoA as the Li^+ salt. The purity of the nucleotide product was evaluated by proton-decoupled ^{13}C NMR, which indicated only two resonances at 198.5 and 208.9 ppm, attributable to C1 thioester carbonyl and C3 keto carbons respectively (Figure 1C).

[l,3,5-^{13}C] HMG-CoA. [1,3,5-^{13}C]HMG-CoA was enzymatically synthesized by condensing [1,3-^{13}C]acetoacetyl-CoA with [1-^{13}C]acetyl-CoA in the presence of catalytic amounts of purified recombinant avian HMG-CoA synthase (*16*). [1,3-^{13}C]Acetoacetyl-CoA (13 μmol) and [1-^{13}C]acetyl-CoA (12 μmol) were incubated with 1 mg of HMG-CoA synthase (1 unit/mg) at 30 °C in 100 mM Tris-HCl, pH 8.2, containing 0.10 mM EDTA. After 3 h, additional [1,3-^{13}C]acetoacetyl-CoA (1.0 μmol) and HMG-CoA synthase (0.2 mg) were added to drive the reaction to completion. After 6 h, the reaction monitored by HPLC was found to be >90% complete. The product was purified by DEAE-Sephadex chromatography (1 × 45 cm column; 1 L gradient ranging from 20 to 300 mM LiCl in 3 mM HCl). The fractions containing the product were brought to near dryness by rotary evaporation and the nucleotide was recovered by precipitation from cold methanol/acetone (1:3). Proton-decoupled ^{13}C NMR evaluation of the isolated product indicated only three resonances at 73.5, 182.9, and 202.9 ppm, attributable to C-3 hydroxyl (not shown), C-5 carboxyl, and C-1 thioester carbonyl carbons, respectively (Figure 1D).

Purification and Assay of HMG-CoA Synthase. Recombinant avian cytosolic HMG-CoA synthase was expressed and purified as previously described (*17*). Protein concentration was routinely determined by the procedure of Bradford (*18*) using bovine serum albumin as the standard. For more exact protein concentration estimates, an extinction coefficient of 1.25 mL mg^{-1} cm^{-1} at 280 nm was used. This value was determined from amino acid composition analysis of HMG-CoA synthase. Protein concentration estimates from Bradford's method are 1.6-fold lower than values determined from the extinction coefficient and, thus, Bradford estimates were accordingly corrected. For measurement of the overall condensation reaction (*3*), 200 mM acetyl-CoA was added to the reaction mixture (30 °C) containing 100 mM Tris-HCl, pH 8.2, 100 mM EDTA, 50 mM acetoacetyl-CoA, and appropriately diluted HMG-CoA synthase (approximately 6 μg of enzyme in 1.0 mL final volume). The reaction rate was monitored by the acetyl-CoA-dependent decrease in absorbance at 300 nm, due to depletion of the enolate of acetoacetyl-CoA as the reaction with acetyl-CoA proceeds. Samples prepared in $H_2{}^{18}O$ and D_2O were assayed by the same procedure and gave identical results.

Purification of C378G Zoogloea ramigera β-Ketothiolase. C378G *Z. ramigera* β-ketothiolase, a mutant enzyme that lacks the general base catalyst and accumulates the acetyl-

S-enzyme intermediate, was chosen for the ^{13}C NMR experiments. The thiolase C378G-encoding plasmid was a generous gift of Professor Anthony Sinskey (MIT). Recombinant forms of β-ketothiolase were obtained from large-scale fermentations in LB medium. After the mutant plasmid had been transformed into *Escherichia coli* BL21, a single colony from a freshly streaked plate was used to inoculate a 5 mL starting culture (LB) containing the appropriate antibiotics. Large-scale fermentation was carried out in a 2500 mL Fernbach flasks. Incubation was carried out at 37 °C until OD_{600} reached 3.0. Expression of the mutant thiolase was induced by the addition of IPTG (1 mM) to a growing culture and incubation was continued for 5 h. Cells were harvested by centrifugation and stored frozen at −80 °C. The *E. coli* cells were lysed in a French pressure cell. In our hands, recombinant β-ketothiolase could not be purified to homogeneity by the published procedure (*19*), which relies heavily on a red-dye resin chromatography step. Our modification involved initial chromatography of the 100000*g* supernatant from the French pressure cell disruption step on Pharmacia Fast-Q anion-exchange resin (gradient elution performed with 75−250 mM NaCl in 20 mM Tris-HCl, pH = 8.2). The thiolase-containing fractions were brought to 1.0 M in ammonium sulfate and loaded onto a phenyl-agarose column. Elution of thiolase protein in homogeneous form was accomplished by use of a reverse gradient (1.0−0.4 M ammonium sulfate in 10 mM sodium phosphate, pH =7.0). Thiolase-containing fractions were pooled, dialyzed against 20 mM Tris-HCl, pH = 8.2, and supplemented with glycerol (20%) prior to storage at −80 °C. From an IPTG induced 0.5 L culture approximately 100 mg of purified β-ketothiolase could be obtained. SDS−polyacrylamide gel electrophoresis of the purified enzyme shows a single band of protein, corresponding to a molecular mass of ∼40 kDa.

NMR Methodology. D_2O (99.99 atom % D) was purchased from MSD Isotopes. $H_2{}^{18}O$ (95+ atom % ^{18}O; Mound-Monsanto) was a generous gift of Dr. C. Kennedy (Medical College of Wisconsin). ^{13}C NMR (proton-decoupled) experiments were performed on a Bruker AC-300 instrument operating at 75.469 MHz for ^{13}C. All spectra were recorded at 21 °C. All reported chemical shifts were referenced to TMS. A sweep width of 16 000 Hz was used, and 16K data points were collected. Signal acquisition employed a 35° pulse angle and a 2 s delay between transients. A typical spectrum of ^{13}C-enriched acetyl-CoA, measured in samples with a 2:1 substrate/enzyme site ratio, required 1.5−5 h of data collection (1500−5000 transients). HMG-CoA synthase and β-ketothiolase samples were buffer exchanged into 10 mM sodium phosphate, pH 7.0, by use of Centricon-25 membrane cones. After concentration to a site concentration of about 1 mM, the samples were lyophilized and dissolved (without significant loss of activity) in an appropriate volume of either deionized water supplemented with 10% D_2O for internal lock, or 100% D_2O, or $H_2{}^{18}O$ supplemented with 10% D_2O for internal lock prior to running the spectra. Two independently exchanged samples were run consecutively and a standard error for differential isotope shift values was calculated. For spectra shown in the figures, the collected data were zero-filled to 64K points and then processed with 5 Hz line broadening to improve the signal-to-noise ratio.

Table 1: Summary of ^{18}O-Induced Isotope Shifts on the [^{13}C] Resonances of HMG-CoA, Ac-CoA, Acetyl-S-WT Synthase, and Acetyl-S-C378G Thiolase Reaction Intermediates

complex		$\Delta\,[\delta_{(H2^{16}O)} - \delta_{(H2^{18}O)}]^a$ ppm
HMG-CoA in buffer	C1	0.008 ± 0.001
	C3	0.004 ± 0.001
	C5	0.026 ± 0.005
Ac-CoA in buffer	C1	0.005 ± 0.001
	C2	0.006 ± 0.001
acetyl-S-WT synthase	C1	0.055 ± 0.005
	C2	0.009 ± 0.002
acetyl-S-C378G thiolase	C1	0.004 ± 0.001
	C2	0.006 ± 0.001

[a] Standard error for differential isotope shift values was calculated from two independently exchanged and consecutively run samples.

RESULTS

Synthesis of ^{13}C-Labeled Acyl-CoA Derivatives. The synthetic methods outlined under Experimental Procedures provided quantitative yields of the labeled CoA derivatives. The purity of the synthesized compounds was assessed by ^{13}C NMR spectroscopy. Figure 1 shows the proton-decoupled ^{13}C NMR spectra of acetyl-CoA, acetoacetyl-CoA, and HMG-CoA with ^{13}C enrichment at various positions. The ^{13}C NMR spectra of [1-^{13}C]- and [1,2-^{13}C]acetyl-CoA show a resonance (a doublet in the case of [1,2-^{13}C]acetyl-CoA) at 204 ppm corresponding to C1 (Figure 1A,B) and, in the case of [1,2-^{13}C]acetyl-CoA, a doublet at 33 ppm corresponding to C2 (not shown). The ^{13}C NMR spectrum of [1,3-^{13}C]-acetoacetyl-CoA shows two resonances at 198.5 and 208.9 ppm, attributable to C1 thioester carbonyl and C3 keto-carbons respectively (Figure 1C). In the case of enzymatically synthesized [1,3,5-^{13}C]HMG-CoA, the C1 thioester carbonyl resonates at 202.9 ppm, the C3 hydroxyl carbon is at 73.5 ppm (not shown), and the C5 carboxyl resonance appears at 182.9 ppm (Figure 1D).

^{18}O-Induced Differential Isotope Shift ^{13}C NMR Spectroscopy. To confirm the magnitude of ^{13}C resonance shift upon incorporation of an ^{18}O substituent, [5-^{18}O]-enriched [l,3,5-^{13}C]HMG-CoA was synthesized by reacting catalytic amounts of HMG-CoA synthase with its [^{13}C]-enriched substrates (2 mM each) in $H_2{}^{18}O$. This incorporates one ^{18}O into the HMG-CoA's free carboxylate, which should exhibit a C−O bond order of 1.5 due to resonance averaging of one single and one double bond. Proton-decoupled ^{13}C NMR spectra of [l,3,5-^{13}C]HMG-CoA synthesized in $H_2{}^{18}O$ vs $H_2{}^{16}O$ are shown in Figure 2A. Substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ resulted in an upfield shift of 0.026 ± 0.005 ppm for C5 (182.25 ppm) of [l,3,5-^{13}C]HMG-CoA, as measured after 5 h of signal accumulation (Table 1). The magnitude of the observed upfield shift is within the range expected for a C−O bond order of 1.5 (*9*). Resonances corresponding to C1 (202.9 ppm) and C3 (73.5 ppm) of HMG-CoA showed insignificant upfield shifts of 0.008 ± 0.001 ppm for the C1 thioester carbon and 0.004 ± 0.001 ppm for the C3 carbon after 5 h of signal accumulation. Since no shift should be observed for peaks due to C1 or C3 of HMG-CoA (no solvent oxygen will label these positions under the conditions employed for HMG-CoA synthesis), these values provide an indication of experimental error.

Proton-decoupled ^{13}C NMR spectra of 2 mM [1,2-^{13}C]acetyl-CoA in buffer/$H_2{}^{18}O$ vs buffer/$H_2{}^{16}O$ are shown

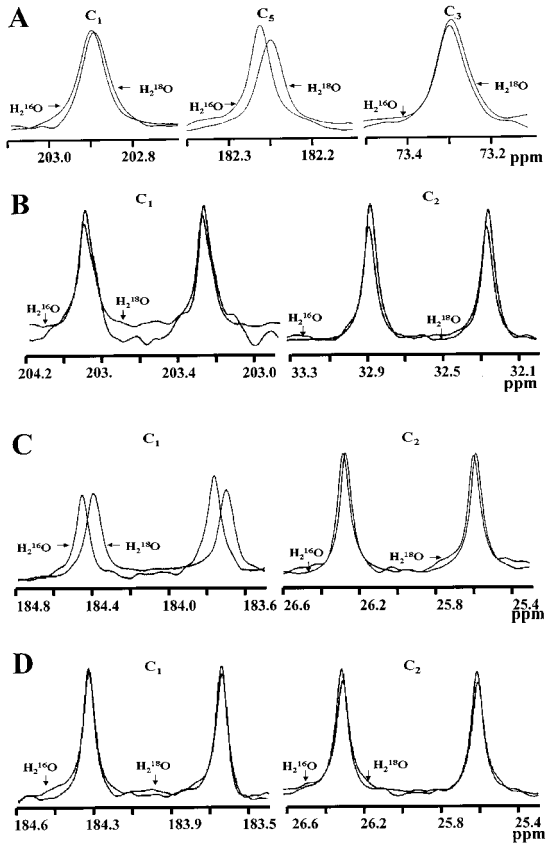

FIGURE 2: ^{18}O isotope shifts in ^{13}C NMR spectra. Samples were prepared as described under Experimental Procedures and contained (A) [l,3,5-^{13}C]HMG-CoA synthesized in $H_2{}^{18}O$ vs $H_2{}^{16}O$, (B) 2 mM [1,2-^{13}C]acetyl-CoA in buffer/$H_2{}^{18}O$ vs buffer/$H_2{}^{16}O$, (C) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in $H_2{}^{18}O$ vs $H_2{}^{16}O$, or (D) β-ketothiolase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in $H_2{}^{18}O$ vs $H_2{}^{16}O$.

in Figure 2B. Substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ resulted in insignificant upfield shifts of 0.005 ± 0.001 ppm for the C1 thioester carbon and 0.006 ± 0.001 ppm for the C2 methyl carbon of acetyl-CoA, measured after 5 h of signal accumulation. These results demonstrate that no uncatalyzed exchange labeling occurs.

The ability of HMG-CoA synthase's acetyl-S-enzyme to reversibly form/collapse a tetrahedral intermediate was tested by reconstituting freeze-dried enzyme in $H_2{}^{18}O$ vs $H_2{}^{16}O$ and adding [1,2-^{13}C]acetyl-CoA, appropriately dissolved in $H_2{}^{18}O$ or $H_2{}^{16}O$ (Figure 2C). Use of doubly labeled [l,2-^{13}C]-acetyl-CoA affords an internal control, since the 26 ppm C2 methyl peak can be observed in $H_2{}^{18}O$ and $H_2{}^{16}O$ experiments to verify that no significant upfield shifts are observed for this carbon, which contains no C−O bond. For the resonance corresponding to the C1 thioester carbon of acetyl-S-enzyme (184.3 ppm), the substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ resulted in an upfield shift of 0.055 ± 0.005 ppm, as measured after 3 h of signal accumulation (Figure 2C). The magnitude of the shift did not increase upon further signal accumulation. In contrast to the observations on C1, the substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ had very little effect on the resonance corresponding to the C2 methyl carbon (26 ppm), as expected. After 3 h of signal accumulation, the observed upfield shift was 0.009 ± 0.002 ppm (Figure 2C), a value that remained unchanged upon further signal accumulation and reflects experimental error.

Table 2. Summary of ^2H-Induced Isotope Shifts on the [^{13}C] Resonances of HMG-CoA, Ac-CoA, Acetyl-S-WT Synthase, and Acetyl-S−C378G Thiolase Reaction Intermediates

complex		$\Delta[\delta_{(H2O)} - \delta_{(D2O)}]^{a,b}$ ppm
HMG-CoA in buffer	C1	0.035 ± 0.005
	C3	0.110 ± 0.015
	C5	0.020 ± 0.005
Ac-CoA in buffer	C1	0.030 ± 0.005
	C2	0.010 ± 0.005
acetyl-S-WT synthase	C1	$0.128 \pm 0.015 \rightarrow 0.186 \pm 0.015$
	C2	$0.021 \pm 0.006 \rightarrow 0.021 \pm 0.006$
acetyl-S-WT synthase[c]	C1	$0.165 \pm 0.015 \rightarrow 0.165 \pm 0.015$
	C2	$0.029 \pm 0.015 \rightarrow 0.029 \pm 0.015$
acetyl-S-WT synthase[d]	C1	0.092 ± 0.008
	C2	0.015 ± 0.005
acetyl-S-C378G thiolase	C1	0.080 ± 0.007
	C2	0.015 ± 0.005

[a] Standard error for differential isotope shift values was calculated from two independently exchanged and consecutively run samples.[b] For acetyl-S-WT synthase and acetyl-S-WT synthase preincubated in D_2O, ^2H-induced shifts are shown after 1 and 3 h of signal accumulation. The rest of the data reported are after 3, 5, or 10 h of signal accumulation (see text). [c] Freeze-dried HMG-CoA synthase (1 mM) was dissolved in D_2O and incubated for 5 h prior to the addition of [1,2-^{13}C]acetyl-CoA (2 mM) and signal accumulation. [d] HMG-CoA synthase (1 mM) and [1,2-^{13}C]acetyl-CoA (2 mM) were dissolved in a mixture of D_2O/H_2O (50/50 v/v).

β-Ketothiolase also forms an NMR-detectable acetyl-S-enzyme (*4*) but does not actively react with water to catalyze thioester hydrolysis during its normal catalytic cycle. Reaction of C378G β-ketothiolase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in $H_2{}^{18}O$ vs $H_2{}^{16}O$ resulted in the ^{13}C NMR spectra shown in Figure 2D. For the resonance corresponding to the C1 thioester carbon (184.3 ppm), the substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ resulted in an upfield shift of 0.004 ± 0.001 ppm, measured after 10 h of signal accumulation (Figure 2D). The magnitude of the shift did not increase upon further signal accumulation. The substitution of $H_2{}^{18}O$ for $H_2{}^{16}O$ had a similarly minimal effect on the resonance corresponding to the C2 methyl carbon (26 ppm). After 10 h of signal accumulation, the observed upfield shift was 0.006 ± 0.001 ppm (Figure 2D) and remained unchanged upon further signal accumulation. The observed upfield shifts for the C1 and C2 resonances reflect experimental error.

^2H-Induced Differential Isotope Shift ^{13}C NMR Spectroscopy. To confirm the magnitude of any ^{13}C shift expected upon incorporation of a ^2H substituent, [l,3,5-^{13}C]HMG-CoA was synthesized as described under Experimental Procedures. Dissolving freeze-dried [l,3,5-^{13}C]HMG-CoA in D_2O at neutral pH (pD = pH + 0.4) results in exchange of one deuteron into the HMG-CoA's C3 hydroxyl group. The ^2H-induced ^{13}C upfield shift (Table 2) corresponding to a β-effect on C3 was calibrated by this experiment. No shift should be observed for resonances due to C1 or C5 of HMG-CoA, since these carbons have no exchangeable hydrogens at neutral pH. Proton-decoupled ^{13}C NMR spectra of freeze-dried [l,3,5-^{13}C]HMG-CoA dissolved in D_2O vs H_2O are shown in Figure 3 A. Substitution of D_2O for H_2O resulted in an upfield shift of 0.110 ± 0.015 ppm for C3 (73.5 ppm) of [l,3,5-^{13}C]HMG-CoA, measured after 5 h of signal accumulation. The magnitude of the observed shift (Table 2) is consistent with other reported estimates for a deuterium-induced β-effect (*8*). Resonances corresponding to C1 (202.9 ppm) and C5 (182.9 ppm) of HMG-CoA showed insignificant upfield shifts of

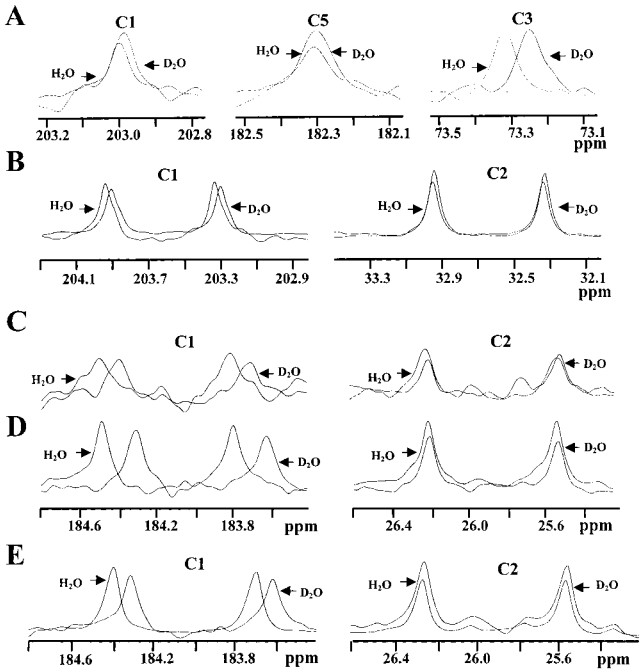

FIGURE 3: ^2H isotope shifts in ^{13}C NMR spectra. Samples were prepared as described under Experimental Procedures and contained (A) [l,3,5-^{13}C]HMG-CoA in D_2O vs H_2O, (B) 2 mM [1,2-^{13}C]acetyl-CoA in buffer/D_2O vs buffer/H_2O, (C) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O (spectra were acquired after 1 h of signal accumulation), (D) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O (spectra were acquired after 3 h of continuous signal accumulation), or (E) β-ketothiolase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O.

0.035 ± 0.005 ppm for C1 thioester carbon and 0.020 ± 0.005 ppm for C5 carboxyl carbon, measured after 5 h of signal accumulation. These values most likely represent a combination of effects from bulk magnetic susceptibility and/or experimental error.

Proton-decoupled ^{13}C NMR spectra of 2 mM [1,2-^{13}C]acetyl-CoA in buffer/D_2O vs buffer/H_2O are shown in Figure 3B. Substitution of D_2O for H_2O resulted in insignificant upfield shifts of 0.030 ± 0.005 ppm for C1 thioester carbon and 0.010 ± 0.005 ppm for C2 methyl carbon, measured after 5 h of signal accumulation. Since neither C1 thioester nor C2 methyl carbons of free acetyl-CoA contain exchangeable hydrogens, the small observed upfield shifts for both carbons most likely represent a combination of any bulk magnetic susceptibility effect and/or experimental error. These observations indicate that no uncatalyzed deuterium exchange occurs in buffered solutions of acetyl-CoA to produce any shift in the C1 or C2 acetyl resonances.

The ability of HMG-CoA synthase's acetyl-S-enzyme to reversibly form/collapse a tetrahedral intermediate was tested by reconstituting freeze-dried enzyme in D_2O vs H_2O and adding freeze-dried [1,2-^{13}C]acetyl-CoA appropriately dissolved in either D_2O or H_2O. Reaction of wild-type HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O resulted in the ^{13}C NMR spectra shown in Figure 3C,D. For the resonance corresponding to the C1 thioester carbon (184.3 ppm), the substitution of D_2O for H_2O resulted in an upfield shift of 0.128 ± 0.015 ppm after 1 h of signal accumulation (Figure 3C). The magnitude of the upfield shift increased to 0.186 ± 0.015 ppm after 3 h of signal accumulation (Figure 3D). The magnitude of the shift did not increase upon further signal accumulation. The observed shift of 0.186 ± 0.015 ppm is large in comparison with many values reported for the deuterium-induced β-effect. On the other hand, the substitution of D_2O for H_2O had very little effect on the resonance corresponding to the C2 methyl carbon (26 ppm), as expected. After 1 h of signal accumulation the observed upfield shift was 0.021 ± 0.006 ppm (Figure 3C), a value that remained unchanged after 5 h of signal accumulation (Figure 3D). The observed upfield shift for the C2 methyl carbon may be attributable to a combination of effects from bulk magnetic susceptibility and experimental error.

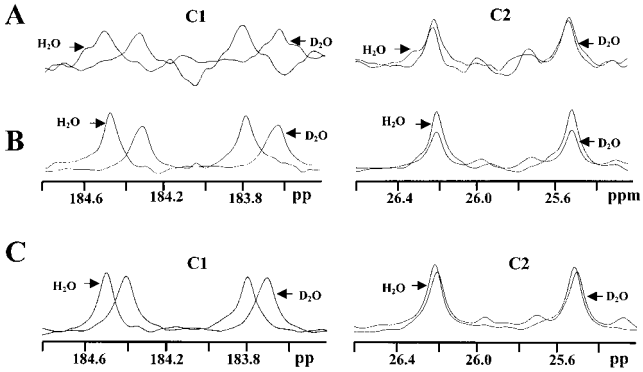

FIGURE 4: Effect of mixed solvent and preincubation in D_2O on ^{13}C NMR spectra. Samples were prepared as described under Experimental Procedures. (A) Sample contained HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O. Freeze-dried HMG-CoA synthase was dissolved in D_2O and incubated for 5 h prior to the addition of [1,2-^{13}C]acetyl-CoA and signal accumulation. Spectra were acquired after 1 h of signal accumulation. (B) Sample contained HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O. Experimental conditions are same as for spectra A. Spectra were acquired after 3 h of continuous signal accumulation. (C) Sample contained HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in a mixture of D_2O/H_2O (50/50 v/v) vs H_2O. Spectra were acquired after 3 h of signal accumulation.

Detection of a time-dependent ^2H-induced ^{13}C isotope shift for the signal corresponding to the C1 thioester carbon of acetyl-CoA is indicative of an interaction with a slowly exchangeable proton (deuteron) of a side chain/amide backbone of an amino acid residue at the active site. To test this hypothesis, freeze-dried HMG-CoA synthase (1 mM) was dissolved in D_2O and incubated for 5 h prior to the addition of [1,2-^{13}C]acetyl-CoA (2 mM). This time should be sufficient enough to fully deuterate a solvent-exposed side chain/backbone of an amino acid residue, an assumption that seems reasonable for a residue situated in an active site that is vacant prior to delivery of substrate. Proton-decoupled ^{13}C NMR spectra were recorded after 1 and 3 h of continuous signal accumulation. The resulting spectra, compared to those obtained at the same time intervals for enzyme mixed with acetyl-CoA in H_2O, are shown in Figure 4A. After 1 h of signal accumulation (Figure 4A), the resonance corresponding to the C1 thioester carbon (184.3 ppm) shows an upfield shift of 0.165 ± 0.015 ppm. The magnitude of the upfield shift remains unchanged after 3 h of continuous signal accumulation (Figure 4B) and most likely represents the limiting value under these experimental conditions. The value of 0.165 ± 0.015 ppm is similar to the one obtained after 3 h of spectra accumulation in the experiment when the enzyme

was not preincubated in D_2O prior to the addition of acetyl-CoA. The magnitude of the shift is consistent with the reported value for the deuterium-induced β-effect. Preincubation of enzyme in D_2O prior to the addition of acetyl-CoA and spectra accumulation had very little effect on the resonance corresponding to the C2 methyl carbon (26 ppm), as expected. After 1 h of spectra accumulation, the observed upfield shift was 0.028 ± 0.006 ppm (Figure 4A), a value that remained unchanged after 3 h of signal accumulation (Figure 4B).

An effect of mixed solvent (D_2O/H_2O, 50/50 v/v) on the observed deuterium-induced ^{13}C isotope shift was studied. Reaction of wild-type HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in a mixture of D_2O/H_2O (50/50, v/v) vs H_2O resulted in ^{13}C NMR spectra shown in Figure 4C. For the resonance corresponding to the C1 thioester carbon (184.3 ppm), the substitution of D_2O/H_2O mixture for H_2O resulted in an upfield shift of 0.092 ± 0.008 ppm, measured after 3 h of signal accumulation (Figure 4C). The magnitude of the shift did not increase upon further signal accumulation. A shift of 0.092 ± 0.008 ppm represents roughly half of the value obtained in the experiment performed in 100% D_2O. It appears that a decrease in the observed deuterium-induced ^{13}C isotope shift correlates with the percentage of H_2O in the mixture. Once again, very little effect is observed on the resonance corresponding to the C2 methyl carbon (26 ppm), as expected. After 3 h of signal accumulation the observed upfield shift was 0.015 ± 0.005 ppm (Figure 4 C).

C378G β-ketothiolase forms an acetyl-S-enzyme reaction intermediate but will not catalyze condensation to form acetoacetyl-CoA. Reaction of C378G β-ketothiolase (1 mM) with [1,2-^{13}C]acetyl-CoA (2 mM) in D_2O vs H_2O resulted in ^{13}C NMR spectra shown in Figure 3E. For the resonance corresponding to the C1 thioester carbon (184.3 ppm), the substitution of D_2O for H_2O resulted in an upfield shift of 0.080 ± 0.007 ppm after 3 h of signal accumulation (Figure 3E). The magnitude of the shift did not increase upon further signal accumulation. The value of 0.080 ± 0.007 ppm is approximately half that expected for a deuterium-induced β-effect. The substitution of D_2O for H_2O had very little effect on the resonance corresponding to the C2 methyl carbon (26 ppm), as expected. After 3 h of signal accumulation the observed upfield shift was 0.015 ± 0.005 ppm (Figure 3E), a value that remained unchanged upon further signal accumulation. The observed upfield shift for the C2 methyl carbon most likely represents a combination of an effect from bulk magnetic susceptibility and an experimental error.

Solvent Accessibility to the Active Sites of Acetyl-S-Enzyme Reaction Intermediates. Vinarov et al. (*4*) suggested that HMG-CoA synthase and β-ketothiolase possess very hydrophobic reaction centers. If that is the case, then diffusion of bulk water into the active site after solvent delivers acetyl-CoA, which then binds as a prelude to formation of the acetyl-S-enzyme reaction intermediate, should be very limited. The following experiments were carried out to test this hypothesis. Spectra of 3 mM [1,2-^{13}C]acetyl-CoA in 10 mM potassium phosphate buffer (pH = 7.0) were acquired in the absence and presence of 0.5 mM Mn^{2+} (Figure 5A,B). The paramagnetic metal ion, Mn^{2+}, has a prominent effect on the line width (significant broadening) of the ^{13}C resonances corresponding to the C1 thioester carbonyl carbon (203 ppm) and a detectable but more modest effect on the C2 methyl carbon (33 ppm) of acetyl-CoA in buffer (Figure 5B). If formation of the binary acetyl-CoA·enzyme Michaelis complex or subsequent production of the acetyl-S-enzyme reaction intermediate results in a sequestration of these reactive components that limits access of bulk water and Mn^{2+} to the active site, then any Mn^{2+} broadening effect would be minimized. To test this prediction, paired samples of wild-type HMG-CoA synthase (1 mM) were mixed with [1,2-^{13}C]acetyl-CoA (3 mM) and incubated for 3 h. One sample was then supplemented with 0.5 mM Mn^{2+}. Proton-decoupled ^{13}C NMR spectra (1 h of signal accumulation) are shown in Figure 5C,D. The presence of 0.5 mM Mn^{2+} in the reaction mixture has no effect on the intensity and line width of the resonances corresponding to the C1 thioester carbonyl carbon (184 ppm) and C2 methyl carbon (26 ppm) of the acetyl-S-enzyme reaction intermediate. On the other hand, resonances corresponding to the C1 thioester carbonyl carbon (203 ppm) and C2 methyl carbon (33 ppm) of free acetyl-CoA (or any acetyl-CoA in a Michaelis complex) are significantly broadened (to baseline level) in the presence of 0.5 mM Mn^{2+} (Figure 5D). Signal accumulation for an additional 4 h results in the spectra shown in Figure 5E,F. The minimal decrease in intensity and increase in line broadening of ^{13}C resonances corresponding to the C1 thioester carbonyl carbon (184 ppm) and C2 methyl carbon (26 ppm) of the acetyl-S-enzyme reaction intermediate, suggests that, over a span of 5 h, there is very slow exchange of solvent in and out of the active site.

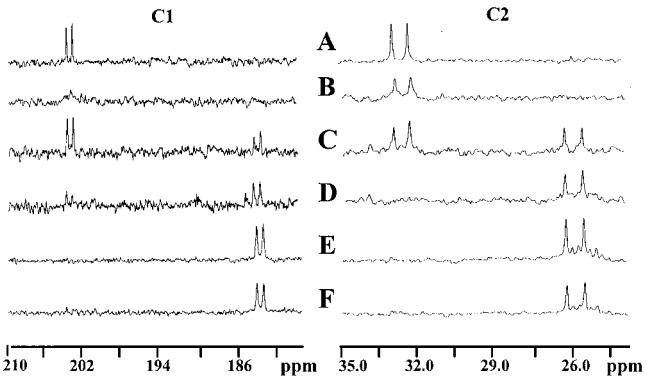

FIGURE 5: Effect of Mn^{2+} on ^{13}C resonances of free [^{13}C]acetyl-CoA and [^{13}C]acetyl-S-enzyme reaction intermediate. Samples were prepared as described under Experimental Procedures and contained (A) 3 mM [1,2-^{13}C]acetyl-CoA in buffer, (B) 3 mM [1,2-^{13}C]acetyl-CoA in buffer in the presence of 0.5 mM Mn^{2+}, (C) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (3 mM) (spectra were acquired after 1 h of signal accumulation), (D) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (3 mM) in the presence of 0.5 mM Mn^{2+} (spectra were acquired after 1 h of signal accumulation), (E) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (3 mM) (spectra were acquired after 3 h of continuous signal accumulation), or (F) HMG-CoA synthase (1 mM) with [1,2-^{13}C]acetyl-CoA (3 mM) in the presence of 0.5 mM Mn^{2+} (spectra were acquired after 5 h of continuous signal accumulation).

DISCUSSION

Bioorganic model chemistry (*7*, *20*) supports the possibility that acetyl-S-enzyme intermediates such as those observed for HMG-CoA synthase and C378G β-ketothiolase (*4*) may be expected to reversibly form upfield-resonating tetrahedral

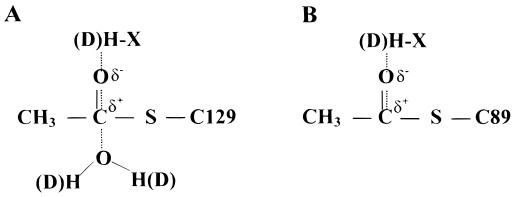

FIGURE 6: Proposed interactions of acetyl-S-enzyme reaction intermediate at the active site of HMG-CoA synthase and β-ketothiolase.

carbon adducts by addition of a solvent hydroxyl group or an active-site nucleophile across the carbonyl group. While no upfield signals attributable to such adducts have yet been detected, it seemed that the transient existence of such a species might be tested by a differential isotope shift approach. In principle, the deuterium labeling approach could detect an upfield shift due to a β-effect upon addition of D_2O (or OD^-) to the carbonyl carbon to form a thiohemiacetal (Figure 6A). In studies on sugars, Pfeffer et al. (*8*) report β-effect shifts in the 0.12−0.23 ppm range. In work on a pepsin-bound substrate analogue, Schmidt et al. (*11*) observed a 0.36 ppm shift and attributed the effect to a tetrahedral diol adduct containing a carbon that is β-linked to two deuterium atoms. However, if the 184 ppm resonance did reflect rapid exchange of a major peak due to a sp^2-hybridized carbon with a minor upfield peak attributable to a sp^3-hybridized carbon, the averaged contribution of the latter species would be too small to account for the significant deuterium shift observed for the 184 ppm peak.

Thus, a mechanism other than formation of a tetrahedral adduct must be invoked to account for the observed deuterium-induced shift. The carbonyl oxygen bonded to a sp^2-hybridized carbon could hydrogen-bond to a donor that has been deuterated by prior exchange with solvent to produce an isotope shift (Figure 6B). A single β-effect would result, accounting for a substantial part of the upfield shift measured for the acetyl-S-enzyme reaction intermediate of HMG-CoA synthase. The smaller upfield shift observed for thiolase (which, as discussed below, does not seem attributable to a solvent-derived heavy atom) may be entirely due to such hydrogen bonding.

The time dependence of a significant portion of the deuterium differential isotope shift observed for HMG-CoA synthase's reaction intermediate deserves comment. Such a component seems attributable to an interaction with an active site amino acid [e.g., (D)HX in Figure 6], since any effect from the solvent in which the protein is dissolved should not be time-dependent. This report indicates that slow changes in solvent composition at the HMG-CoA synthase active site seem unlikely. Such an assertion is prompted by the experimental observation that, once acetyl-S-enzyme has formed, the active site is inaccessible[2] to small solvent-borne ions such as paramagnetic Mn^{2+}. An active site that excludes bulk solvent from the reactive thioester-containing reaction

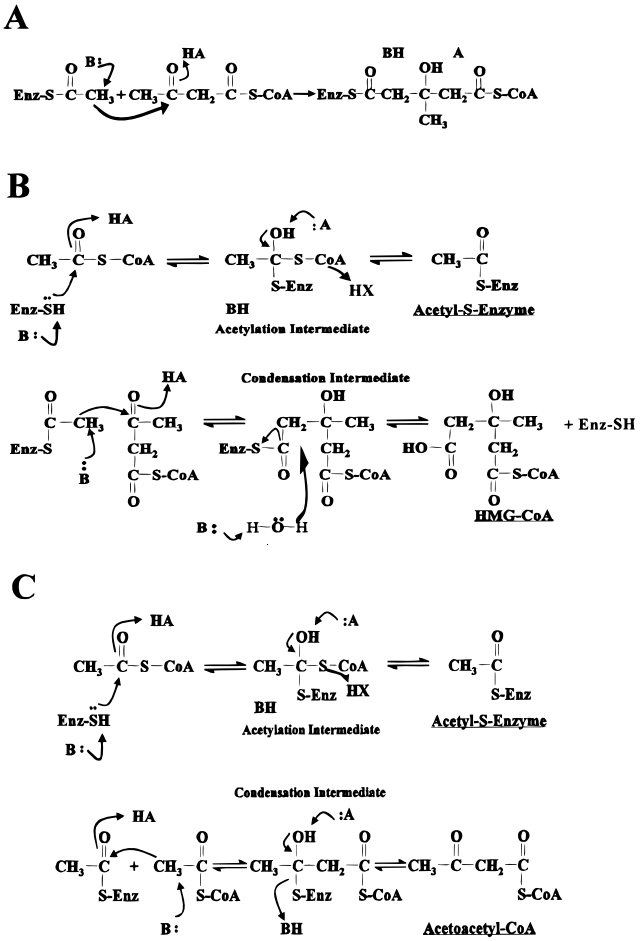

FIGURE 7: Reaction mechanisms for HMG-CoA synthase and β-ketothiolase. (A) Minimal mechanism for HMG-CoA synthase. (B) Expanded mechanism for HMG-CoA synthase indicating reaction intermediates and possible roles for acid/base catalysts. (C) Mechanism for β-ketothiolase indicating reaction intermediates and possible roles for acid/base catalysts.

intermediates may have evolved to optimize the efficiency of HMG-CoA production.

On the basis of deuterium effects, ambiguity over whether HMG-CoA synthase can convert acetyl-S-enzyme into a tetrahedral adduct persists. This issue is, however, resolved by the observation of an ^{18}O-dependent isotope shift. The magnitude of the effect is comparable to that reported by Schmidt et al. (*11*), who attribute their observation to the two oxygen atoms in a tetrahedral diol adduct of a pepsin−substrate analogue complex. However, the 184 ppm ^{13}C resonance of HMG-CoA synthase's reaction intermediate suggests a carbonyl character. In this case, the magnitude of the ^{18}O shift simply reflects substantial double-bond character in the carbonyl group.

No ^{18}O shift is observed for thiolase, which in the course of a normal reaction cycle does not catalyze the addition of water across a thioester bond (Figure 7 C), that would involve formation of a diol-containing adduct. A question arises concerning whether such a diol-containing tetrahedral adduct (Figure 8) is only a transition-state intermediate or a more stable intermediate with a finite lifetime. Isotope effect data on acyl transfer reactions of *p*-nitrophenyl acetate with various nucleophiles (*21*) have been interpreted (*22*) to suggest that acyl transfer occurs via a "concerted" mechanism in which the tetrahedral adduct forms only as a transition-

[2] The paramagnetic broadening of NMR lines can be attributed to scalar (through-bond) as well as dipolar (through-space) effects. For example, in the case of Mn^{2+} addition to acetyl-CoA, both C1 and C2 (to which Mn^{2+} is unlikely to ligand) exhibit broadened resonances. In the case of Mn^{2+} addition to acetyl-S-enzyme, while extensive liganding of the carbonyl oxygen to active-site ligands may minimize through-bond interaction with cation, a dipolar effect should still be apparent if Mn^{2+} has access to the active site. No such effect is measurable.

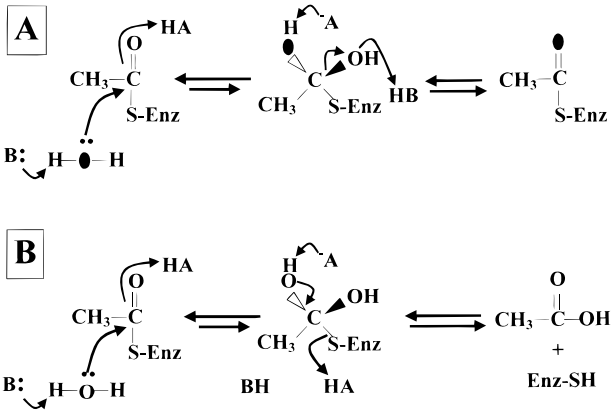

FIGURE 8: Mechanism for ^{18}O exchange (A) and hydrolysis (B) of the acetyl-S-enzyme reaction intermediate of HMG-CoA synthase.

state intermediate, rapidly dissipated since nucleophilic attack and leaving group departure occur as part of the same step. For this to occur, the enzyme would have to accommodate an attacking nucleophile on one face of the carbonyl carbon as well as leaving group expulsion from the other face. Perhaps the ^{18}O exchange observed for HMG-CoA synthase (Figure 8A) reflects an active site that can simultaneously accommodate these spatial requirements. In the context of the concerted mechanism, failure of thiolase to exhibit ^{18}O exchange might be due to inability to meet these steric requirements. Alternatively, the tetrahedral diol-containing adduct may represent a more stable intermediate [i.e., a "stepwise" mechanism is operative (*22*)]. In such a case, rotational mobility around the C−S bond would allow repositioning of the oxygens in the diol adduct, easily accounting for ^{18}O exchange when collapse to re-form the sp^2 -hybridized carbonyl occurs. This would account for the data measured for HMG-CoA synthase, which forms a particularly stable acetyl-S-enzyme and might sufficiently stabilize a diol-containing adduct to the extent that the stepwise mechanism [discounted on the basis of isotope effect data on a model system (*21*)] becomes operative. If thiolase operated by a stepwise mechanism, constraints on rotational mobility of the diol adduct would have to be invoked to account for selective expulsion of the solvent-derived oxygen when the sp^2-hybridized carbonyl reforms. Under such motional constraints, the carbonyl resonance for the thiolase intermediate might be expected to exhibit a wider line width than measured for the comparable resonance of the HMG-CoA synthase intermediate. Thus, proposing a stepwise mechanism seems unattractive for a variety of reasons. Alternative explanations such as absence of solvent or inefficient reaction of solvent across the carbonyl remain to be considered in accounting for the thiolase data.

In contrast to the dissipation of the acetyl-S-enzyme reaction intermediate of HMG-CoA synthase by a hydrolysis partial reaction, formation of the acetyl-S-enzyme involves no solvent oxygen. Thus, strictly speaking, involvement of a tetrahedral adduct has only been demonstrated for acetyl group hydrolysis (Figure 8B), although the homology with hydrolysis that releases product HMG-CoA is clear. Analogous formation of a tetrahedral adduct during production of acetyl-S-enzyme seems quite likely but remains to be directly demonstrated. In the context of hydrolysis, isolated acetyl-S-enzyme exhibits a rate of hydrolysis ($k_{hydrolysis} = 0.0036$ s^{-1}; K. Chun, unpublished observations) that is 10-fold lower than the rate measured under steady-state conditions in the presence of excess acetyl-CoA and the CoASH product formed during the reaction. Even the slower rate translates into multiple turnovers during the course of acquisition of a ^{13}C NMR spectrum. Thus, while the time constraints of the NMR experiment preclude straightforward comparison of $k_{exchange}$ and $k_{hydrolysis}$, certainly the rate of hydrolysis is rapid enough to be compatible with the observed ^{18}O exchange. Interestingly, the turnover number of HMG-CoA synthase for the overall productive condensation and release of HMG-CoA is ~ 4 s^{-1}, 2 orders of magnitude faster than steady-state hydrolysis of acetyl-CoA and 3 orders of magnitude faster than hydrolysis of the isolated acetyl-S-enzyme intermediate. Clearly the enzyme is optimized for productive hydrolytic release of the condensation product and efficiently shields the early acetyl-S-enzyme intermediate from premature dissipation via hydrolysis. We believe that, albeit slow in rate, hydrolysis of acetyl-CoA and acetyl-S-enzyme mimics the productive terminal hydrolytic release of product. Indeed, preliminary mutagenesis work (K. Chun, unpublished work) has identified mutants that exhibit parallel defects in acetyl-S-enzyme formation, acetyl-CoA hydrolysis, and HMG-CoA production, supporting such a hypothesis.

In the case of HMG-CoA synthase, a minimal mechanism for catalysis has been based on analogy with the citrate synthase reaction, which involves no acyl-S-enzyme intermediate. Such a mechanism (Figure 7A) might explicitly involve the participation of only two key residues: (1) a general base that deprotonates the C2 methyl group of acetyl-S-enzyme to support its attack on acetoacetyl-CoA and (2) a general acid that protonates the C3 carbonyl of acetoacetyl-CoA, producing the hydroxyl group of product HMG-CoA. Results presented in this paper implicate previously undetected tetrahedral intermediates in the reaction pathway of HMG-CoA synthase and indicate that the minimal mechanism is no longer tenable. These new observations justify consideration of a more detailed scheme shown in Figure 7B.

While the differential isotope shift technique can be a very powerful tool for establishing the identity of reaction intermediates, it obviously cannot stand alone. HMG-CoA synthase mutants that are defective in acetylation of C129 have been identified (K. Chun, personal communication). Characterization of such enzymes and analysis by NMR or other biophysical approaches may provide a more direct test of tetrahedral adduct formation during this early stage of the reaction.

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