Detection of Acetyl-*S*-Enzyme Reaction Intermediates of Hydroxymethylglutaryl-CoA Synthase and *β*-Ketothiolase by ^{13}C NMR

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3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) 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] In this report we describe the use of ^{13}C NMR, a tool which has been profitably used to investigate enzyme−inhibitor adducts[4,5] but rarely applied to reaction intermediates,[6] to detect this covalent acetyl-enzyme intermediate. Both acetyl carbon resonances exhibit large upfield shifts; this may be explained, in part, by the hypothesis that the acetyl group is situated in a very hydrophobic environment. These observations are extended by an investigation of *β*-ketothiolase, which displays ^{13}C NMR shifts for an acetyl-enzyme intermediate that are similar to those observed with HMG-CoA synthase. The data suggest that sequestration of acyl-enzymes may be a common feature in those enzymatic C−C condensation reactions that involve covalent reaction intermediates.

[1-^{13}C]Acetyl-CoA in buffered aqueous solution exhibits a chemical shift (204 ppm; Figure 1A) typical of a thioesterified carboxyl group. At a 3:1 ratio of [1-^{13}C]acetyl-CoA/active sites, despite the high molecular weight (116000) of dimeric avian cytosolic HMG-CoA synthase, a new resonance peak (184 ppm; Figure 1B) attributable to the [^{13}C]-acetylated enzyme is clearly observable. Experiments with mutant forms of the enzyme suggest that this signal is attributable to the covalent acetyl-*S*-enzyme reaction intermediate. Mutation of H264 produces enzymes (e.g., H264N, H264A) which are unimpaired in terms of acetylation by acetyl-CoA but exhibit enhanced acyl-CoA hydrolysis.[7] An experiment performed using a 2:1 ratio of substrate/H264A sites (Figure 1C) produces a prominent 184 ppm peak due to acetyl-*S*-enzyme. The elevated intrinsic acyl-CoA hydrolase activity eliminates unbound substrate over the period of signal accumulation and the corresponding 204 ppm signal; a resonance peak for the hydrolysis product, [1-^{13}C]acetate, is observed at 175 ppm (data not shown). The conservative C129S mutation perturbs neither binding of acyl-CoA metabolites nor formation of a Michaelis complex with acetyl-CoA, but without the C129 sulfhydryl, no covalent acylation of the enzyme occurs,[8] and no ^{13}C signal attributable to such an intermediate should appear. In fact, no 184 ppm peak is observed (Figure 1D) in a 2:1 mixture of [1-^{13}C]acetyl-CoA and C129S. Signal from C129S-bound substrate is not appreciably shifted; it overlaps the peak attributable to residual unbound acetyl-CoA, producing at 204 ppm a signal slightly broadened (∼17 Hz) in comparison with the peak width (10 Hz) of [1-^{13}C]acetyl-CoA in buffer (Figure 1A).

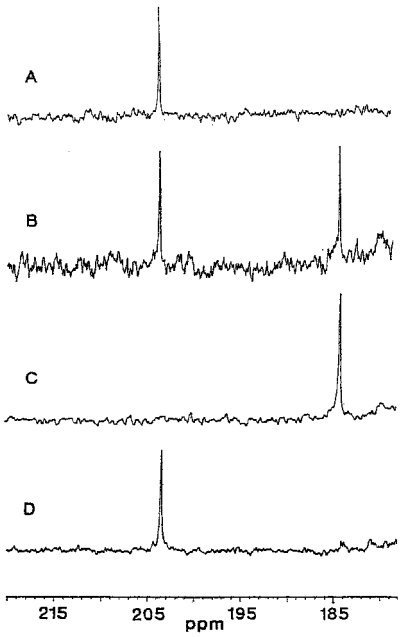

Figure 1. [1-^{13}C]Acetyl-CoA binding to wild-type and mutant avian cytosolic HMG-CoA synthases. ^{13}C NMR spectra (180−215 ppm, referenced to TMS) were measured at 21 °C using a Bruker-AC300 spectrometer operating at 75.47 MHz for ^{13}C. Samples, buffered with 10 mM potassium phosphate (pH 7.0), contained: (A) 2.0 mM [1-^{13}C]acetyl-CoA; (B) 2.4 mM [1-^{13}C]acetyl-CoA and 0.8 mM wild-type HMG-CoA synthase; (C) 1.6 mM [1-^{13}C]acetyl-CoA and 0.8 mM H264A HMG-CoA synthase; (D) 1.6 mM [1-^{13}C]acetyl-CoA and 0.8 mM C129S HMG-CoA synthase. Enzyme site concentration is calculated by using a subunit molecular weight of 57 600. Buffer contained 20% D_2O for internal lock. FIDs were processed with 5 Hz line-broadening; spectra A−D were obtained with 1500, 2000, 5000, and 5000 transients, respectively. A 2000 transient experiment requires approximately 1.5 h of acquisition time. The acetyl-*S*-enzyme reaction intermediate is quite stable in the absence of the second substrate, acetoacetyl-CoA, and can be isolated by gel filtration or SDS-PAGE.[3] At pH 7.0, intrinsic hydrolysis of acetyl-*S*-enzyme is slow[3,8] for wild-type enzyme, and the slight excess of free metabolite re-acetylates any free enzyme-SH produced. In the case of the H264A experiment (spectrum C), the elevated intrinsic hydrolysis activity of this mutant[7] accounts for depletion of the slight excess of unbound metabolite during the 5000 transient experiment.

The hypothesis that formation of a covalent acetyl-*S*-enzyme is required in order for the prominent upfield shift to be observed suggested extension of these studies to another enzyme that forms this type of reaction intermediate. *β*-Ketothiolase acetylates C89[9] during the reversible interconversion of acetyl-CoA with acetoacetyl-CoA. Elimination of the general base catalyst, C378, precludes condensation to form, at equilibrium, a mixture of acetyl- and acetoacetyl-CoA while allowing accumulation of the

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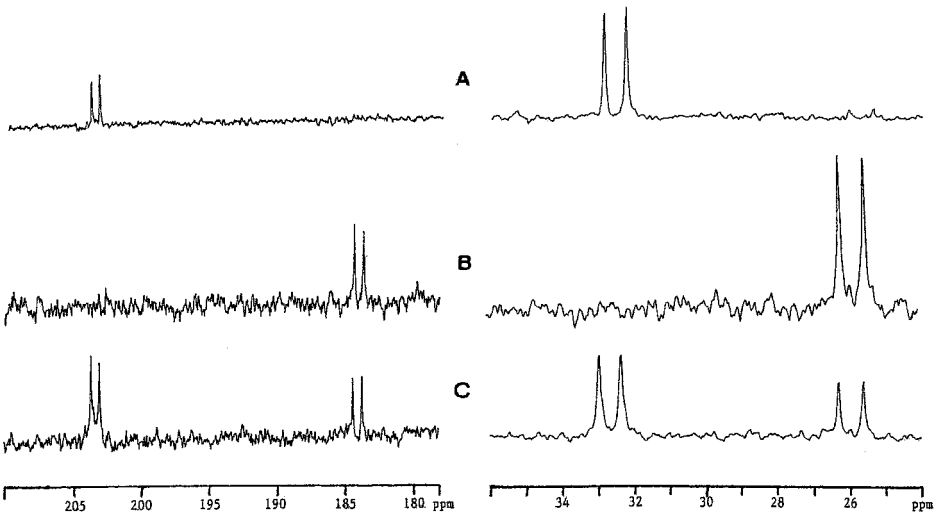

Figure 2. [1,2-^{13}C]Acetyl-CoA binding to HMG-CoA synthase and C378G *Z. ramigera* β-ketothiolase. Downfield (left panel: 180−210 ppm) and upfield (right panel: 24−36 ppm, referenced to TMS) regions of ^{13}C NMR spectra, measured as described for Figure 1, are shown. Samples, buffered with 20 mM potassium phosphate (pH 7.0) and containing 20% D_2O for internal lock, contained: (A) 2.0 mM [1,2-^{13}C]acetyl-CoA; (B) 2.0 mM [1,2-^{13}C]acetyl-CoA and 1.0 mM HMG-CoA synthase; (C) 2.0 mM [1,2-^{13}C]acetyl-CoA and 1.0 mM C378G thiolase (concentration based on subunit molecular weight of 40 000). Spectra A−C were obtained with 1500, 2500, and 2200 transients, respectively; a 2500 transient experiment requires slightly less than 2 h acquisition time. In spectrum B, only trace amounts of free metabolite are detectable due to its depletion during the course of spectral acquisition; this is attributable to HMG-CoA synthase's intrinsic acyl-CoA hydrolase activity.

covalent acetyl-*S*-enzyme.[10] These traits suggested the utility of C378G thiolase for our investigation. A series of experiments was performed using the doubly labeled substrate [1,2-^{13}C]acetyl-CoA. Upon comparison of ^{13}C spectra in the absence or presence of HMG-CoA synthase, upfield shifts of the signals attributable to both C1 (left panels; $\Delta\delta = 20$ ppm) and C2 (right panels; $\Delta\delta = 7$ ppm) of acetyl-*S*-enzyme are observed (Figure 2A, 2B). A similar experiment with C378G *Zoogloea ramigera* thiolase demonstrates upfield ^{13}C chemical shifts (C1: $\Delta\delta = 20$ ppm, C2: $\Delta\delta = 7$ ppm; Figure 2C) comparable to those observed with HMG-CoA synthase, indicating that the covalent reaction intermediates provide a self-consistent explanation for these data.

The 20-ppm upfield shift observed for the acetyl C1 represents a large effect and probably reflects multiple contributions. One possibility (D. Vinarov, preliminary observations) is that acetyl-*S*-enzyme formation involves transient production of a species in which C1 is linked to both the sulfur atoms of CoASH and C129. The tetrahedral carbon which would result may be expected to resonate around 100 ppm.[5,6] Rapid exchange between such a 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 in the observed signal. However, experiments with the model compound *N*,*S*-diacetylcysteamine suggest that a significant part of the effect may be due to a change in dielectric constant when the acetyl group is transferred from Coenzyme A in aqueous solution to a hydrophobic active site in the protein. Natural abundance ^{13}C spectra of *N*,*S*-diacetylcysteamine in water or benzene indicate that, regardless of whether the acetyl carboxyl exists in thioester or amide linkage, a substantial (8 ppm) upfield shift is observed in the low dielectric constant solvent. Thus, in the enzyme experiment, while there must be solvent access to the active site to permit substrate delivery, after enzyme acylation and CoASH release, a conformational change in the protein may result in sequestration of the reactive intermediate in a hydrophobic environment where abortive thioester hydrolysis is minimized.

There are mechanistic similarities between the reactions catalyzed by HMG-CoA synthase, β-ketothiolase, and β-ketoacyl-ACP synthase. Moreover, at a structural level, both β-ketothiolase and β-ketoacyl-ACP synthase exhibit a similar protein fold.[11] In the context of our model compound results it may be significant that, for β-ketoacyl-ACP synthase, the environment of the acylated active site cysteine has been characterized as predominantly hydrophobic. The structural and mechanistic similarities between these lipogenic enzymes provide a perspective that may guide future work on identification of the other factors which contribute to the unusual chemical environment of acyl-S-enzyme intermediates that each of these proteins produces.

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