Biochemistry

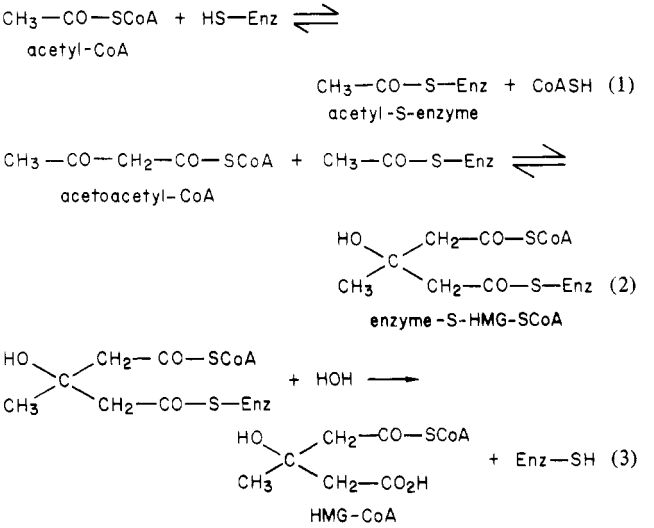
Volume 18, Number 3 February 6, 1979

3-Hydroxy-3-methylglutaryl Coenzyme A Synthase: Use of a Spin-Labeled Probe to Study Acyl Coenzyme A Binding†

Henry M. Miziorko,* M. Daniel Lane, and Stuart W. Weidman

ABSTRACT: R·CoA (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA), a spin-labeled acyl-CoA, binds tightly (K_D = 79 μM) and stoichiometrically with respect to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase active sites. Specificity in R·CoA binding is further suggested by kinetic data which show that R·CoA is a competitive inhibitor (K_i = 108 μM) with respect to acetyl-CoA. The components of the electron spin resonance (ESR) spectrum due to enzyme-bound R·CoA indicate strong immobilization of the radical; a rotational correlation time of approximately 5×10^{-8} s is estimated. Binding of R·CoA to the enzyme results in a tenfold enhancement of its effect on the longitudinal relaxation rate of water protons, which is also consistent with a strongly immobilized radical. Invariance of the tenfold enhancement with varying saturation of enzyme with R·CoA and linearity in a Scatchard plot of binding data suggest that R·CoA binds to identical, independent enzyme subunits. ESR data indicate that bound R·CoA can be displaced by each of the enzyme substrates or products. This observation is compatible with the postulate that there is one CoA binding pocket per active site. The R·CoA–enzyme complex was titrated with acetoacetyl-CoA and the displacement of R·CoA was followed by measuring the longitudinal relaxation rate of water protons. The data demonstrate that this substrate binds very tightly, even when forming an abortive complex by occupying the acetyl-CoA site. Additional titrations suggest that enzyme binds HMG-CoA and acetyl-CoA tightly but has considerably lower affinity for CoASH.

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[1] and CoASH. Previous studies on the hepatic mitochondrial enzyme (Miziorko et al., 1975, 1976; Miziorko & Lane, 1977) support a three-step sequence (reactions 1–3) as the description of the overall reaction. The existence of the acetyl-S-enzyme and enzyme–S-HMG-CoA intermediates predicted by this scheme has been demonstrated. Moreover, these intermediates are kinetically competent to participate in the overall reaction (Miziorko & Lane, 1977). Detailed studies of the enzyme's interaction with substrates and products would yield useful information, facilitating both further testing of the three-step reaction sequence and also an evaluation of the importance of various acyl-CoA derivatives in regulating the activity of this ketogenic enzyme. The affinity of liver synthase for acetoacetyl-CoA is reportedly very high (K_m < 5 μM) and, therefore, signal-to-noise limitations in the spectrophotometric and radioisotopic assays preclude a standard kinetic analysis (Reed et al., 1975). The enzyme's ability to slowly catalyze an abortive hydrolysis of acyl-CoA (Miziorko et al., 1975) rules out the possibility of using conventional equilibrium



† From the Department of Biochemistry, the Medical College of Wisconsin, Milwaukee, Wisconsin 53226 (H.M.M.), the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (M.D.L.), and the Departments of Preventive Medicine and Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri (S.W.W.). *Received July 19, 1978.* Supported in part by U.S. Public Health Service Grants AM-21491 (H.M.M.), AM-14575 (M.D.L.), and AM-13351, NHLI Contract 72-2906-L, and National Science Foundation Grant PCM 74-21871 (S.W.W.).

[1] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; R·CoA, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA; CoASH, coenzyme A; PRR, proton relaxation rate; EPR, electron paramagnetic resonance; ESR, electron spin resonance.

0006-2960/79/0418-0399$01.00/0

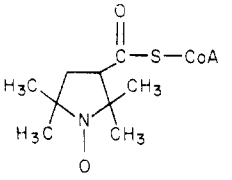
dialysis techniques to acquire binding data. Thus, a technique is required which facilitates a more rapid estimate of acyl-CoA binding before a detailed investigation of the interaction between HMG-CoA synthase with tight binding substrates and products can be attempted.

R·CoA (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl-CoA) proved to be useful in probing the active site of citrate synthase, which catalyzes a condensation reaction analogous to the HMG-CoA synthase catalyzed reaction (Weidman et al., 1973). Thus, it seemed logical to explore the possibilities that R·CoA would serve as a suitable probe for the latter enzyme and that EPR techniques would be useful for rapid estimates of enzyme bound acyl-CoA. This report demonstrates that R·CoA interacts with avian liver HMG-CoA synthase in a highly specific manner, facilitating the study of the binding of individual substrates and products to this enzyme. Analysis of the EPR and NMR data obtained with the R·CoA–enzyme complex permits estimation of the relative immobilization of enzyme-bound R·CoA, evaluation of the specificity of the CoA binding site, and a prediction of relative substrate and product affinities and of multiple tight binding modes for acetoacetyl-CoA. A preliminary account of this work has appeared (Miziorko & Weidman, 1978).

Experimental Section

Materials

Homogeneous mitochondrial HMG-CoA synthase was prepared by the method of Reed et al. (1975). Citrate synthase was purchased from Boehringer-Mannheim. R·CoA (I) was synthesized and purified as described by Weidman et al.



I

(1973). CoASH was purchased from P-L Biochemicals. The acyl-CoA compounds used in PRR titrations were prepared by the method of Simon & Shemin (1953) using the appropriate acid anhydride, except in the case of the acetoacetyl-CoA preparation, where diketene was used. HMG anhydride was synthesized by the method of Goldfarb & Pitot (1971). All other reagents were of the highest purity commercially available.

Methods

HMG-CoA synthase was assayed as described by Reed et al. (1975). Methods for the measurement of synthase catalyzed hydrolysis of acetyl-CoA have also been described (Miziorko et al., 1975). R·CoA concentration is determined by the procedure of Weidman et al. (1973).

EPR Measurements. The EPR spectra of 0.030–0.050-mL samples containing R·CoA were measured in quartz capillaries or micro flat cells using Varian E-4 or E-109 EPR spectrophotometers. The binding of spin label to HMG-CoA synthase was typically monitored by comparing the peak-to-peak amplitude of the high-field line of a sample containing R·CoA and enzyme to the amplitude observed with a solution containing an equal concentration of R·CoA in buffer (Weidman et al., 1973). The spectrum of enzyme-bound R·CoA was observed by increasing instrumental gain and modulation amplitude as indicated.

PRR Measurements. The longitudinal relaxation rate of water protons was measured at 24.3 MHz using an NMR Specialties PS60W pulsed NMR spectrometer as previously described (Mildvan & Engle, 1972). Calculation of observed enhancement (ϵ^*) and binary enhancement (ϵ_b) was performed using the approach of Mildvan & Weiner (1969).

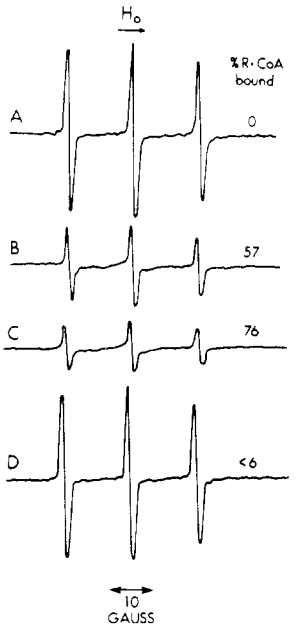


FIGURE 1: EPR spectra of R·CoA in the presence of variable concentrations of HMG-CoA synthase sites. R·CoA, 182 μM, in 20 mM potassium phosphate buffer, pH 7.0, was brought to a concentration of 0 (A), 211 (B), or 364 μM (C) HMG-CoA synthase sites. The displacement of enzyme-bound R·CoA shown in D was effected by bringing sample B to a final concentration of 250 μM acetyl-CoA or 250 μM acetoacetyl-CoA, or by bringing sample C to a final concentration of 1 mM HMG-CoA or 1.8 mM CoASH. Spectra were recorded under identical conditions of gain and modulation amplitude (1.0 G), designed to optimize display of the signal due to free R·CoA. Temperature was 23 °C.

Results and Discussion

EPR, PRR, and Kinetic Studies on the Binding of R·CoA to HMG-CoA Synthase. Inspection of the EPR spectra of R·CoA solutions containing varying concentrations of HMG-CoA synthase reveals a decrease in the amplitudes of the three nitroxide spectral lines with increasing enzyme site concentration (Figures 1A–C). In contrast to the results reported for R·CoA binding to citrate synthase (Weidman et al., 1973), approximately equal estimates for R·CoA binding to HMG-CoA synthase were obtained by comparing amplitudes of either the high- or low-field lines of sample spectra to the corresponding spectral lines observed with a control solution of the spin label. This observation suggests that bound radical is strongly immobilized and that there is little contribution to the amplitude of low- and high-field peaks from the signal due to the bound species. Thus, the amplitudes of the high- or low-field lines are useful parameters for quantitatively estimating the binding of R·CoA to enzyme (Weiner, 1969). Enzyme was titrated with various concentrations of R·CoA, covering a saturation range from 10 to 80% (calculated assuming 53 000 daltons per HMG-CoA synthase active site (Reed et al., 1975). When the binding data are displayed in a Scatchard plot (Figure 2) and subjected to linear regression analysis, the solid theoretical line, shown fit to the data, is obtained. The theoretical fit indicates a K_D = 79 μM for the enzyme–R·CoA complex and a stoichiometry of 1.02 mol of R·CoA bound per mol of enzyme monomer. The linearity of the Scatchard display suggests that R·CoA binds to identical, independent subunits.

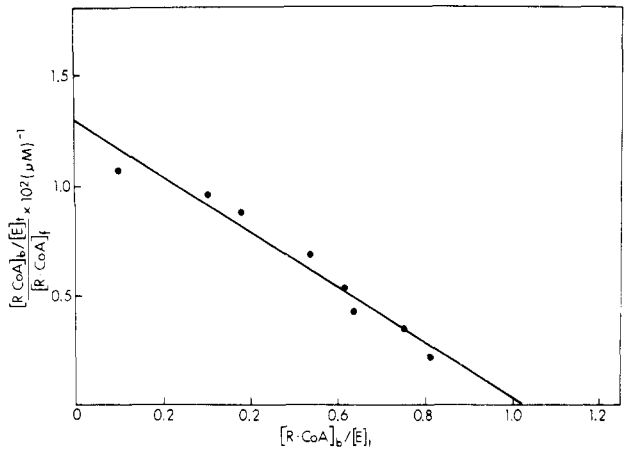


FIGURE 2: Scatchard plot of the EPR data for R·CoA binding to HMG-CoA synthase. Linear regression analysis yields the solid line shown fit to the data and indicates that 1.0 R·CoA binds per HMG-CoA synthase active site with a K_D = 79 ± 9 μM.

Specificity in the binding of R·CoA to enzyme is indicated not only by a stoichiometry of one label per active site but also by the observation that R·CoA is a competitive inhibitor with respect to acetyl-CoA. To avoid complications in analyses of the kinetic data, acetoacetyl-CoA was omitted from the reaction mixtures in this experiment and the inhibition by R·CoA of synthase catalyzed hydrolysis of acetyl-CoA (Miziorko et al., 1975) was measured. At specified times after the initiation of the reaction, aliquots are removed from the incubation mix and the remaining [^{14}C]acetyl-CoA is rapidly converted to [^{14}C]citrate by large amounts of citrate synthase and oxaloacetate. Samples are then acidified and heated to dryness, volatilizing [^{14}C]acetic acid produced by the synthase catalyzed hydrolysis and leaving [^{14}C]citrate. The depletion of acid-stable radioactivity is a measure of hydrolysis. When rates of hydrolysis of acetyl-CoA at varying concentrations of R·CoA are displayed in a Lineweaver–Burk plot (Figure 3), the competitive nature of R·CoA inhibition is clear. A K_i = 108 μM is determined, which agrees reasonably well with the physically measured K_D. Thus, the spin label appears to bind at the acetyl-CoA site.

Occupancy of the acetyl-CoA site raises the question of whether R·CoA merely binds to enzyme or actually acylates HMG-CoA synthase. Using procedures previously devised to trap [^{14}C]acyl-S-enzyme (Miziorko et al., 1975; Miziorko & Lane, 1977), it is not possible to demonstrate formation of a covalent R·S-enzyme adduct. It is possible that the adduct forms but is labile to the trichloroacetic acid precipitation procedure. However, the technique produces normal results with a companion sample of [^{14}C]acetyl-S-enzyme. Control experiments show no loss of EPR signal when R·CoA is incubated in trichloroacetic acid under conditions comparable to those used in the trapping experiment. In experiments in which spin label was displaced from enzyme by the addition of substrates or products (Figure 1D), the EPR spectrum showed high-field and center peak amplitudes in a ratio approaching 0.80. Such a ratio would be expected if the displaced label was in the form of the CoA thioester rather than the free acid, which exhibits a spectrum with high-field and center peaks in a ratio of 0.95. Thus, the displacement experiments also suggest that R·CoA binds noncovalently at the acetyl-CoA site. Such an observation is not surprising, since it has been demonstrated that acetoacetyl-CoA, which binds at the acetyl-CoA site when it functions as a substrate inhibitor, fails to acylate HMG-CoA synthase (Miziorko & Lane, 1977).

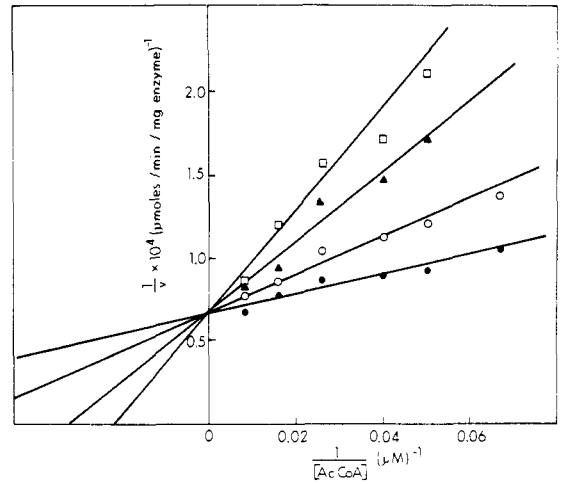


FIGURE 3: Kinetic determination of the inhibitor constant for R·CoA. Reaction mixtures contained potassium phosphate, pH 7.0 (20 mM), EDTA (0.2 mM), HMG-CoA synthase (22 μg), and [1-^{14}C]-acetyl-CoA (5400 cpm/nmol), as indicated, in a total volume of 0.2 mL. R·CoA levels were: 0 (●), 125 (○), 250 (▲), or 375 μM (□). Temperature was 30 °C. The reaction was started by addition of enzyme to the buffered samples; 0.06-mL aliquots were withdrawn at 15-min intervals and incubated for 15 min in 0.1 M Tris-Cl, pH 8.2, with 0.4 mM oxaloacetate and 20 μg of citrate synthase. Incubation mixes were acidified with an equal volume of 6 N HCl and heated to dryness. Acid-stable radioactivity due to [^{14}C]citrate was used as a measure of nonhydrolyzed [^{14}C]acetyl-CoA in the original samples. Analysis of the data indicates K_i = 108 ± 23 μM.

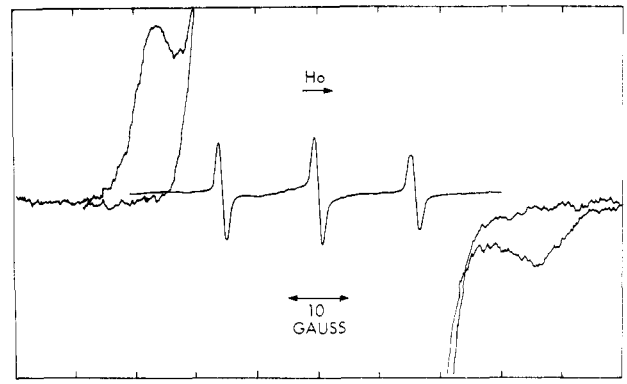


FIGURE 4: EPR spectrum of R·CoA bound to HMG-CoA synthase. The sample contained 220 μM HMG-CoA synthase sites and 103 μM R·CoA in 20 mM potassium phosphate buffer, pH 7.0. The high- and low-field peaks due to immobilized radical are observable at high gain and modulation amplitude (6.3 G). The central portion of the spectrum is obscured by the large signal due to residual unbound R·CoA. The high gain, high modulation amplitude spectrum showing no sign of the high- and low-field peaks due to immobilized radical is obtained by displacing R·CoA with a CoA-containing ligand (cf. Figure 1D). A spectrum of the original sample, recorded at 21-fold lower gain and at a 1.0-G modulation amplitude, is shown to indicate the position of the signal due to free R·CoA. Temperature was 23 °C.

While the enzyme-bound R· moiety is not covalently attached to enzyme, it is, nevertheless, strongly immobilized. When instrumental gain and modulation amplitude are increased, the EPR spectrum displayed in Figure 4 is observed. The central portion of the signal due to enzyme-bound R·CoA is obscured by the large signal from residual free spin label. However, the low- and high-field extremes of the bound R·CoA spectrum are clearly visible with a 60-G separation between the two peaks, placing them beyond the region where there is signal from the high- and low-field peaks due to free R·CoA. Comparison with spectra reported by Hsia & Piette (1969) suggests that the bound spin label has an approximate ro-

Table I: Binary Enhancement (ϵ_b) Calculated at Varying Saturation of HMG-CoA Synthase with R·CoA[a]

$[E\text{-}R\cdot CoA]/[E]_{tot}$	ϵ_b
0.10	9.0
0.54	9.6
0.63	10.7
0.75	10.9
0.81	11.0
	$\bar{\epsilon}_b = 10.2 \pm 0.9$

[a] Solutions containing 0.22 mM HMG-CoA synthase sites in 20 mM potassium phosphate buffer, pH 7.0, were brought to R·CoA concentrations ranging from 0 to 0.52 mM. The longitudinal relaxation times (T_1) of water protons in these samples and in samples containing comparable concentrations of R·CoA in buffer were measured at 24.3 MHz as described by Mildvan & Engle (1972). ϵ_b was calculated using eq 4. Distribution of R·CoA between free and bound forms was determined by EPR measurements on the same samples used for T_1 experiments. Temperature was 22 °C.

tational correlation time of $2\text{–}7 \times 10^{-8}$ s, much longer than the 10^{-10} s expected for free R·CoA (Weidman et al., 1973). If the rigid limit EPR spectrum of R·CoA is assumed to be similar to that published for 2,2,5,5-tetramethyl-3-carboxamidopyrrolidinyl-1-oxyl (Stone et al., 1965), then the observed high-field peak of the immobilized R·CoA spectrum falls 1.5 G short of the rigid limit. According to the treatment of McCalley et al. (1972), this indicates a rotational correlation time of 5×10^{-8} s for enzyme-bound R·CoA, supporting the approximate estimate listed above. Thus, the rotational correlation time for the bound R· moiety approaches the correlation time of 10^{-7} s calculated for the HMG-CoA synthase dimer using the Stokes–Einstein equation and assuming a spherical protein. It seems, then, that little mobility is left to the acyl group of the CoA derivative which binds at the acetyl-CoA site. Such limited mobility of the acyl group would be advantageous if it is necessary to momentarily bring the acetyl and acetoacetyl moieties into close proximity in order for the enzyme to catalyze the deprotonation of the methyl group of acetyl-S-enzyme and the condensation reaction.

Immobilization of enzyme-bound R·CoA is also indicated by the enhancement of the effect of the radical on the water proton relaxation rate. The longitudinal molar relaxivity of free R·CoA in 20 mM potassium phosphate buffer (pH 7.0), measured at 24.3 MHz and 21 °C, was found to be 313 ± 13 M^{-1} s^{-1}, in reasonable agreement with the value recently reported by Fung et al. (1976). Binding of R·CoA to HMG-CoA synthase enhances the relaxivity considerably. The enhancement parameter ϵ_b, due to bound R·CoA, can be calculated from the experimentally observed ϵ^* by correcting for the concentration of free R·CoA in solution (as determined by ESR measurements made on the PRR samples) using the formula described by Mildvan & Weiner (1969).

$$\epsilon^* = \frac{(R\cdot CoA)_f}{(R\cdot CoA)_t}\epsilon_f + \frac{(R\cdot CoA)_b}{(R\cdot CoA)_t}\epsilon_b$$

In the above equation, the subscripts f, b, and t refer to free, bound, and total R·CoA; ϵ_f is defined as 1.0. The calculated values of ϵ_b (Table I) yield an average of 10.2, which is the highest ϵ_b reported for a binary enzyme–R·CoA complex (Weidman et al., 1973; Fung et al., 1976). While ϵ_b is a function not only of the degree of immobilization of bound R·CoA but also of the access of water to the radical, the magnitude of ϵ_b is consistent with strong immobilization of the nitroxide. Moreover, the observed binary enhancement can be entirely accounted for by invoking the change in correlation time for free vs. bound R·CoA. No change in the number of water ligands need be postulated, although a small effect cannot be ruled out. When ϵ_b is examined as a function of the saturation of enzyme with R·CoA, very little systematic dependence is observed. This observation is consistent with the postulate of independent, identical synthase monomers which was advanced on the basis of linearity in the Scatchard plot of binding data.

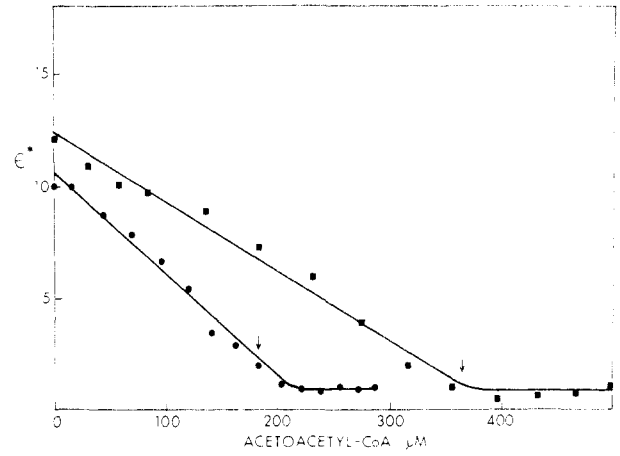


FIGURE 5: Titration of R·CoA–HMG-CoA synthase with acetoacetyl-CoA. A plot of the enhancement (ϵ^*) of the water proton relaxation rate, due to enzyme-bound R·CoA, vs. acetoacetyl-CoA concentration is shown. Titrations were performed on samples containing 91 μM R·CoA and either 182 μM (●) or 364 μM (■) enzyme sites. Temperature was 23 °C. Arrows indicate [acetoacetyl-CoA]/[enzyme sites] = 1.0.

Interaction of CoA Derivatives with Spin-Labeled HMG-CoA Synthase. If R·CoA binds at the acetyl-CoA site, as suggested by its ability to function as a competitive inhibitor (Figure 3), displacement of spin label by other metabolites which can occupy the acetyl-CoA site is predicted. Upon mixing of an enzyme-bound R·CoA sample with either substrate, i.e., acetyl-CoA or acetoacetyl-CoA, or with either product, i.e., HMG-CoA or CoASH, there is a displacement of bound R·CoA, as indicated by an increase in the EPR signal amplitude due to free R·CoA (Figure 1D) and by a disappearance of the spectral peaks due to bound R·CoA (Figure 4). Comparison of the spectra (Figure 1D) with the nonenzymic control (Figure 1A) indicates that the interaction of each of these metabolites with enzyme can cause complete displacement of R·CoA. The ability of acetyl-CoA and HMG-CoA to displace enzyme-bound R·CoA is reasonable if the synthase exhibits a ping-pong pattern for substrate addition and product release (Cleland, 1963). However, complete displacement of spin label upon addition of acetoacetyl-CoA or CoASH was not entirely anticipated. The initial EPR results suggested that PRR experiments in which the synthase-R·CoA complex is titrated with CoASH or various acyl-CoAs would provide additional information on the interaction of these compounds with enzyme.

When acetoacetyl-CoA is used for the titration, a linear decrease in ϵ^* is observed until a stoichiometry for acetoacetyl-CoA/enzyme = 1 is reached (Figure 5). At this concentration of acetoacetyl-CoA, the titration is essentially complete, with an end point that closely approaches the value of $\epsilon^* = 1$ that would be predicted for a solution of unbound R·CoA. Performing titrations with varying ratios of [R·CoA]/[synthase] (e.g., 1:2, 1:4; cf. Figure 5) confirms that the decrease in ϵ^* is complete when [acetoacetyl-CoA]/[synthase] = 1.0 instead of when [acetoacetyl-CoA] equals [R·CoA-enzyme]. Assuming that the three-step reaction sequence (eq 1–3) accurately describes enzymatic synthesis

of HMG-CoA, productive binding of acetoacetyl-CoA requires occupancy of the acetyl-CoA site. The stoichiometry measured in the PRR titrations indicates that acetoacetyl-CoA is not behaving as a second substrate, but is acting as a substrate inhibitor, binding at the acetyl-CoA site and displacing R·CoA. The possibility that acetoacetyl-CoA stimulates hydrolysis of a spin-labeled acyl-S-enzyme to form the unbound free acid form of R· appears unlikely on the basis of stoichiometry as well as line shape of the EPR spectrum in the displacement experiments. Although acetoacetyl-CoA is binding in an abortive mode, the PRR titrations indicate that such binding is very tight. The linear decrease in ϵ^* and the sharp break in the titration curves suggest that the affinity for acetoacetyl-CoA in this binding mode is appreciably higher than that of enzyme for R·CoA (K_D = 79 μM). While the curves in Figure 5 yield useful information on stoichiometry, they are not well suited for the extraction of precise K_D values for the enzyme–acetoacetyl-CoA interaction. However, a K_D in the range of 10^{-6} to 10^{-5} M for the abortive binding of acetoacetyl-CoA would be compatible with the PRR data and would suggest that productive binding of this metabolite as the second substrate would be a very high affinity process. This prediction is consistent with the observations that standard kinetic approaches are insufficiently sensitive to directly measure a K_m for acetoacetyl-CoA (Reed et al., 1975).

The multiple binding modes of acetoacetyl-CoA are easily explained by the postulate that HMG-CoA synthase contains only one CoA binding pocket per active site (Miziorko & Lane, 1977). If acetoacetyl-CoA binds to unacetylated synthase with reasonably high affinity (cf. eq 1), substrate inhibition would be expected because acetyl-CoA binding is hindered. Such substrate inhibition has, in fact, been reported at low levels (>20 μM) of this metabolite (Reed et al., 1975). Upon acetylation of an active site cysteine (Miziorko et al., 1975) and release of CoASH, the enzyme is in a conformational state which is characterized by a very high affinity for the acetoacetyl-CoA. Such binding is compatible with an indirect estimate of $K_m = 10^{-9}$ M for acetoacetyl-CoA (Fritz & Lee, 1974). Apparent differences between K_m and K_i for acetoacetyl-CoA suggest that changes in the acyl binding pocket, or in a single CoA binding site, or in both occur upon acetylation of enzyme. At present, the data are insufficient to distinguish which of these factors results in the enzyme's enhanced affinity for acetoacetyl-CoA in the productive binding mode.

The hypothesis that there is only one CoA binding pocket per active site is also compatible with the observed displacement of R·CoA by other CoA derivatives. Titrations of enzyme-bound R·CoA with either HMG-CoA, acetyl-CoA, or CoASH produce displacement of bound R-CoA at low concentrations (<300 μM) of titrant (data not shown). This observation suggests that all of these compounds are capable of binding at a similar site. While all of these derivatives displace R·CoA, the concentrations of titrant required to decrease ϵ^* to a value approaching 1 differ appreciably and suggest that enzyme has high affinity for acetoacetyl-CoA and HMG-CoA, a slightly weaker affinity for acetyl-CoA, and a poor affinity for CoASH. The kinetic observation that CoASH poorly inhibits synthase activity (Reed et al., 1975) is consistent with the PRR data. In titrations with these CoA derivatives, the PRR approach again provides information on stoichiometry and also indicates that no ternary complexes (R·CoA–enzyme–CoA derivative) are detectable. The enzyme's affinity for acetyl-CoA and HMG-CoA is much greater than the affinity for R·CoA, precluding the use of PRR for determining quantitative K_D values. However, the PRR titrations do confirm the original EPR observations that both substrates and both products will displace R·CoA from synthase. In addition they suggest that, under physiological conditions, competition for the acetyl-CoA site will occur. Thus, synthase activity in vivo may vary markedly with differing [acyl-CoA]/[CoA] levels.

Experiments are in progress to evaluate the suitability of acetoacetyl-CoA analogues, which have decreased affinity for synthase, for use as substrates in more detailed studies aimed at testing whether the hepatic mitochondrial enzyme exhibits the expected ping-pong kinetic pattern. Such an investigation may provide estimates of binding constants for substrates and products that can be compared with K_D values which will be obtained by a continued exploitation of physical techniques. The combined approach will further test the current model for HMG-CoA synthesis and determine more precisely whether varying acyl-CoA and CoA concentrations may modulate hepatic HMG-CoA synthase activity.

Acknowledgments

We are grateful to Dr. Albert S. Mildvan for making available the E-4 EPR spectrometer and PS-60W pulsed NMR spectrometer and to the National Biomedical ESR Center (Milwaukee, WI) for making available the E-109 EPR spectrometer used in these studies.

References

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104.
Fritz, I. B., & Lee, L. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F., & Tygstrup, N., Eds.) p 224, Academic Press, New York.
Fung, C. H., Gupta, R. K., & Mildvan, A. S. (1976) *Biochemistry 15*, 85.
Goldfarb, S., & Pitot, H. C. (1971) *J. Lipid Res. 12*, 512.
Hsia, J. C., & Piette, L. H. (1969) *Arch. Biochem. Biophys. 129*, 296.
McCalley, R. C., Shimshick, E. J., & McConnell, H. M. (1972) *Chem. Phys. Lett. 13*, 115.
Mildvan, A. S., & Weiner, H. (1969) *Biochemistry 8*, 552.
Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26C*, 654.
Miziorko, H. M., & Lane, M. D. (1977) *J. Biol. Chem. 252*, 1414.
Miziorko, H. M., & Weidman, S. W. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1805.
Miziorko, H. M., Clinkenbeard, K. D., Reed, W. D., & Lane, M. D. (1975) *J. Biol. Chem. 250*, 5768.
Miziorko, H. M., Shortle, D., & Lane, M. D. (1976) *Biochem. Biophys. Res. Commun. 69*, 92.
Reed, W. D., Clinkenbeard, K. D., & Lane, M. D. (1975) *J. Biol. Chem. 250*, 3117.
Simon, E. J., & Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.
Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010.
Weidman, S. W., Drysdale, G. R., & Mildvan, A. S. (1973) *Biochemistry 12*, 1874.
Weiner, H. (1969) *Biochemistry 8*, 526.