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Proton Transfer from Acetyl-coenzyme A Catalyzed by Thiolase I from Porcine Heart†

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ABSTRACT: The synthesis of acetoacetyl-coenzyme A (acetoacetyl-CoA) from two molecules of acetyl-CoA catalyzed by thiolase I from porcine heart exhibits only a small primary deuterium isotope effect on V_{max} (1.3 ± 0.1) and V_{max}/K_m (1.1 ± 0.1). With [^{3}H]acetyl-CoA as substrate, there is no change in the radiospecific activity of the reisolated acetyl-CoA during the approach to equilibrium, consistent with the lack of an isotope effect on V_{max}/K_m for the reaction. The radiospecific activity of acetoacetyl-CoA, which is trapped by rapid reduction with NADH in the presence of 3-hydroxyacyl-CoA dehydrogenase, is 0.86 that of the starting acetyl-CoA throughout the approach to equilibrium, requiring a small intramolecular discrimination against tritium in the condensation half-reaction. In the reverse direction, cleavage of [2-^{3}H]acetoacetyl-CoA, all (>97%) of the tritium initially present at C-2 is found in the product, acetyl-CoA, with no significant exchange into water. These results suggest that the rate-determining step of the condensation half-reaction is a step between proton transfer from carbon and release of acetoacetyl-CoA from the enzyme. The release of acetyl-CoA from the enzyme is fast compared to condensation and release of acetoacetyl-CoA, and the base responsible for proton abstraction from acetyl-CoA is not in protonic equilibrium with bulk solution. The general base catalyst responsible for the proton transfer step may be physically or kinetically shielded from bulk solvent. The possible catalytic consequences of proton transfer from carbon in a region restricted from solvent are discussed.

The major mechanism of biological carbon–carbon bond formation involves the enzyme-catalyzed condensation of carbanion-equivalent species (enolate, enol, imine, and enamine) with carbonyl electrophiles. For many of these enzymatic reactions, activation of a hydrogen α to a carbonyl group is achieved through imine formation or involvement of metal ion catalysts, e.g., the aldolases (Hoerecker et al., 1972), and certain sugar isomerases (Rose, 1975). For other enzyme reactions such as those involving carbon–carbon bond formation to the α carbon of acetyl-coenzyme A (AcCoA),[1] there seem to be no such mechanisms of activation of the proton α to the thioester. It is frequently assumed that the proton α to a thioester is sufficiently acidic that activation toward abstraction as a proton is chemically and kinetically trivial (Eggerer & Klette, 1967). This is not so. Estimates of the acidity of acetyl thioesters place the pK_a for the α hydrogen at approximately 20 ± 2 (Fedor & Gray, 1976), similar to the pK_a estimated for acetone (Pearson & Dillon, 1953).

The reaction shown in eq 1 might occur at the active site of an enzyme catalyzing the enolization of AcCoA. If the

$$E\text{–}B^{-}\cdot HCH_2COSCoA \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E\text{–}BH\cdot{}^{-}CH_2COSCoA \quad (1)$$

pK of BH is taken as 7 and the pK of AcCoA is taken as 20, the equilibrium constant for the reaction of eq 1 $K_{eq} = k_f/k_r = 10^{-14}$. If k_r is as fast as possible (10^{13} s^{-1}, a bond vibration) (Jencks, 1980), then with $K = 10^{-14}$, $k_f = 0.1$ s^{-1}. However, the k_{cat} values for many enzymes catalyzing condensation reactions of this type are on the order of 0.1–10^2 s^{-1} (Thibblin & Jencks, 1979). Thus, even if catalysis of proton transfer has evolved above and beyond the normal criteria of "perfection" (Albery & Knowles, 1976), the proton abstraction would still be 10–10^3 times too slow to keep up with enzyme turnover. If k_r is actually less than 10^{13} s^{-1}, the value calculated for k_f would likewise have to be reduced by the same amount. Since this calculation assumes that the enzyme is as catalytically effective as possible, there are only two ways to account for the observed k_{cat} for the enzyme—either the equilibrium constant for carbanion formation is larger than 10^{-14} by at least 10–10^3 or a carbanion intermediate does not exist (Thibblin & Jencks, 1979), and the reaction must proceed by a concerted mechanism or through an enol intermediate.

We were interested in determining the kinetic importance of proton transfer from AcCoA in the overall reaction catalyzed by thiolase I from porcine heart (Staack et al., 1978).

$$2CH_3COSCoA \rightleftharpoons CH_3COCH_2COSCoA + CoASH \quad (2)$$

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[1] Abbreviations used: AcCoA, acetyl-coenzyme A; AcAcCoA, acetoacetyl-coenzyme A; NADH, reduced nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography.

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This reaction is similar in type to those catalyzed by malate synthase (Eggerer & Klette, 1967) and citrate synthase (Eggerer, 1965); however, thiolase provides an opportunity to study the proton transfer reaction in both directions since the reaction (eq 2) can be made to proceed in both directions (Gilbert et al., 1981).

Experimental Procedures

Materials. Coenzyme A and NADH were obtained from P-L Biochemicals, and 3-hydroxyacyl-CoA dehydrogenase was from Sigma. All ^3H- and ^{14}C-labeled materials were from ICN. AcAcCoA and AcCoA were prepared as described previously (Gilbert et al., 1981). Hydroxybutyryl-CoA was synthesized from CoA and β-butyrolactone (Lynen, 1969). Both AcAcCoA and AcCoA preparations were greater than 95% pure by HPLC. The concentration of free CoA was less than 1% in these preparations. All other organic reagents were purified by recrystallization or distillation prior to use. Glass-distilled, deionized water was used for all experiments.

Methods. The concentrations of AcCoA and AcAcCoA, protein concentrations, and thiolase activity were determined as described previously (Gilbert et al., 1981). All spectrophotometric measurements were made by using a Varian 634 double beam recording spectrophotometer with the cell compartment maintained at 25.0 ± 0.1 °C. A Corning Model 130 pH meter with a Radiometer GK2321 combination electrode calibrated by the two-point method was used for pH measurements. HPLC was performed by using a 0.45 × 25 cm reversed-phase octadecylsilane chromatography column (5 μm, ODS from Custom LC, Inc.). Elution was accomplished isocratically with 0.1 M monobasic potassium phosphate containing 5–20% (v/v) methanol depending upon the compounds to be separated. Flow rates were generally 1.0–1.5 mL/min, and detection was by UV absorbance at 260 nm. This system was capable of base-line resolution of CoA, AcCoA, and 3-hydroxybutyryl-CoA.

Liquid scintillation counting was performed with a Beckman LS-333 scintillation spectrometer. Counting efficiencies were determined by adding an internal [^{14}C]toluene or [^3H]toluene standard (from ICN) to representative samples. In double labeling experiments, the ^3H counts per minute were corrected for spillover from the ^{14}C channel. Ratios of ^3H to ^{14}C were chosen to ensure that the spillover correction was small relative to the actual ^3H counts per minute observed.

Enzyme Preparation. Thiolase I was purified from porcine heart by the method of Staack et al. (1978). The enzyme preparation was greater than 95% homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using approximately 100 μg of protein per gel. The specific activity of the purified thiolase I was found to be 9.8–10 units/mg when protein concentrations were estimated by the method of Bradford (1976). The kinetic constants determined for our preparation (Gilbert et al., 1981) agree well with those reported for thiolase I by Staack et al. (1978) and differ significantly from the kinetic constants of thiolase II from porcine heart (Staack et al., 1978).

Primary Deuterium Isotope Effects. The AcCoA and [$^2H_3$]AcCoA were prepared from the same stock solution of CoASH by the reaction of the CoASH with a small excess (10%) of acetic anhydride or [$^2H_6$]acetic anhydride in argon-degassed 0.1 M potassium bicarbonate, pH 7.5–8.0. The appropriate concentration of AcCoA or [$^2H_3$]AcCoA was added to a 1.0-mL cuvette containing 0.1 mM NADH and 6.9 units/mL 3-hydroxyacyl-CoA dehydrogenase in 0.1 M potassium phosphate buffer, pH 6.82, 25.0 °C. The absorbance at 340 nm due to NADH was monitored for 1–3 min to check for any background oxidation of the NADH (none was observed). The reaction was initiated by adding thiolase at a final concentration of 4.0 milliunits/mL. The initial velocity of NADH oxidation was observed at 340 nm (ϵ = 6.23 × 10^3 M^{-1} cm^{-1}) (Louie & Kaplan, 1970). Measurements were performed in duplicate or triplicate, alternating between AcCoA and [$^2H_3$]AcCoA. Doubling the concentration of 3-hydroxyacyl-CoA dehydrogenase or halving the concentration of NADH had no effect on the initial velocity.

Tritium Selection Effects in the Direction of AcAcCoA Synthesis. The reaction was initiated by the addition of [^3H,^{14}C]AcCoA (0.04–0.05 mM final concentration) ^3H = 82 Ci/mol ^{14}C = 31 Ci/mol) to 0.1 mL of 0.1 M potassium phosphate buffer, pH 6.82, 25.0 °C, containing 3.2 units/mL dialyzed thiolase, 27 units/mL 3-hydroxyacyl-CoA dehydrogenase, and 10 mM NADH. After the appropriate time of incubation, 0.04 mL of this solution was injected directly into the HPLC. The water, AcCoA, and 3-hydroxybutyryl-CoA were separated by elution with 0.1 M monobasic potassium phosphate containing 10% (v/v) methanol. The flow rate was 1.0 mL/min, and detection was by UV absorbance at 260 nm. Fractions of 1.0 mL were collected. Each fraction was counted in entirety in 10 mL of Hydroscint dioxane-based scintillation cocktail (ICN). The initial $^3H/^{14}C$ ratio of the AcCoA was determined by analysis of a control sample containing no thiolase. Prior to use, the [^3H,^{14}C]AcCoA was lyophylized in 1 mM HCl to reduce the amount of labeled acetate in the sample to levels that did not interfere with the observation of label appearance into water. The labeled AcCoA used in these experiments was greater than 95% radiochemically pure as determined by HPLC. Identical results were obtained with labeled AcCoA which had been purified before the experiment by HPLC. The 3-hydroxybutyryl-CoA formed enzymatically coeluted with 3-hydroxybutyryl-CoA synthesized by a different method (Lynen, 1969).

Trapping of [2-^3H]AcAcCoA by Thiolase and Excess CoASH. A solution of [2-^3H]AcAcCoA was prepared by incubating AcAcCoA (8.1 mM) with [^3H]OH (0.1 Ci/mL) in 0.1 M potassium phosphate buffer, pH 6.82, for 10 min at ambient temperature. Small aliquots of this stock solution ($[AcAcCoA]_{final}$ = 0.16 mM) were added to a solution containing excess CoASH (1.4 mM) and different concentrations of thiolase (0–4.3 units/mL). After incubation for 1.0 min (sufficient time for complete conversion to AcCoA under these conditions), the solution was diluted to 1.0 mL with distilled water, and a small aliquot of [^{14}C]AcCoA (~1000 cpm) was added to correct for any partial recovery of the AcCoA. The entire solution was applied to a small column (0.5 × 0.5 cm) of DEAE-Sephacel which had been equilibrated with distilled water. After the column was repeatedly washed with distilled water until the eluate was free of ^3H (≤200 cpm), the AcCoA was eluted by washing the column with two 0.5-mL aliquots of 0.3 M potassium phosphate, pH 6.8. With this procedure, greater than 75% of the total AcCoA applied to the column could be recovered (based on recovery of applied [^{14}C]AcCoA). If the AcCoA was eluted from the column by continuous washing with 0.3 M potassium phosphate and all fractions were collected and counted, the recovery was greater than 90%. A control experiment in which the [^3H]AcAcCoA was trapped by reduction with 10 mM NADH in the presence of 70 units/mL 3-hydroxyacyl-CoA dehydrogenase was performed in a similar fashion.

Solvent Isotope Effects on the Cleavage of AcAcCoA. Solutions of AcAcCoA (26 μM) and CoASH (50 μM) were prepared in 0.1 M potassium phosphate buffer, 50% base in

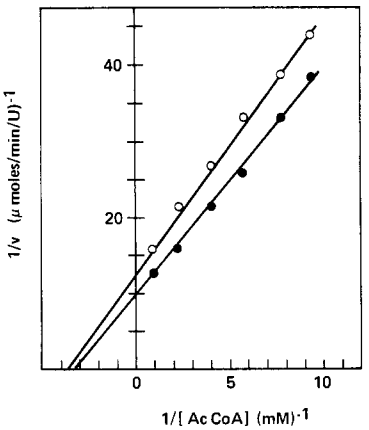


FIGURE 1: Effect of deuterium substitution on the V_{max} and V_{max}/K_m values for AcCoA. Experiments were performed at 25.0 °C, at pH 6.82, in 0.1 M potassium phosphate buffer. The thiolase-catalyzed synthesis of AcAcCoA was coupled to the reduction of AcAcCoA catalyzed by 3-hydroxyacyl-CoA dehydrogenase. All assays contained 0.004 unit/mL dialyzed thiolase, 0.1 mM NADH, and 6.9 units/mL 3-hydroxyacyl-CoA dehydrogenase. (●) AcCoA; (○) [^{2}H]AcCoA.

water and deuterium oxide. The reaction was initiated by the addition of a small aliquot of thiolase. The initial velocity of AcAcCoA cleavage was monitored by following the appearance of the thioester absorbance of AcCoA (Eggerer & Klette, 1967) at 232 nm ($\epsilon = 4.1 \times 10^3$ M^{-1} cm^{-1}). In these experiments, the solvent was greater than 95 mol % deuterium.

Results

By coupling the thiolase-catalyzed synthesis of AcAcCoA to the NADH-dependent reduction of AcAcCoA catalyzed by 3-hydroxyacyl-CoA dehydrogenase

$$2CH_3COSCoA \rightleftharpoons CH_3COCH_2COSCoA + CoASH \quad (3)$$

$$CH_3COCH_2COSCoA + NADH + H^+ \rightleftharpoons CH_3CH(OH)CH_2COSCoA + NAD^+ \quad (4)$$

it is possible to study the thiolase reaction in the thermodynamically unfavorable (Gilbert et al., 1981) direction.

The effect of deuterium substitution at the α carbon of AcCoA on the initial velocity kinetics of the thiolase reaction in the direction of AcAcCoA synthesis is shown in Figure 1. The deuterium isotope effect on V_{max}, $(V_{max})_H/(V_{max})_D = 1.3 \pm 0.1$, is small but detectable. The deuterium isotope effect on V_{max}/K_{Ac}, $(V_{max}/K_{Ac})_H/(V_{max}/K_{Ac})_D = 1.1 \pm 0.1$, is also small and probably not significantly different from 1.0.

As the synthesis of AcAcCoA from AcCoA approaches equilibrium, the fate of a tritium atom initially present at the α carbon of AcCoA can be determined by separating the AcCoA, 3-hydroxybutyryl-CoA, and water by HPLC and determining the amount of tritium label in each species. A typical separation is shown in Figure 2. In this particular sample, the reaction has been allowed to continue approximately 5 times longer than required to reach equilibrium to check for alternate fates of substrate and product. As can be seen from Figure 2, greater than 90% of the original ^{3}H label is found in water, AcCoA, or 3-hydroxybutyryl-CoA. Only a small amount of label (<10%) is found in a peak eluting later than hydroxybutyryl-CoA (probably crotonyl-CoA) even after a comparatively long incubation. At shorter incubation times, greater than 95% of the total label is in the three analyzed species.

In order to accurately follow changes in the specific activity of the ^{3}H label, a double label procedure was utilized by employing [^{3}H,^{14}C]AcCoA as the substrate. An initially surprising result was that the ^{3}H/^{14}C ratio was not constant

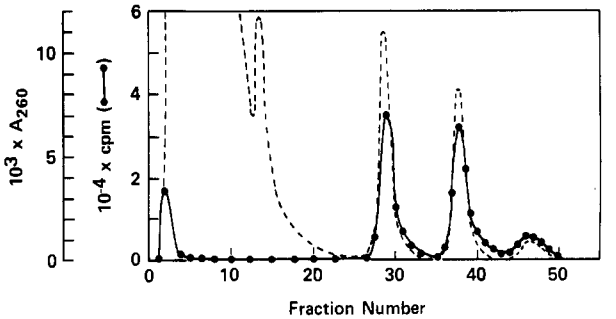


FIGURE 2: Separation of AcCoA and 3-hydroxybutyryl-CoA by HPLC. The separation was performed on a solution of [^{3}H,^{14}C]-AcCoA (initial concentration 0.05 mM) which had been incubated 17 min with thiolase (3.2 units/mL), 3-hydroxyacyl-CoA dehydrogenase (27 units/mL), and NADH (10 mM) at pH 6.82 in 0.1 M potassium phosphate buffer, 25.0 °C. Chromatographic conditions: column, 5 μm octadecylsilane reversed phase; solvent, 0.1 M monobasic potassium phosphate with 10% (v/v) methanol; flow rate, 1.5 mL/min; detection, absorbance at 260 nm, 0.02 AUFS; fraction size, 1.5 mL. (●) ^{3}H cpm; (---) absorbance at 260 nm. The first radioactive peak eluted is [^{3}H]OH, the second is AcCoA, the third is 3-hydroxybutyryl-CoA, and the last is probably crotonyl-CoA which is observed only upon prolonged incubation.

across the AcCoA or hydroxybutyryl-CoA peaks eluted from the HPLC. Several additional experiments have established that this phenomenon most likely results from an isotope effect on the HPLC separation. (1) Rechromatography of the front third of the AcCoA peak produces a similar variation in the ^{3}H/^{14}C ratio. (2) A sample of doubly labeled AcCoA incubated with thiolase and 2-mercaptoethanol under conditions where acetyl transfer from AcCoA to 2-mercaptoethanol occurs almost entirely by a thiolase-catalyzed reaction (Gilbert et al., 1981) shows no (<1%) residual radioactivity in the region where AcCoA elutes on HPLC. If the variation in the ^{3}H/^{14}C ratio were due to an impurity, this impurity must react with 2-mercaptoethanol in a thiolase-catalyzed reaction or a rapid nonenzymatic reaction. (3) The same variation in the ^{3}H/^{14}C ratio occurs across the 3-hydroxybutyryl-CoA peak and is noticed both early and late in the approach to equilibrium. Since the 3-hydroxybutyryl-CoA is derived from AcCoA by two consecutive enzyme-catalyzed reactions, it seems very unlikely that the nonconstant ^{3}H/^{14}C ratio across both AcCoA and 3-hydroxybutyryl-CoA peaks is due to the presence of impurities. In order to avoid complications due to possible isotope effects on the HPLC separation, the ^{3}H/^{14}C ratios for AcCoA and 3-hydroxybutyryl-CoA were determined by collecting fractions over the entire peak, counting each fraction, and summing the ^{3}H and ^{14}C counts per minute. The background levels of ^{3}H and ^{14}C counts per minute were sufficiently low (≤0.5%) compared to the observed counts per minute that counting multiple samples had no significant effect on the precision of the results.

When [^{3}H,^{14}C]AcCoA is incubated for various times with thiolase, NADH, and 3-hydroxyacyl-CoA dehydrogenase, the ^{3}H/^{14}C ratios for AcCoA and 3-hydroxybutyryl-CoA are found to be invariant over the entire approach to equilibrium (Figure 3). The ^{3}H/^{14}C ratio of AcCoA remains constant and equal to the ^{3}H/^{14}C ratio of AcCoA at the beginning of the experiment. The ^{3}H/^{14}C ratio for 3-hydroxybutyryl-CoA is also constant over the entire experiment and is equal to 0.86 of the original ^{3}H/^{14}C ratio for AcCoA. The sum of the ^{14}C counts per minute in AcCoA and 3-hydroxybutyryl-CoA is constant (±5%) over the entire experiment, showing that the original carbon atoms of AcCoA are converted only to 3-hydroxybutyryl-CoA. The ^{3}H/^{14}C ratio of the sum of AcCoA and 3-hydroxybutyryl-CoA declines approximately 6% during

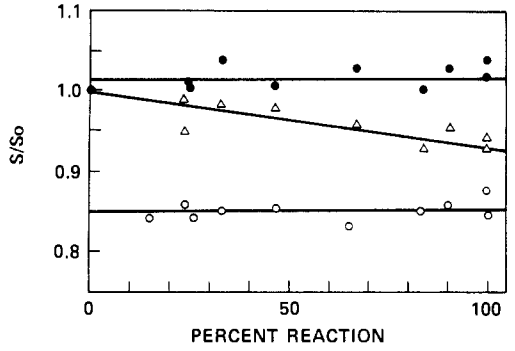


FIGURE 3: Radiospecific activity of AcCoA and 3-hydroxybutyryl-CoA as the thiolase reaction approaches equilibrium. The radiospecific activities, S/S_0, are reported as the ratio of the observed $^3H/^{14}C$ counts per minute to the initial $^3H/^{14}C$ counts per minute for AcCoA before the experiment. Reaction mixtures contained 0.05 mM [^3H,^{14}C]-AcCoA (^3H = 82 Ci/mol, ^{14}C = 31 Ci/mol), 2.7 units/mL dialyzed thiolase, 27 units/mL 3-hydroxyacyl-CoA dehydrogenase, and 10 mM NADH at pH 6.82 in 0.1 M potassium phosphate, 25.0 °C. The percent reaction is the percent of the final equilibrium position (58% 3-hydroxybutyryl-CoA by ^{14}C). (●) AcCoA; (○) 3-hydroxybutyryl-CoA; (△) sum of AcCoA and 3-hydroxybutyryl-CoA.

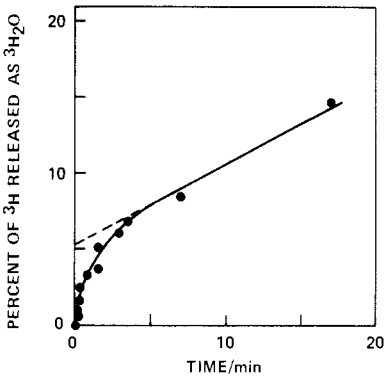


FIGURE 4: Appearance of tritium into water from AcCoA during the approach to the thiolase equilibrium. Experimental conditions are as described in the legend to Figure 3. ^3H release of 100% would correspond to 1.1×10^5 cpm.

the approach to equilibrium, showing that ^3H is being selectively lost from the acyl-CoA derivatives.

During the approach to equilibrium, there is a release of approximately 6% of the total ^3H into [^3H]OH which elutes from the HPLC with the solvent front. The increase in ^3H found in the solvent front is not accompanied by any increase in the ^{14}C so that the increased ^3H is not due to AcCoA hydrolysis. After attainment of equilibrium, there is a progressive washout of ^3H label into water which is due to substrate–product interconversion at equilibrium (Figure 4). Extrapolation of the slow ^3H washout back to zero time shows that 6% of the total ^3H label is lost upon reaching equilibrium (Figure 4).

The loss of ^3H to solvent is not due to non-enzyme-catalyzed washout of ^3H from the intermediate AcAcCoA, which is a relatively acidic carbon acid (pK = 9.0) (Gilbert et al., 1981). At saturating concentrations of NADH, the k_{cat}/K_m for the reduction of AcAcCoA by 3-hydroxyacyl-CoA dehydrogenase is 1.7×10^7 M^{-1} s^{-1} (Schifferdecker & Schulz, 1974; Noyes & Bradshaw, 1973). Due to the unfavorable equilibrium constant for AcAcCoA synthesis and the favorable equilibrium constant for AcAcCoA reduction, the steady-state concentration of AcAcCoA is vanishingly small. A concentration of 28 units/mL 3-hydroxyacyl-CoA dehydrogenase corresponds to a molar concentration of 2.4×10^{-6} M (Schifferdecker & Schulz, 1974; Noyes & Bradshaw, 1973). Since the concentration of 3-hydroxyacyl-CoA dehydrogenase is in excess of the concentration of AcAcCoA, the second-order rate constant for reduction of AcAcCoA will be given by k_{cat}/K_m. The pseudo-first-order rate constant for the reduction is then given by (k_{cat}/K_m)[3-hydroxyacyl-CoA dehydrogenase] and is equal to 41 s^{-1}. This is approximately 500 times faster than the rate constant for non-enzyme-catalyzed washout of ^3H from AcAcCoA under the conditions of our experiment.

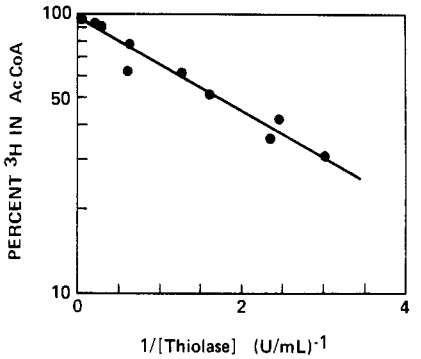


FIGURE 5: Trapping of tritium from [2-^3H]AcAcCoA in AcCoA as a function of thiolase concentration. Incubations contained 0.16 mM [2-^3H]AcAcCoA (3.1×10^5 cpm/μmol), 1.4 mM CoASH, and different concentrations of thiolase in 0.1 M potassium phosphate buffer, pH 6.82, 25.0 °C. The 100% point corresponds to the amount of ^3H found when the AcAcCoA was incubated with 10 mM NADH and 70 units/mL 3-hydroxyacyl-CoA dehydrogenase under the same conditions.

In the direction of AcAcCoA cleavage, the fate of a ^3H label specifically incorporated at C-2 of AcAcCoA can be determined. Since AcAcCoA is a relatively acidic carbon acid, the C-2 protons undergo a non-enzyme-catalyzed exchange with solvent. Thus, [2-^3H]AcAcCoA can be prepared by incubation of AcAcCoA with [^3H]OH. When [2-^3H]AcAcCoA is incubated with either high concentrations of thiolase (4.3 units/mL) and excess CoASH or 3-hydroxyacyl-CoA dehydrogenase (70 units/mL) and excess NADH, the isolated AcCoA or 3-hydroxybutyryl-CoA contains exactly the same amount of ^3H. Since the 3-hydroxyacyl-CoA dehydrogenase reaction does not involve any proton transfer to or from C-2 and is at least 10 times faster than the thiolase reaction under these conditions, it may be concluded that in the direction of AcAcCoA cleavage, the thiolase reaction proceeds with no significant washout of label originally at C-2 of AcAcCoA. Decreasing the concentration of thiolase leads to an increased amount of washout of the ^3H label in the isolated AcCoA. In the absence of enzyme, there is no significant label (<3%) isolated in the AcAcCoA.

By varying the concentration of thiolase, it is possible to observe a partitioning of the ^3H label between quantitative trapping as AcCoA at high thiolase concentrations and non-enzyme-catalyzed washout at low thiolase concentrations (Figure 5). By comparing the known half-life for the thiolase-catalyzed cleavage of AcAcCoA under these conditions to the extent of label trapped as AcCoA, it is possible to estimate that the rate constant for the non-enzyme-catalyzed washout of ^3H from C-2 of AcAcCoA is approximately 0.08 s^{-1} at pH 6.82 in 0.1 M potassium phosphate, 25.0 °C.

When the thiolase-catalyzed cleavage of AcAcCoA in the presence of CoASH is observed in 0.1 M potassium phosphate buffer, 50% base, 25.0 °C, in water and deuterium oxide, there is a small solvent isotope effect on the velocity of AcAcCoA disappearance. Under the conditions of this experiment, both AcAcCoA and CoASH are at saturating concentrations so that the observed solvent isotope effect is the effect on V_{max}. The value of the solvent isotope effect under these conditions is $k_{HOH}/k_{DOD} = 1.3 \pm 0.1$.

Discussion

Thiolase I from porcine heart catalyzes the reversible synthesis of AcAcCoA from two molecules of AcCoA. Although the cleavage of AcAcCoA is strongly favored thermodynamically (Gilbert et al., 1981), the synthesis reaction can be observed by coupling the synthesis of AcAcCoA to the NADH-dependent reduction of AcAcCoA to 3-hydroxybutyryl-CoA. Like other thiolases, the thiolase I catalyzed reaction proceeds through an acetyl enzyme intermediate (Gilbert et al., 1981).

$$\text{AcCoA} + \text{E} \rightleftharpoons \text{E–Ac} + \text{CoASH} \quad (5)$$

$$\text{E–Ac} + \text{AcCoA} \rightleftharpoons \text{AcAcCoA} + \text{E} \quad (6)$$

In the direction of AcAcCoA synthesis from two molecules of AcCoA, there is a small primary deuterium isotope effect on V_{max} ($k_H/k_D = 1.3 \pm 0.1$). This is consistent with the previous observation that for thiolase I the slowest half-reaction in the direction of AcAcCoA synthesis is the formation of the acetyl enzyme from AcCoA and free enzyme (eq 5) (Gilbert et al., 1981). This transacylation reaction would not be expected to exhibit a large secondary kinetic isotope effect (Kovach et al., 1980).

In the direction of AcAcCoA synthesis from AcCoA, there is no tritium selection isotope effect. During the conversion of [2-^{3}H]AcCoA to 3-hydroxybutyryl-CoA, the radiospecific activity of the reisolated AcCoA remains constant throughout the approach to equilibrium. A constant specific activity of AcCoA throughout the approach to equilibrium requires that the enzyme utilizes tritium-containing molecules at the same rate as nontritiated molecules. Since tritium selection isotope effects measure the isotope effect on V_{max}/K_m (Northrup, 1975), the lack of any discrimination against tritium is consistent with the lack of a significant primary deuterium isotope effect on V_{max}/K_m.

For molecules such as AcCoA in which the tritium label is statistically distributed between three hydrogens of a torsiosymmetric methyl group, it is possible to detect an intramolecular discrimination between abstraction of tritium compared to abstraction of a proton from the same methyl group (Chung & Walsh, 1976). When a tritium-containing molecule of AcCoA binds to the acetyl enzyme, there will be a 33% chance that the tritium atom will be species presented to the general base for abstraction. If the actual proton transfer step were rate limiting in this half-reaction, there would be a large discrimination against abstraction of tritium, and the specific activity at C-2 of the product 3-hydroxybutyryl-CoA would approach that of the starting AcCoA. The observation of an intramolecular tritium discrimination isotope effect is possible even if a step before proton transfer were rate determining in the condensation half-reaction. If methyl group rotation is very rapid and if proton transfer is slower than the following steps, hydrogen abstraction will be favored over tritium abstraction from the same methyl group. Although methyl group rotation in the enzyme–substrate complex is likely to be rapid, it is not known if this is a valid assumption. If methyl group rotation and release of AcCoA from the enzyme–substrate complex were both slow, the hydrogen isotope which would be abstracted would be determined by the orientation of the methyl group on binding of AcCoA. In this case, abstraction of tritium would be statistical, and the specific activity at C-2 of the hydroxybutyryl-CoA would approach 67% of the specific activity of the starting AcCoA. If a step following the proton transfer event were slow, the proton transfer will be at equilibrium, and a small net equilibrium tritium isotope effect and/or rapid washout of tritium from AcCoA into water would be observed.

The 3-hydroxybutyryl-CoA product of the reaction is derived from two molecules of AcCoA, so that tritium-bearing carbon atoms are located at both C-2 and C-4. The carbon atom at C-4 is derived from the acetyl enzyme intermediate (Gilbert et al., 1981; Gehring & Lynen, 1972) so that there should be no tritium isotope effect affecting the specific activity of tritium at this carbon (Kovach et al., 1980). If any intramolecular discrimination against tritium is observed, it must occur only at C-2. If there is an infinite discrimination against tritium abstraction at C-2, the specific activity of the isolated 3-hydroxybutyryl-CoA should be equal to that of the original AcCoA at both C-2 and C-4. If there is no discrimination against tritium abstraction at C-2, and tritium loss at C-2 is statistical (33%), the total specific activity of the isolated 3-hydroxybutyryl-CoA would be reduced 16.7% (33%/2) compared to that of the starting AcCoA since there are two tritium-bearing carbons in the hydroxybutyryl-CoA. In the absence of any tritium discrimination isotope effect, the specific activity of the 3-hydroxybutyryl-CoA should be 83.3% of the original specific activity of the starting AcCoA.

The actual intramolecular tritium discrimination isotope effect $(k_H/k_T)_I$ may be calculated from the relationship

$$(k_H/k_T)_I = \frac{[\text{Ac}]_0}{6([\text{Ac}]_0 - [\text{HB}]_0)} \quad (7)$$

where $[\text{Ac}]_0$ is the ratio of ^{3}H to ^{14}C of the AcCoA at zero time and $[\text{HB}]_0$ is the ratio of ^{3}H to ^{14}C for the isolated hydroxybutyryl-CoA extrapolated to zero time. (The extrapolation of [HB] to zero time is not necessary in this system since there is no overall tritium discrimination isotope effect, and the specific activities of AcCoA and hydroxybutyryl-CoA do not change with time.) The factor of 6 in the denominator of eq 7 is necessary to compensate for the fact that only one-sixth of the total number of hydrogens in the methyl groups of the two AcCoA substrate molecules actually contain tritium.

Since the specific activity of the isolated hydroxybutyryl-CoA is 0.86 ± 0.01 that of the initial AcCoA, the intramolecular tritium discrimination isotope effect for the reaction is 1.19 ± 0.09. A similar calculation can be made on the basis of the amount of ^{3}H released to water during the approach to equilibrium. At equilibrium, 5.5% of the total ^{3}H in the system has been released as [^{3}H]OH, and 58% of the total ^{14}C is contained in hydroxybutyryl-CoA. Of the molecules of hydroxybutyryl-CoA formed, 11% have lost tritium label, and the specific activity of the hydroxybutyryl-CoA should be 89% that of the initial AcCoA. Application of eq 7 to this result gives $(k_H/k_T)_I = 1.5$, in reasonable agreement with the value determined by measuring the specific activity of the hydroxybutyryl-CoA. (It should be recalled that in this particular system the difference between no isotope effect and an infinite isotope effect would only be a difference of 17% in the actual specific activity of the hydroxybutyryl-CoA.)

The small intramolecular tritium discrimination isotope effect shows that the abstraction of tritium from the α carbon of AcCoA in the condensation half-reaction is almost statistical. This requires either that in the condensation half-reaction (eq 6) proton transfer is fast relative to methyl group rotation and/or release of AcCoA from the enzyme or that the proton abstraction is at equilibrium and a step following proton transfer is rate limiting.

These two possibilities may be distinguished by examining the fate of a ^{3}H label initially at C-2 of AcAcCoA as the AcAcCoA is cleaved in the presence of CoASH to two molecules of AcCoA. The results show that in this direction all

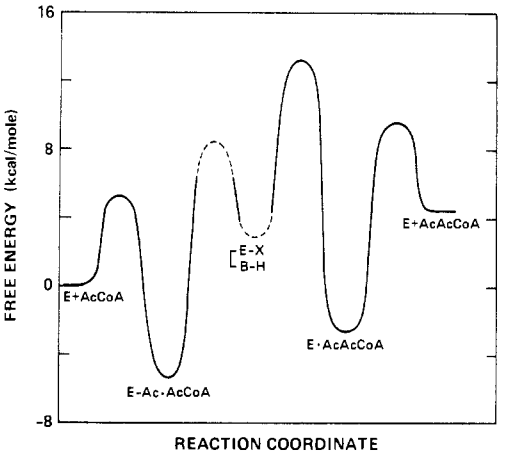


FIGURE 6: Reaction coordinate diagram for the condensation half-reaction catalyzed by thiolase I. Relative free energies shown were calculated from the values of k_{cat} and k_{cat}/K_m described by Gilbert et al. (1981), assuming that binding steps occur at a diffusion-controlled rate ($k = 10^8$ M^{-1} s^{-1}) and that the substrate K_m values reflect the thermodynamic dissociation constants. The E–X intermediate shown represents a species in which the proton abstracted from AcCoA resides on the general base catalyst (BH). Energy levels shown by dotted lines are arbitrary but qualitatively correct.

(>97%) of the label initially at C-2 of AcAcCoA appears in the product AcCoA. This means that every time the carbon–carbon bond is cleaved, and protonation (from solvent) occurs at AcCoA, the AcCoA is released from the enzyme. If proton abstraction followed by condensation and release of AcAcCoA (the reverse direction in this case were fast compared to release of AcCoA from the enzyme, a significant fraction of the tritium originally at C-2 of AcAcCoA would exchange with solvent through the reverse reaction. (Remember there is only a small discrimination against tritium abstraction in this direction.) Thus, the energy barrier for proton abstraction followed by condensation and release of AcAcCoA from the enzyme must be larger than the energy barrier for release of AcCoA. The highest energy transition state in the condensation half-reaction must occur between proton abstraction from AcCoA and release of AcAcCoA from the enzyme (Figure 6). The lack of a significant intramolecular discrimination against tritium abstraction does not result from slow release of the AcCoA from the enzyme or from immobilization of methyl group rotation.

In the direction of AcAcCoA cleavage, there is only a small solvent deuterium isotope effect on V_{max} ($k_{HOH}/k_{DOD} = 1.3$). Although solvent deuterium isotope effects may be complicated by the effects of deuterium oxide solvent on protein structure, a rather large solvent deuterium isotope effect might be expected if proton transfer to carbon were involved in the rate-determining transition state. Fedor & Gray (1976) have observed a large ($k_{H_2O}/k_{D_2O} \geq 6$) solvent isotope effect on the isomerization of vinylacetyl thioesters under conditions where general acid-catalyzed protonation on carbon is the rate-determining step. In the direction of AcAcCoA cleavage under V_{max} conditions where the ground state is some enzyme–substrate complex, the highest energy transition state between the predominant ground state and the release of AcCoA does not involve proton transfer to carbon.

For the condensation of the acetyl enzyme with AcCoA, proton transfer to or from carbon does not pose a kinetically significant energy barrier to the overall condensation half-reaction (Figure 6). In the direction of AcAcCoA synthesis, the proton transfer step must be faster than some subsequent step so that the proton transfer is at equilibrium. If the proton transfer step is at equilibrium, then it is reasonable to ask why there is no rapid enzyme-catalyzed exchange of the protons of AcCoA with solvent. The lack of a rapid exchange of the protons of AcCoA with solvent, even though the proton transfer step is actually at equilibrium on the enzyme, requires that the general base catalyst which abstracts the proton is not in protonic equilibrium with bulk solvent. The failure of the general base catalyst to equilibrate with bulk solvent could be the consequence of a kinetic or steric effect.

For a general acid catalyst of arbitrary pK 7, the rate constant for proton transfer to water may be estimated.

$$BH + HOH \underset{k_r}{\overset{k_f}{\rightleftharpoons}} B^- + H_3O^+ \qquad (8)$$

The equilibrium constant for the reaction of eq 8 is given by $k_f/k_r = K_a$ where k_f and k_r are defined as shown and K_a is the acid dissociation constant of BH. In the thermodynamically favorable direction, the rate constant for this reaction will be diffusion controlled (Eigen, 1964) so that $k_r = 10^{10}$ M^{-1} s^{-1}. The rate constant k_f for proton transfer from BH to water is given by $k_f = k_r K_a = 10^{10} \times 10^{-7} = 10^3$ s^{-1}. However, most experiments are performed in buffered solutions rather than pure water. In the presence of buffer species, the proton transfer from the protonated general base catalyst to a buffer species will be almost diffusion controlled ($k = 10^9$ M^{-1} s^{-1}) (Eigen, 1964). At a total buffer concentration of 0.1 M, the observed rate constant for proton transfer between the enzyme and buffer would be approximately 10^8 s^{-1}. If the net rate constant (Cleland, 1975) of the step following proton abstraction from AcCoA is much greater than 10^8 s^{-1}, the lifetime of enzyme-bound species which could undergo proton exchange with solvent would be sufficiently short that no exchange of protons with solvent would be observed. The lack of observable exchange between AcCoA and solvent could result from a kinetic effect even though the proton transfer step is at equilibrium and the base catalyst on the enzyme is in contact with bulk solvent (including buffer species).

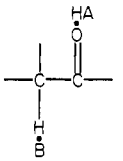
A slow equilibrium of the protonated general base catalyst with solvent could also result if the proton transfer between AcCoA and the general base catalyst occurs in a region of the active site not in physical contact with solvent or buffer species. Such a steric barrier would greatly decrease the rate constant for equilibration of a protonated general base with solvent protons, so that equilibration of protons between AcCoA and the medium would be slow even though the proton abstraction is at equilibrium on the enzyme.

Since the inability to detect rapid substrate–water proton exchange for thiolase and other enzymes catalyzing enolization of carbon acids (Eggerer, 1965; Eggerer & Klette, 1967; Stubbe et al., 1977; Bloch, 1970) may result from inaccessibility of water to the active site, the catalytic advantages of excluding bulk solvent from the active site of an enzyme catalyzing proton transfer from a carbon acid should be considered. Unlike proton transfer between Bronsted acids and bases (Eigen, 1964), proton transfer from a carbon acid to a Bronsted base may not occur through a bridging water molecule (Goodall & Long, 1968). The transition state for proton transfer to and from carbon acids appears to be significantly less highly solvated by water than the ground states (Gilbert, 1980). For the non-enzyme-catalyzed enolization of carbon acids, a significant fraction of the activation energy may be required to desolvate reactant species (Gilbert, 1980).

For proton transfer between a nitroalkane carbon acid and a carboxylate anion, changing the solvent from water to dimethyl sulfoxide increases the second-order rate constant for the reaction by approximately 10^5 in both directions (Keeffe et al., 1979). Although the equilibrium constant for the re-

action in dimethyl sulfoxide is similar to the equilibrium constant for the reaction in water, the transition state is stabilized (relative to the ground states) on transfer from water to dimethyl sulfoxide. If enzymes catalyzing proton transfer from carbon were to provide a means of selectively desolvating the ground-state reactants, the activation energy of the reaction could be reduced significantly, leading to a large rate acceleration.

If we consider the enolization of a carbon acid at the active site of an enzyme catalyst, it would be possible to accelerate the enolization by general base-catalyzed proton abstraction from carbon and/or general acid-catalyzed proton donation to the developing negative charge on oxygen.



(9)

Such bifunctional catalysis is detected in the non-enzyme-catalyzed enolization of acetone in aqueous solution as evidenced by the observation of a second-order dependence on buffer concentration (Hegarty & Jencks, 1975; Dawson & Spivey, 1930).

If such catalytic mechanisms are operative, an additional catalytic advantage may result from restricting access of solvent to the active site. If the general base catalyst (B, eq 9) were anionic (a carboxylate anion), decreasing the polarity of the environment and restricting access to bulk water would increase the effective pK of this general base and increase the rate constant for enolization. The pK_a of acetic acid increases dramatically when the solvent is changed from water to dimethyl sulfoxide (from 4.6 to 12) even though dimethyl sulfoxide is a more basic solvent of reasonably high dielectric constant (Clare et al., 1966). Similar effects may be envisioned for the general acid catalyst (HA, eq 9), but these effects should operate in the opposite direction, i.e., effecting a decrease in the pK of the acid catalyst. Thus, the general acid catalyst should be cationic (lysine or histidine). In this case, desolvation upon binding substrate would lead to a decrease in the pK of the general acid catalyst, increasing the rate constant for enolization.

It is interesting to note that the active-site base in triosephosphate isomerase (Hartman, 1971), mandelate racemase (Fee et al., 1974), glucose-6-phosphate isomerase (O'Connell & Rose, 1973), enolase (O'Connell & Rose, 1970), and 2-keto-3-deoxy-6-phosphonogluconate aldolase (Meloche, 1973) may be a carboxylate anion. The X-ray crystal structure of the triosephosphate isomerase–dihydroxyacetone phosphate complex (Alber et al., 1981) shows that an active-site glutamate residue occupies a position appropriate for the general base catalyst which abstracts the proton from the substrate dihydroxyacetone phosphate. The carbonyl group of the dihydroxyacetone phosphate is near a histidine residue, although it is not certain at this time if the histidine residue is actually close enough to serve as a general acid catalyst. In addition, the active site in the ES complex is significantly shielded from contact with bulk solvent.

It is not certain at the present time whether the proton abstraction catalyzed by thiolase (or any other enzyme catalyzing the enolization of carbon acids) leads to the formation of a carbanion intermediate. Although a free carbanion would be a very unstable intermediate, selective solvation and desolvation at the active site could provide enough stabilization to allow the existence of such an intermediate. Regardless of whether the reaction proceeds through a carbanion or enol intermediate or is concerted, solvation effects at the active site could make large contributions to catalysis.

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