Elementary Steps in the Reaction Mechanism of Chicken Liver Fatty Acid Synthase: Acetylation–Deacetylation†

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ABSTRACT: The kinetics of the reaction of acetyl coenzyme A (AcCoA) with fatty acid synthase has been studied with a modified quenched-flow technique in 0.1 M potassium phosphate (pH 7.0), 0.5 mM ethylenediaminetetraacetic acid, and 10% glycerol (w/v) at 23 °C. The kinetics of the deacetylation of the isolated acetylated enzyme by CoA also was studied. An overall mechanism consistent with the data is

$$\text{AcCoA} + \text{E} \overset{K_1}{\rightleftharpoons} \text{E·AcCoA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{EAc·CoA} \overset{K_3}{\rightleftharpoons} \text{EAc} + \text{CoA}$$

where E represents the enzyme. The equilibrium dissociation constants, K_1 and K_3, were estimated to be 85 and 70 μM, respectively, and the rate constants k_2 and k_{-2} are 43 and 103 s^{-1}, respectively. The maximum number of acetyl groups bound to the enzyme in terms of this mechanism is 3.8 (mol/mol). This mechanism also is consistent with the amount of acetylated enzyme formed during titrations of the enzyme and radioactive AcCoA with CoA. The spontaneous hydrolysis of the enzyme at 23 °C has a rate constant of 4.7×10^{-4} s^{-1}. The acetyl groups on the native enzyme are rapidly removed by hydroxylamine. However, 0.39 of the acetyl groups remains after treatment with hydroxylamine if the enzyme is first denatured in 4 M urea. This suggests that the acyl binding sites on the native enzyme are an unstable acetyl oxygen ester and an acetyl thio ester. Destruction of the thioesterase activity of the enzyme through chemical modification of the enzyme does not alter the rate of spontaneous hydrolysis of the acetyl-enzyme nor its reactivity toward hydroxylamine.

The fatty acid synthase from chicken liver is a multienzyme complex that catalyzes the synthesis of palmitic acid from acetyl-CoA (AcCoA),[1] malonyl-CoA, and NADPH. The synthesis is initiated by the acetyl group and involves the condensation of seven malonyl groups on to the growing chain. Each condensation reaction is followed by two reduction steps involving NADPH and an intermediate dehydration of an alcohol. The overall reaction is

$$\text{AcCoA} + 7\text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow 14\text{NADP}^+ + \text{palmitic acid} + 8\text{CoA} + 7\text{CO}_2 + 6\text{H}_2\text{O}$$

Early studies (Jacob et al., 1968; Chesterton et al., 1968; Phillips et al., 1970a,b) demonstrated that intermediates of fatty acid synthesis are covalently bound to the enzyme complex on three different sites: (1) 4′-phosphopantetheine, (2) cysteine, and (3) threonine or serine. Other evidence supporting multiple acylation sites has been reported recently (Cardon & Hammes, 1982, 1983). Reviews of the overall reaction mechanism are available (cf. Volpe & Vagelos, 1973; Bloch & Vance, 1977), and a structural model for the positioning of the catalytic sites on two identical polypeptide chains has been proposed (Stoops & Wakil, 1981). Steady-state kinetic studies of the pigeon liver (Nixon et al., 1968; Kumar et al., 1970; Katiyar et al., 1975) and the chicken liver (Cox & Hammes, 1983) enzymes have been reported, but kinetic studies of the individual steps in the reaction mechanism remain to be done.

In this paper, the acetylation of the enzyme by AcCoA and the deacetylation of the acetylated enzyme by CoA have been studied with a modified quenched-flow technique. The mechanism involves the relatively rapid binding of AcCoA or CoA to the enzyme followed by acetylation or deacetylation, respectively, on the enzyme. The rate constants for the acetylation and deacetylation have been determined. In addition, the rate of spontaneous hydrolysis of the acetylated enzyme has been determined, and the nature of the acetyl-enzyme linkage has been explored with hydroxylamine.

Materials and Methods

Chemicals. AcCoA, malonyl-CoA, CoA, NADPH (type X), dithiothreitol, tosyl fluoride, and poly(ethylene glycol) (M_r 8 000) were obtained from Sigma, and enzyme-grade ammonium sulfate was from Schwarz/Mann. 2,4-Dinitrophenyl acetate was from Eastman Kodak. [^{14}C]AcCoA and [^{3}H]-AcCoA were from Amersham and ICN, respectively. The purity of the radioactive compounds was found to be ≥95% as judged by thin-layer chromatography on Eastman Kodak 6065 cellulose with fluorescent indicator with the solvent system isobutyric acid–1 N ammonium hydroxide–0.1 M EDTA (50:30:1) and by high-performance liquid chromatography on a C-18 reverse-phase column eluted with acetonitrile–10 mM ammonium acetate, pH 4.5 (4.6:95.4). All other reagents were high-purity commercial grades, and all solutions were prepared with deionized distilled water.

Fatty Acid Synthase Preparation. Fatty acid synthase from chicken liver was prepared and assayed as previously described (Cardon & Hammes, 1982). The only change in the preparation was to precipitate the enzyme by bringing the solution to 15% saturation with poly(ethylene glycol) after the DE-52 column, rather than using an ammonium sulfate precipitation. All precipitations were performed under nitrogen. The specific activity of the enzyme was approximately 1.6 μmol of NADPH/(min mg) at 25 °C. Protein concentrations were determined by using an extinction coefficient for fatty acid synthase of 4.82×10^5 M^{-1} cm^{-1} (Hsu & Yun, 1970).

When required, the enzyme was modified with tosyl fluoride as previously described (Cardon & Hammes, 1982).

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[1] Abbreviations: AcCoA, acetyl coenzyme A; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid.

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Kinetics of Enzyme Acetylation and Deacetylation. The time course of the acetylation and deacetylation of fatty acid synthase was studied with a quenched-flow apparatus described elsewhere (Akiyama, 1980; Cash & Hess, 1981). Conventional usage of this instrument caused extensive denaturation of the enzyme; therefore a special operating procedure was devised. The apparatus was used as a continuous mode with two different incubation tubes (Teflon tubing, 4.9 and 16.8 cm long) with a large internal diameter (1.5 mm). The large diameter and the short length were essential for the prevention of enzyme denaturation. The sample delivery time was varied to obtain different reaction times by restriction of the nitrogen flow (25 psi) used to drive the syringe plungers. In a conventional operating mode the pistons are driven at the same constant speed for all experiments, and the length of the tubes is varied to achieve different reaction times. The disadvantage of the nonconventional usage is that the dead time, t_D, which is an instrumental characteristic related to the difference in the time required to mix the reagents and the time required to quench the mixed reagents, changes as the sample delivery time changes. The time of reaction, t_R, is related to the dead time and the time required to deliver a fixed volume of sample, t_S, by the relationship (Cash & Hess, 1981)

$$t_R = t_S(V_i/V) - t_D \quad (1)$$

where V_i is the volume of the incubation tubing, V is the total volume injected into the tubing (0.80 mL), and t_D is a function of t_S. The dependence of t_D on t_S was determined with a standard reaction, the hydrolysis of 0.4 mM dinitrophenyl acetate by 0.02–0.4 M NaOH in 10% glycerol (w/v) at 23 °C; the reaction was quenched with concentrated HCl (final pH 2.0; Cash & Hess, 1981). The dead time varied from −5 to −45 ms as the sample delivery time varied from about 0.1 to 2.0 s. In the kinetic experiments, delivery times longer than 1 s were not used, and t_R varied from about 20 to 500 ms with the two incubation tubes used. Points at t_R = 30 s were obtained by manual pushing of the syringes in the quenched-flow apparatus.

The retention of enzymatic activity was found to depend on the amount of volume injected, the diameter and length of the incubation tube, the glycerol concentration, and the temperature. The temperature was found to be an especially critical variable. The enzyme was almost completely denatured at 0 °C and approached its maximum stability around room temperature; the enzyme became increasingly unstable as the temperature was raised above ~25 °C. The type of mixer used (T or 8 jet tangential) and delivery time did not appreciably alter the enzyme stability. Conditions were optimized by trial and error procedures. The kinetic experiments were carried out at 23 °C with 10% (w/v) glycerol present. With the instrument conditions described above, <15% of the overall enzyme activity is lost.

The kinetics of enzyme acetylation were studied by mixing 0.4 mL of enzyme (0.25–0.45 μM) in 0.2 M potassium phosphate (pH 7.0), 1 mM EDTA, and 10% glycerol (w/v) with 0.4 mL of the desired concentration of radioactive AcCoA (15–25 cpm/pmol) in distilled deionized water and 10% glycerol (w/v) at 23 °C. After mixing in the quenched-flow apparatus, the reaction was quenched by addition of 0.4 mL of concentrated $HClO_4$ to a final concentration of about 0.56 M. The precipitate was stored on ice for 30–150 min and collected by filtration on a Whatman GF/F glass-fiber filter. The acetylated enzyme was found to be stable during this storage period. The filter was washed with five 4.5-mL aliquots of ice-cold 0.56 M $HClO_4$, followed by two washes with 4.5 mL of 95% ethanol at room temperature. The dry filter was incubated with 10 mL of aqueous counting scintillant (Amersham) for at least 12 h before the radioactivity was determined with a Beckman LS-255 scintillation counter. The background radioactivity was determined at each concentration of AcCoA by a control experiment in which the total concentration of AcCoA was increased 25–500-fold, but the amount of radioactivity remained the same. The radioactivity in excess of that calculated for the dilution of radioactivity was assumed to be due to nonspecific binding of AcCoA to the enzyme. Use of this background correction typically decreased the observed extent of binding by a few percent.

The kinetics of the spontaneous hydrolysis of acetylated enzyme was studied in the following manner. The enzyme was mixed with radioactive AcCoA to final concentrations of 0.2 μM enzyme and 60 μM AcCoA in about 3.5 mL of 0.2 M potassium phosphate (pH 7.0) and 1 mM EDTA. The solution was then passed through six 5-mL Sephadex G-50 (fine) centrifuge columns (Penefsky, 1977), and the solution was incubated at the desired temperature. At times between 4 min and 4 h, 200-μL aliquots were quenched with 1 mL of 0.56 M ice-cold perchloric acid. The precipitate was then assayed for radioactivity as described above.

The kinetics of the deacetylation of acetylated enzyme by CoA also was studied with the quenched-flow method at 23 °C. The acetylated enzyme was prepared by passing 0.45 mL of enzyme (~8 μM) through a 5.6-mL Sephadex G-50 column equilibrated with 60 μM radioactive AcCoA in 0.2 M potassium phosphate (pH 7.0) and 1 mM EDTA at 23 °C. The enzyme was eluted with the same solution, and the fraction containing acetylated enzyme was collected rapidly and passed through two 3-mL Sephadex G-50 centrifuge columns to remove the excess AcCoA. Control experiments indicated this procedure eliminated all detectable radioactivity that was not enzyme bound. The enzyme was then brought to a concentration of 0.3–0.4 μM in 0.2 M potassium phosphate (pH 7.0), 1 mM EDTA, and 10% glycerol (w/v). The enzyme was loaded in the quenched-flow apparatus and mixed with CoA in 10% glycerol. Approximately 8 min passed between the acetylation and the first mixing in the quenched-flow apparatus. The quenched-flow experiment was carried out exactly as described for the acetylation kinetics and takes about 10–15 min for one time course.

The concentrations of CoA and AcCoA were determined by using an extinction coefficient of 14 600 M^{-1} cm^{-1} at 260 nm (Dugan & Porter, 1970).

Titration of Enzyme–AcCoA with CoA. The amount of acetylated enzyme found at various concentrations of AcCoA and CoA was determined by mixing 60 or 120 μM radioactive AcCoA (40 cpm/pmol), 0.3 μM of enzyme, and 0–2.6 mM CoA in 0.2 M potassium phosphate (pH 7.0) and 1 mM EDTA. The total volume was 0.15 mL. The mixture was incubated for 15 s at room temperature after addition of the last component, the enzyme, and quenched with 1 mL of 0.56 M ice-cold $HClO_4$. The precipitate was analyzed for radioactivity as previously described.

Reaction of Acetylated Enzyme with NH_2OH. The reactivity of the acetyl-enzyme with NH_2 was determined under two sets of conditions. In one case, the radioactive acetylated enzyme was prepared as for the spontaneous hydrolysis experiment, and ~0.1 mL of ~0.2 μM acetylated enzyme in 0.2 M potassium phosphate (pH 7.0) and 1 mM EDTA was reacted with 10 μL of 2.0 M neutralized NH_2OH for ~15 min at 23 °C. The reaction mixture was quenched with 1 mL of 0.56 M ice-cold $HClO_4$, and the precipitate was analyzed for radioactivity as previously described. In the second case,

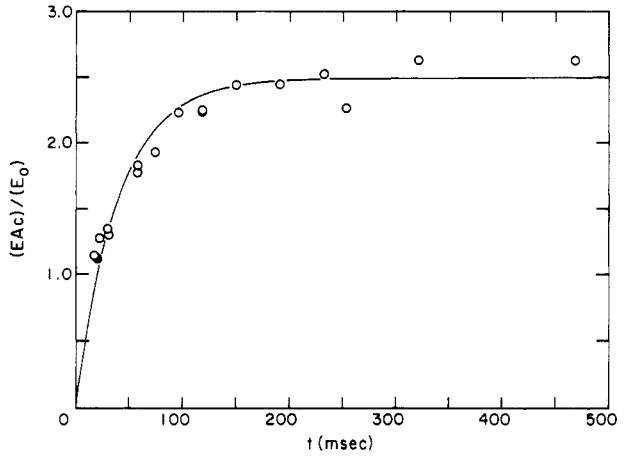

FIGURE 1: Plot of the amount of acetylated fatty acid synthase, (EAc), divided by the amount of total enzyme, (E_0), mol/mol, vs. time in 0.1 M potassium phosphate (pH 7.0), 0.5 mM EDTA, and 10% glycerol (w/v) at 23 °C. The concentrations of enzyme and [^{3}H]-AcCoA after being mixed in the quenched-flow apparatus were 0.17 and 28.6 μM, respectively. The curve is the best fit to eq 2; the best-fit parameters are A = 2.50 and k_{obsd} = 24.9 s^{-1}.

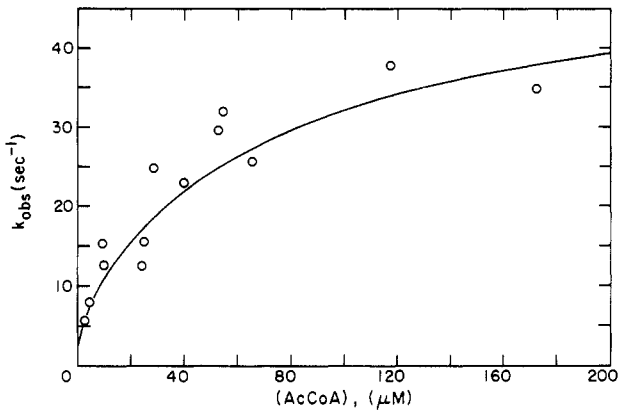

FIGURE 2: Plot of the rate constant for acetylation of fatty acid synthase by AcCoA, k_{obsd}, vs. the concentration of AcCoA. The experimental conditions were the same as in the legend to Figure 1. The enzyme concentration was 0.18 μM (±11%). The curve was calculated with eq 4 and the best-fit parameters obtained by simultaneously fitting the data in Figures 2 and 3 to eq 4 and 5. The best-fit parameters are K_1 = 85 μM, k_2 = 42.9 s^{-1}, and k_{-2}/K_3 = 7.7 μM^{-1} s^{-1}.

the acetylated enzyme was mixed with an equal volume of 8 M urea before reaction with NH_2OH and analysis for radioactivity.

Results

The rate of acetylation of fatty acid synthase by acetyl-CoA was studied over a range of AcCoA concentrations (2.8–172 μM) and an enzyme concentration, (E_0), of 0.15–0.21 μM in 0.1 M potassium phosphate (pH 7.0) and 0.5 mM EDTA at 23 °C. Under these conditions, the acetylation reaction followed first-order kinetics, and the time course was fit to the equation

$$(EAc)/(E_0) = A(1 - e^{-k_{obsd}t}) \quad (2)$$

by a nonlinear least-squares analysis. In this equation EAc represents the acetylated enzyme, E_0 is the total amount of enzyme, A and k_{obsd} are parameters, and t is the time. A typical time course and a curve calculated according to eq 2 are shown in Figure 1. The dependence of A and k_{obsd} on the total concentration of AcCoA is illustrated in Figures 2 and 3. The dependence of k_{obsd} on substrate concentration,

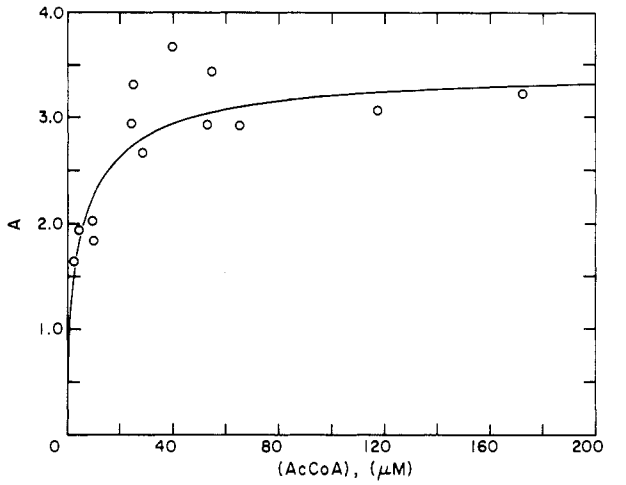

FIGURE 3: Plot of the maximum fraction of acetylated enzyme, A, as defined by eq 2 vs. the concentration of AcCoA. The experimental conditions were the same as in the legend to Figure 2. The curve was calculated with eq 5 and the best-fit parameters obtained by simultaneously fitting the data in Figures 2 and 3 to eq 4 and 5. The best-fit parameters are K_1 = 85 μM, k_2 = 42.9 s^{-1}, k_{-2}/K_3 = 7.7 μM^{-1} s^{-1}, and n = 3.84.

namely, the approach to a limiting value at high concentrations of AcCoA, suggests that the mechanism involves rapid formation of an enzyme–substrate complex, followed by the rate-determining acetylation. The decrease in A observed at low concentrations of AcCoA indicates that reversal of the acetylation occurs. A mechanism consistent with the data is

$$E + AcCoA \underset{}{\overset{K_1}{\rightleftharpoons}} E\cdot AcCoA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAc\cdot CoA \overset{K_3}{\rightleftharpoons} EAc + CoA \quad (3)$$

where the k_i's are rate constants and the K_i's are equilibrium dissociation constants. The reversal of the acetylation reaction would not be expected to follow first-order kinetics. However, because the reversal rate is relatively slow and does not contribute greatly to the overall rate, only first-order kinetics are observed. The apparent first-order rate constant (k_{obsd}) can be approximated by linearizing the rate equation for the reverse reaction in the neighborhood of equilibrium; furthermore because of the low enzyme concentration used, the concentration of the species EAc·CoA is negligible. For this case

$$k_{obsd} = \frac{k_2}{1 + K_1/(AcCoA)} + 2(k_{-2}/K_3)(EAc)_\infty \quad (4)$$

$$A = \frac{nk_2/[1 + K_1/(AcCoA)]}{(k_{-2}/K_3)(EAc)_\infty + k_2/[1 + K_1/(AcCoA)]} \quad (5)$$

where the subscript ∞ denotes the equilibrium concentration and n is the maximum number of acetyl groups that can bind to the enzyme. However, $(EAc)_\infty = A(E_0)$. In the kinetic experiments (E_0) = 0.18 μM ± 11% and, therefore, can be assumed to be constant. The data were fit to eq 4 and 5 simultaneously by a nonlinear least-squares analysis, after eq 5 was first solved for A with $(EAc)_\infty = A(E_0)$. The curves in Figures 2 and 3 have been calculated with eq 4 and 5 and the best-fit parameters n = 3.84 (±0.42), K_1 = 85 (±40) μM, k_2 = 42.9 (±5.9) s^{-1}, and k_{-2}/K_3 = 7.7 (±5.2) μM^{-1} s^{-1}. The values of n and k_2 are well-defined. However, the value of k_{-2}/K_3 can be considered only as a rough estimate since the near equilibrium approximation gives the maximum possible contribution of the reverse reaction. The fit of the data is rather insensitive to the value of K_1 so that the value of K_1 depends significantly on the extent of reversal. For example,

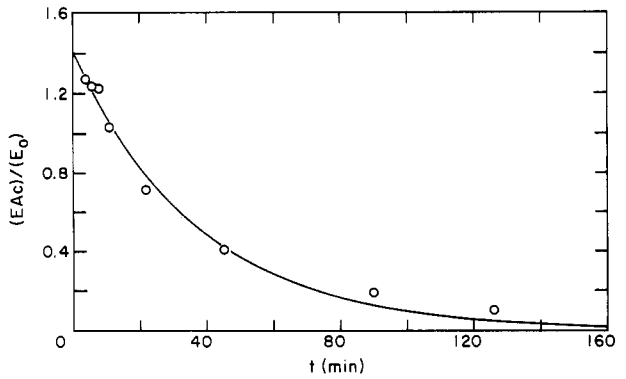

FIGURE 4: Plot of the amount of acetylated fatty acid synthase, (EAc), divided by the amount of enzyme, (E_0), mol/mol, vs. time in 0.2 M potassium phosphate (pH 7.0), 1 mM EDTA, and 0.2 μM ^{3}H-acetylated enzyme at 23 °C. The curve is the best fit to $(EAc)/(E_0) = A \exp(-kt)$; the best-fit parameters are $A = 1.43$ and $k = 4.5 \times 10^{-4}$ s^{-1}.

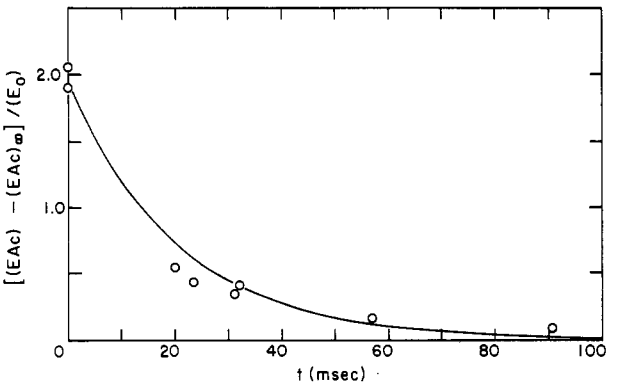

FIGURE 5: Plot of the amount of acetylated fatty acid synthase, (EAc), minus the amount at equilibrium, $(EAc)_\infty$, divided by the amount of total enzyme, (E_0), mol/mol, vs. time in 0.1 M potassium phosphate (pH 7.0), 0.5 mM EDTA, and 10% glycerol (w/v) at 23 °C. The final concentrations of ^{3}H-acetylated enzyme and CoA after being mixed in the quenched flow were 0.18 and 53.7 μM, respectively. The curve is the best fit to eq 6. The best-fit parameters are $A' = 1.98$ and $k'_{obsd} = 49.1$ s^{-1}.

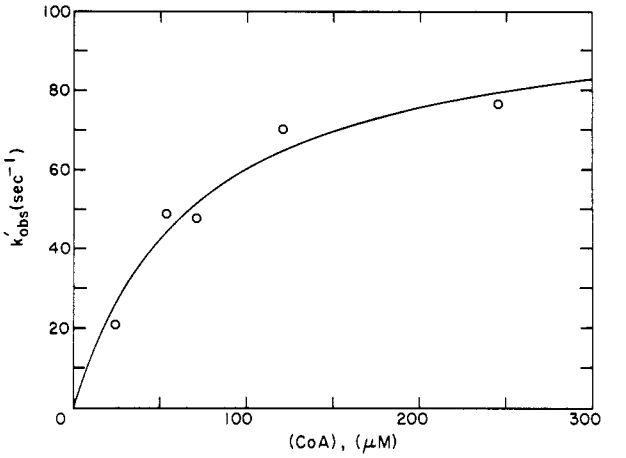

FIGURE 6: Plot of the rate constant for deacetylation of fatty acid synthase by CoA, k'_{obsd}, vs. the concentration of CoA. The experimental conditions were the same as in Figure 5; the concentration of enzyme was 0.165 μM (±11%). The curve is the best fit to eq 7; the best-fit parameters are $K_3 = 71$ μM, $k_{-2} = 103$ s^{-1}, and $k_2/K_1 \simeq 0$.

if k_{-2} is set equal to zero, the dependence of k_{obsd} on the AcCoA concentration is fit reasonably well, and $K_1 = 16$ μM.

The rate of hydrolysis of the acetyl group bound to the enzyme in the absence of CoA and AcCoA was measured under conditions similar to those used in studying the kinetics of enzyme acetylation by AcCoA. A typical time course is shown in Figure 4. The rate of hydrolysis followed first-order kinetics, and nonlinear least-squares analysis of the data gives a first-order rate constant of $(4.70 \pm 0.56) \times 10^{-4}$ s^{-1} at 23 °C (average of four determinations) and $(3.80 \pm 0.62) \times 10^{-5}$ s^{-1} at 0 °C (average of three determinations). The initial stoichiometry ranged from 1.0 to 2.5 acetyl/enzyme (mol/mol). This hydrolysis is not due to the thioesterase activity of the enzyme since the rate of hydrolysis is not altered when this activity is inhibited by modification of the enzyme with tosyl fluoride. Since the rate constants for hydrolysis are much less than the value of $(k_{-2}/K_3)(E_0)$ in the acetylation experiments, spontaneous hydrolysis is not of importance during the quenched-flow experiments.

The rate of deacetylation of the acetylated enzyme by CoA also has been studied with the quenched flow under the same conditions as used for the acetylation reaction. The number of these experiments was limited by the high cost of the radioactive AcCoA used in preparation of the acetylated enzyme. In addition, the spontaneous hydrolysis of the enzyme is a complicating feature of this experiment. However, since the rate constant for this process is known, the extent of spontaneous hydrolysis is known. In analyzing the quenched-flow deacetylation experiments, the amount of spontaneous hydrolysis was corrected for by multiplying the amount of observed acetylated enzyme by $e^{kt'}$ where k is the rate constant for spontaneous hydrolysis and t' is the time that has passed since the acetylated enzyme was isolated. In order to minimize this correction, the experiments were completed within 25 min. A typical plot of the time course of deacetylation by CoA is presented in Figure 5. At low CoA concentrations the amount of acetylated enzyme does not approach zero at long times because an equilibrium is established between the AcCoA and CoA present. The data were fit to the rate law

$$(EAc)_t - (EAc)_\infty = A' \exp(-k'_{obsd} t) \qquad (6)$$

where the concentration of acetylated enzyme at $t = \infty$ was only appreciable at the lowest CoA concentration. The curve in Figure 5 has been calculated according to eq 6. The values of k'_{obsd} are shown as a function of the concentration of CoA in Figure 6. While the data are limited, the rate constant appears to approach a limiting value at high concentrations of CoA. The mechanism of eq 3 can be used to interpret the data. Linearization of the rate equation as before gives

$$k'_{obsd} = \frac{k_{-2}}{1 + K_3/(CoA)} + (k_2/K_1)[(AcCoA)_\infty + (E)_\infty] \qquad (7)$$

In this case, $(AcCoA)_\infty + (E)_\infty$ is approximately constant and equal to ~0.5 μM. The data in Figure 6 were fit to eq 7, and the best-fit parameters obtained were $k_2/K_1 \approx 0$, $k_{-2} = 103$ (±13) s^{-1}, and $K_3 = 71$ (±22) μM. The value of $(k_2/K_1)[(E)_\infty + (AcCoA)_\infty]$ calculated from the constants determined from the kinetics of acetylation is ~0.3 s^{-1}. This is much less than the first-order rate constants observed for deacetylation, consistent with the finding that k_2/K_1 cannot be determined in the deacetylation experiments. The parameters for deacetylation give $k_{-2}/K_3 \sim 1.4$ μM^{-1} s^{-1}, which is 5 times smaller than inferred from the acetylation kinetics. In view of the approximate analysis and the fact that k_{-2}/K_3 is poorly determined in the acetylation experiments, this discrepancy is not unreasonable.

A further test of the mechanism was carried out by titrating the enzyme (0.3 μM) with CoA (0–2.6 mM) in the presence of 60 or 120 μM radioactive AcCoA in 0.2 M potassium

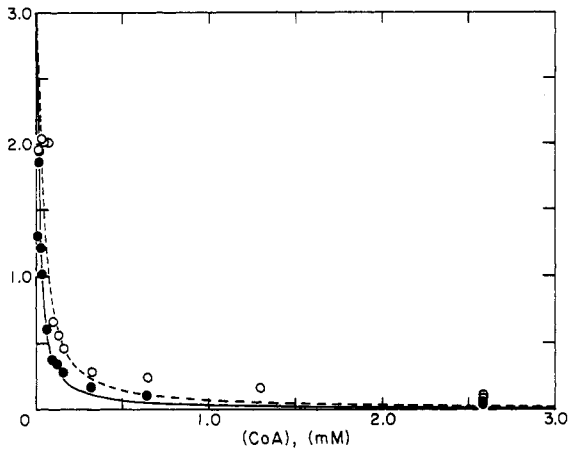

FIGURE 7: Plot of the fraction of acetylated enzyme (mol/mol), r, vs. the concentration of CoA. The data represent a titration of the enzyme (0.3 μM) in 0.2 M potassium phosphate (pH 7.0), 1 mM EDTA, and 60 (●) or 120 (○) μM [^{3}H]AcCoA with CoA at 23 °C. The curves are the fits to eq 9, calculated as described in the text.

phosphate (pH 7.0) and 1 mM EDTA at 23 °C. The results are presented in Figure 7 as a plot of the amount of acetylated enzyme vs. the CoA concentration. Quantitative analysis of these results requires that the inhibition of acetylation by CoA and of deacetylation by AcCoA be taken into account. The minimal mechanism that can be considered for two identical polypeptide chains and a maximum acetylation stoichiometry of 4 is

$$\begin{aligned}
&E + AcCoA \underset{}{\overset{K_1}{\rightleftharpoons}} E\cdot AcCoA \overset{K_2}{\rightleftharpoons} \\
&\qquad EAc\cdot CoA \overset{K_3}{\rightleftharpoons} EAc + CoA \\
&EAc + AcCoA \overset{K_1}{\rightleftharpoons} EAc\cdot AcCoA \overset{K_2}{\rightleftharpoons} \\
&\qquad E(Ac)_2\cdot CoA \overset{K_3}{\rightleftharpoons} E(Ac)_2 + CoA \\
&\qquad E + CoA \overset{K_3}{\rightleftharpoons} E\cdot CoA \\
&E(Ac)_2 + AcCoA \overset{K_1}{\rightleftharpoons} E(Ac)_2\cdot AcCoA \qquad (8)
\end{aligned}$$

For this mechanism, the dissociation constants for the binding of CoA and AcCoA have been assumed to be independent of the acetylation state of the enzyme, as has the equilibrium between acetylation and deacetylation on the enzyme surface (K_2). The fraction of enzyme acetylated, r, for this mechanism is

$$\begin{aligned}
r = {} & [1 + (CoA)/K_3 + (AcCoA)/K_1] \times \\
& [1 + 2K_3K_2(AcCoA)/[K_1(CoA)]]/ \\
& \{1 + (AcCoA)(1 + K_2)/K_1 + (CoA)(1 + 1/K_2)/K_3 + \\
& K_1(CoA)/[K_2K_3(AcCoA)] + \\
& K_2K_3(AcCoA)/[K_1(CoA)] + \\
& K_1(CoA)^2/[K_2K_3^2(AcCoA)] + \\
& K_2K_3(AcCoA)^2/[K_1^2(CoA)]\} \qquad (9)
\end{aligned}$$

Obviously the data are insufficient to determine K_1, K_2, and K_3. Instead K_1 and K_3 were assumed to be 85 and 70 μM (from the kinetic data), and the data were fit with one parameter, K_2. The curves in Figure 7 are calculated with the value of K_2 obtained from the analysis, 0.35. The ratio of rate constants k_2/k_{-2} from the kinetic data is 0.42. This agreement is better than might be expected and indicates the kinetic parameters are self-consistent.

In order to explore the nature of the acetyl-enzyme bonding, the acetyl-enzyme was reacted with 0.2 M neutralized NH_2OH in 0.2 M potassium phosphate (pH 7.0) and 1 mM EDTA at 23 °C. Hydroxylamine cleaves thio esters but not typical oxy esters. All of the acetyl groups are removed from the enzyme within 15 min for a range of stoichiometries of 1–2.5 acetyl groups/enzyme. The enzyme is not appreciably denatured by this treatment. If the acetylated enzyme is mixed with an equal volume of 8 M urea under the same conditions, the stoichiometry of acetylation is not altered. If this denatured enzyme is then reacted with 0.2 M neutralized NH_2OH, only a portion of the acetyl groups are removed. The fraction of acetyl groups remaining is 0.384 ± 0.018 (five determinations) over the same range of acetyl stoichiometry as above. Essentially the same results were obtained when the thioesterase activity of the enzyme was eliminated by modification with tosyl fluoride: the fraction of acetyl groups remaining was 0.392 ± 0.018 (four determinations). The simplest interpretation of these findings is that the distribution of thio and oxygen esters is the same on the native and denatured enzymes but that the stable oxygen ester found on the denatured enzyme is hydroxylamine sensitive on the native enzyme. This sensitivity is due either to an instability of the oxygen ester on the native enzyme or to transfer of the acetyl group from oxygen to sulfur during treatment of the native enzyme with hydroxylamine.

Discussion

The mechanism for the acetylation of fatty acid synthase by AcCoA is more complex than anticipated. The defining of a mechanism is made more difficult by the susceptibility of the enzyme to denaturation in the quenched flow and the limitation in the number of experiments due to the high cost of radioactive AcCoA. Nevertheless, the use of air-flow restriction valves and wide bore tubing in the quenched flow have permitted the study of a fragile multienzyme complex. Both the acetylation of the enzyme by AcCoA and the deacetylation by CoA clearly involve relatively rapid binding of AcCoA and CoA to the enzyme. The limiting first-order rate constants for acetylation and deacetylation, ~40 and ~100 s^{-1}, are well-defined, and the maximum stoichiometry for acetylation appears to be 4 acetyl groups/molecule (2/polypeptide chain). A similar stoichiometry has been previously reported (Stern et al., 1982). The dissociation constants characterizing the binding of AcCoA and CoA to the enzyme are not well-defined by the data: they lie in the range 20–80 μM. However, the kinetic parameters are consistent with the equilibrium titrations carried out.

The number of CoA and AcCoA binding sites on the enzyme has not been fixed by this work. If two binding sites are present per polypeptide chain, the mechanism of eq 8 is applicable. This mechanism predicts first-order kinetics only if the second acetylation (or deacetylation) is rapid relative to the first. Even if the rates are identical for the first and second acetylation (or deacetylation), deviation from first-order kinetics would be expected. However, if any deviations occur, they are too small to be detected. The approximate data analysis used, therefore, seems most appropriate.

The rate constant for acetylation, 40 s^{-1}, can be compared with the overall turnover number of the enzyme under comparable conditions, ~0.8 s^{-1}, in terms of palmitic acid and a single polypeptide chain (Cox & Hammes, 1983). The priming rate obviously is not rate determining. Even if equilibration between enzyme, CoA, and acetyl-CoA occurs (cf. Stern et al., 1982), the relaxation time for equilibration according to eq 3 is <0.5 s for CoA and AcCoA concentrations of 1 μM and would be even less at higher concentrations. The Michaelis constant for AcCoA from steady-state kinetic studies is ~0.8 μM. If a sequential addition of substrates and rapid

equilibration of substrate binding are assumed, the equilibrium dissociation constant calculated from the Michaelis constant is $\sim 0.8(40/0.8) = 40\ \mu M$, which is within the range derived from the quenched-flow data.

The maximum number of acetyl groups bound to the enzyme appears to be 4/enzyme. The experiments with hydroxylamine indicate that at all degrees of acetylation, two classes of sites are occupied. One acetyl group is bound as an oxygen ester that either is unstable in the native structure or is rapidly transferred to a sulfhydryl site, while the second is a sulfhydryl site. The equilibrium constant for acetylation between these sites appears to be close to unity; the fraction of oxygen ester is ~ 0.38. However, this value could be influenced by complex kinetic phenomena occurring when the enzyme is treated with hydroxylamine. The oxygen ester has the correct characteristics to be the "loading" site previously postulated (Jacob et al., 1968; Phillips et al., 1970a,b). Previous work with a fluorescent CoA derivative has indicated the presence of at least four types of acylation sites on the enzyme and also has suggested the presence of an oxygen ester loading site (Cardon & Hammes, 1983).

The rate of deacylation by CoA is quite rapid. Deacylation by CoA has been postulated to be a control feature for fatty acid synthase (Sedgwick & Smith, 1981; Stern et al., 1982). Equilibration between AcCoA, CoA, malonyl-CoA, and the enzyme very likely plays a role in the physiological reaction. The thioesterase activity of the enzyme is not involved in the hydrolysis of the acetylated enzyme, and nonenzymatic hydrolysis of the acetylated enzyme appears to be too slow to be of physiological significance.

Studies in progress are aimed at further elucidation of the elementary steps in the mechanism of action of fatty acid synthase.

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Registry No. AcCoA, 72-89-9; CoA, 85-61-0; hydroxylamine, 7803-49-8; fatty acid synthase, 9045-77-6.

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