Elementary Steps in the Reaction Mechanism of Chicken Liver Fatty Acid Synthase: β-Ketoacyl Reductase and Enoyl Reductase†

Jean A. H. Cognet and Gordon G. Hammes*

Department of Chemistry, Cornell University, Ithaca, New York 14853
Received June 6, 1984

ABSTRACT: The following reactions catalyzed by chicken liver fatty acid synthase have been studied with the stopped-flow method in 0.1 M potassium phosphate (pH 7.0) and 1 mM ethylenediaminetetraacetic acid at 25 °C by monitoring the change in NADPH fluorescence: the transfer of acetoacetyl from acetoacetyl coenzyme A to the enzyme, reduction of the enzyme-bound acetoacetyl by NADPH (β-ketoacyl reductase), and reduction of enzyme-bound D-hydroxybutyryl/crotonyl by NADPH (enoyl reductase). The first two reactions were studied by mixing enzyme–NADPH with acetoacetyl-CoA under conditions where the kinetics can be analyzed as two consecutive pseudo-first-order processes: a mechanism consistent with the acetoacetyl-CoA dependence of the pseudo-first-order rate constant associated with formation of the acetoacetyl-enzyme is a relatively rapid binding of substrate to the enzyme, with a dissociation constant of 650 μM, followed by formation of covalently bound acetoacetyl, with a rate constant of 10.2 s^{-1}. The acetoacetyl-enzyme is reduced by enzyme-bound NADPH with a rate constant of 20 s^{-1}, and the NADPH binding is characterized by a dissociation constant of 5.3 μM. Reduction of the D-hydroxybutyryl-/crotonyl-enzyme was studied by mixing NADPH with enzyme that was equilibrated with D-hydroxybutyryl-CoA or crotonyl-CoA; the rate constant for reduction of an equilibrium mixture of D-hydroxybutyryl- and crotonyl-enzyme is 36.6 s^{-1}. Steady-state kinetic studies of the reduction of acetoacetyl-CoA and crotonyl-CoA by NADPH also have been carried out. The results obtained indicate the steady-state reductions proceed primarily through intermediates in which the substrate is not covalently bound, whereas the single turnover reduction of covalently bound intermediates is observed in the transient studies. Acetoacetyl-CoA and crotonyl-CoA can serve as primers for steady-state fatty acid synthesis if malonyl-CoA is a cosubstrate. A theoretical and experimental analysis of the products obtained by reduction of the isolated acetoacetyl-enzyme with limited [^{3}H]NADPH shows the ratio of hydroxybutyryl- to butyryl-enzyme is determined by the initial ratio of the concentration of NADPH to that of covalently bound substrate and the ratio of turnover numbers for β-ketoacyl synthase and enoyl reductase. The ratio of turnover numbers obtained from the product analyses is in good agreement with that obtained from the stopped-flow kinetics. The theoretical treatment can be utilized for any sequence of two reactions involving a common substrate.

The fatty acid synthase from chicken liver is a multienzyme complex that catalyzes the synthesis of palmitic acid from acetyl coenzyme A (AcCoA),[1] MalCoA, and NADPH. The synthesis is primed by the covalent transfer of an acetyl group to the enzyme from AcCoA, probably mediated by an hydroxyl loading site on the enzyme (Wakil et al., 1983; Cardon & Hammes, 1982, 1983). Malonyl is transferred to the enzyme in a similar fashion. The correct positioning of the acetyl and malonyl group on the enzyme appears to be established by rapid equilibration of these groups with enzyme and CoA (Stern et al., 1982; Cognet et al., 1983; Soulié et al., 1983). The condensation of these groups results in formation of an acetoacetyl-enzyme intermediate with the concomitant release of CO_2. The enzyme-bound acetoacetyl is subsequently reduced to D-hydroxybutyryl by NADPH; D-hydroxybutyryl is dehydrated to form crotonyl, and the carbon–carbon double bond is reduced by a second NADPH to give enzyme-bound butyryl. This cycle is repeated 7 times by successive condensation of malonyl on to the growing hydrocarbon chain until palmitic acid is released by a thioesterase. The overall reaction is

$$\text{AcCoA} + 7\text{MalCoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{CH}_3(\text{CH}_2\text{CH}_2)_7\text{COOH} + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O} + 7\text{CO}_2$$

Fatty acid synthases have been reviewed extensively (Volpe & Vagelos, 1973; Bloch & Vance, 1977; Wakil et al., 1983). Chicken fatty acid synthase contains two identical polypeptide chains of M_r 250 000. Steady-state kinetic studies (Katiyar et al., 1975; Cox & Hammes, 1983) have explored the overall synthesis. Transient kinetic studies have been carried out of the acetylation and deacetylation of the enzyme by AcCoA and CoA, (Cognet & Hammes, 1983), of the binding of NADPH to the enzyme, and of the formation and reduction of the acetoacetyl-enzyme with AcCoA, MalCoA, and NADPH as substrates (Cognet et al., 1983; Yuan & Hammes, 1983). In this work, the transfer of acetoacetyl from AcacCoA to the enzyme, the reduction of enzyme-bound acetoacetyl by NADPH, and the reduction of enzyme-bound D-hydroxybutyryl and crotonyl by NADPH have been studied with the stopped-flow method. Steady-state kinetic studies of the re-

† This work was supported by grants from the National Institutes of Health (GM 13292) and the National Science Foundation (PCM 8120818).

[1] Abbreviations: CoA, coenzyme A; AcCoA, acetyl-CoA; MalCoA, malonyl-CoA; AcacCoA, acetoacetyl-CoA; HBCoA, D-β-hydroxybutyryl-CoA; CrotCoA, crotonyl-CoA; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-0290$01.50/0

ductions also have been carried out. Finally, an analysis of the reaction products of the sequential reduction by β-ketoacyl synthase and enoyl reductase with limited NADPH has permitted a determination of the ratio of the two turnover numbers.

Materials and Methods

Chemicals. AcCoA, MalCoA, AcacCoA, CrotCoA, β-NADPH (type X), dithiothreitol, D-glucose 6-phosphate, D-glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, type XXI), MOPS, Tris, acetyl phosphate, and phosphotransacetylase were obtained from Sigma. AG1-X4, an anion-exchange resin (200–400 mesh), was from Bio-Rad. L(+)-Cysteine hydrochloride was from Eastman Kodak. Silica gel plates for thin-layer chromatography (80 × 40 cm) were from Macherey-Nagel. Commercial CoA thio esters were purified by high-performance liquid chromatography on a C-18 reverse-phase column eluted with the following solvent systems: AcacCoA with methanol–10 mM potassium phosphate, pH 7.0 (9:91), or with methanol–10 mM MOPS, pH 7.0 (9:91); CrotCoA with methanol–10 mM potassium phosphate, pH 7.0 (20:80), or with methanol–10 mM MOPS, pH 7.0 (17:83); MalCoA with methanol–10 mM ammonium acetate, pH 4.5 (9:91). All other chemicals were high quality commercial grades, and all solutions were prepared with distilled, deionized water.

Fatty Acid Synthase Preparation. The enzyme was prepared from chicken livers and assayed as previously described (Cardon & Hammes, 1982; Cognet & Hammes, 1983). The preparation was scaled up 2-fold by doubling all the volumes used in the first set of precipitations. The final pellet was redissolved in ≈15 mL of 20 mM potassium phosphate (pH 7.5)–1 mM EDTA, containing 10% glycerol (w/v), and was applied to a DE-52 column (1.5 × 11 cm), and the column was washed with the same buffer. The enzyme was eluted with a 20–150 mM potassium phosphate (pH 7.5)–10% glycerol–1 mM EDTA, linear gradient (total volume 120 mL). Application of the enzyme and elution were carried out very slowly at a flow rate ≤0.3 mL/min. Fractions with a specific activity greater than 1.5 μmol of NADPH/min·mg) were collected, and dithiothreitol was added to 10 mM for storage at −20 °C. This variation yielded large quantities of enzyme (≈250 mg) at high concentrations (≥6 mg/mL), with specific activities as high as 2.2 μmol of NADPH/(min·mg). The final precipitation and column chromatography on Bio-Gel A-1.5 used in the original preparation were not necessary. The 10 mM dithiothreitol and 10% glycerol (w/v) necessary for storage were removed prior to all experiments by passage through 3-mL Sephadex G-50 (fine) centrifuge columns preequilibrated with the desired buffer, 0.1 M potassium phosphate or 0.1 M MOPS and 1 mM EDTA (pH 7.0) (Penefsky, 1977). The specific activity of the enzyme after the centrifuge columns was 1.6–1.8 μmol of NADPH/(min·mg). The protein concentration was determined by measurement of the absorbance at 280 nm and by use of an extinction coefficient for fatty acid synthase of 4.82×10^5 M^{-1} cm^{-1} (Hsu & Yun, 1970).

Preparation of D-β-Hydroxybutyryl-CoA. HBCoA was prepared by a modification of an earlier procedure (Anderson & Hammes, 1984): 500 μL of 2.4 mg of fatty acid synthase/mL in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0), 60 μL of 7.5 mM NADPH, 150 μL of 73 mM AcacCoA, 26 μL of 430 mM D-glucose 6-phosphate, and 4 μL of an ammonium sulfate suspension of D-glucose-6-phosphate dehydrogenase were added to 2.1 mL of 0.1 M Tris-HCl (pH 7.0) at 25 °C. The NADPH absorbance was monitored at 340 nm and reached a plateau in about 25 min. Equivalent additions of AcacCoA and D-glucose 6-phosphate were made each time the NADPH was regenerated. A total of 900 μL of AcacCoA and 156 μL of D-glucose 6-phosphate was added in this fashion. The completion of the reaction was checked by the addition of small amounts of AcacCoA, D-glucose 6-phosphate, or NADPH. The reaction was quenched by acidifying the solution and vortexing with a few drops of chloroform. The precipitated enzyme was eliminated by filtration through a glass wool plug in a Pasteur pipet. The solution was then adjusted to pH 8.0 with solutions of LiOH and Tris and reacted with cysteine (3 mM) at room temperature (total volume ≈7 mL). The disappearance of CrotCoA was monitored at 290 nm. When no further change in absorbance was observed (≈40 min), the solution was titrated to pH 6.5 with HCl, diluted 6–7-fold, and loaded on a 27-mL column of the anion-exchange resin AG1-X4. The column was washed (2.5 mL/min) with 400 mL of 90 mM LiCl–10 mM HCl to remove the cysteine–CrotCoA addition reaction product. The HBCoA was then eluted with 200 mM LiCl–10 mM HCl; the product was identified by its absorbance at 254 nm. The solution collected (130 mL, pH adjusted to 7.0) was lyophilized to 5 mL and loaded on a (1 × 60 cm) Sephadex G-10 column to remove the LiCl and CoA degradation products. The column was eluted with water, and the product was identified by its ultraviolet absorbance. After lyophilization, the final purification was carried out by high-performance liquid chromatography on a C-18 reverse-phase column eluted with 10 mM potassium phosphate (pH 7.0)–methanol (91:9). The purified HBCoA (≈10 mg) was lyophilized to 1 mL and stored at −20 °C.

Steady-State Kinetic and Optical Measurements. The steady-state enzyme assays monitored the oxidation of NADPH at 25 °C by following the decrease in absorbance of NADPH at 340 nm, $\epsilon_{340} = 6220$ M^{-1} cm^{-1} (Horecker & Kornberg, 1948), with a Cary 118 spectrophotometer. The concentrations of CoA thio esters (except for CrotCoA) were determined by using an extinction coefficient of $\epsilon_{260} = 14\,600$ M^{-1} cm^{-1} (Dugan & Porter, 1970). For CrotCoA, $\epsilon_{266} = 22\,600$ (Stadtman, 1957).

Stopped-Flow Measurements. Stopped-flow experiments were performed on a modified Durrum-Gibson stopped-flow spectrophotometer by observing changes in NADPH fluorescence (364–367-nm excitation, >420-nm emission) as described previously (Akiyama & Hammes, 1981; Cognet et al., 1983). For all measurements, the data were filtered electronically with a time constant of 1 ms. The noise to signal ratio had to be less than 0.25% for determination of the rate of reduction of covalently bound CrotCoA and HBCoA by enoyl reductase.

Hydrolysis of the Acetoacetylated Enzyme and Reaction Product Analysis. Commercial AcacCoA was mixed with the enzyme at room temperature to final concentrations of 500 and 8.2 μM, respectively. The mixture (1.4 mL) was rapidly passed through three 3-mL Sephadex G-50 centrifuge columns to eliminate AcacCoA and CoA. The acetoacetyl-enzyme (total enzyme concentration 6.3 ± 0.5 μM) was quickly loaded in a quenched-flow apparatus, which was used only as a rapid mixing device. At various times, 150 μL of the acetoacetyl-enzyme was mixed with an equal volume of tritiated NADPH (0.16–1.95 μM) and allowed to stand for at least 25 s at 25 °C. The NADPH was specifically labeled at the *pro-4S* position (1160 cpm/pmol) and was a generous gift of Dr. V. E. Anderson (Anderson & Hammes, 1984). Thin-layer chromatography with the solvent system ether–glacial acetic acid (96:4) (Cognet et al., 1983) indicated the purity of the

NADPH was ≥91%. To study the kinetics of spontaneous hydrolysis of various enzyme intermediates, 80 μL of the reaction mixture of acetoacetyl-enzyme and NADPH was sampled at various times and quenched in 1 mL of ice-cold $HClO_4$ (0.56 M). The precipitate was stored on ice and collected by filtration on a Whatman GF/F glass-fiber filter. The filter was washed with five 5-mL aliquots of ice-cold $HClO_4$ (0.56 M) followed by two washes of 95% ethanol at room temperature. The dry filter was incubated with 10 mL of aqueous scintillation fluid (Amersham) for at least 24 h before its total radioactivity was determined in a Beckman LS 255 scintillation counter. A control experiment, in which the tritiated NADPH was mixed with the enzyme under the same conditions, showed that no radioactivity remained on the filter.

To determine the proportions of D-hydroxybutyryl- and butyryl-enzyme, a sample (140, 220, or 300 μL) of the reaction mixture was reacted with a benzylamine solution (200, 300, or 400 μL, respectively) and analyzed by high-performance liquid chromatography (Anderson & Hammes, 1984).

Results

Steady-State Kinetics of the Reduction Reactions. The rate law for the steady-state initial velocity, v, of the reduction of the substrate, S (AcacCoA or CrotCoA), by NADPH catalyzed by fatty acid synthase follows Michaelis–Menten kinetics:

$$v/[E]_0 = k_s/(1 + K_s/[S]) \quad (1)$$

In eq 1, k_s is the turnover number in moles of NADPH consumed per mole of enzyme per second, $[E]_0$ is the total enzyme concentration, and K_s is the Michaelis constant. The rate law was determined in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0), at 25 °C, and ~150 μM NADPH (25 times the equilibrium dissociation constant for NADPH). The concentration ranges were 64–970 μM with AcacCoA purified by high-performance liquid chromatography and 96 μM–19.7 mM with commercial AcacCoA, 0.38–7.5 mM with CrotCoA purified by high-performance liquid chromatography, and 1.82–36.4 mM with commercial CrotCoA. Similar results were obtained with the purified and commercial substrates. The results were fit to eq 1 by a nonlinear least-squares analysis; the standard errors are given in parentheses. For the reduction of AcacCoA (primarily to HBCoA), $[E]_0$ was ≈0.05 μM, k_s = 65 (±6) s^{-1}, and K_s = 21.5 (±3.2) mM. For the reduction of CrotCoA, $[E]_0$ was ≈0.18 μM, k_s = 1.8 (±0.3) s^{-1}, and K_s = 40 (±10) mM. (The turnover numbers per polypeptide chain are half the reported values.) The results with CrotCoA should be viewed with some caution because incubation of the enzyme with CrotCoA causes inhibition (both reversible and irreversible) of both the enoyl reductase and synthetic activities of the enzyme. In principle, this might cause an underestimate of the initial velocity although no appreciable deviations from eq 1 were observed.

In 0.1 M MOPS–1 mM EDTA (pH 7.0), 25 °C, the reduction rate of purified AcacCoA over the range 0.185–1.85 mM was ≈65% of the rate obtained in phosphate buffer. The overall synthetic specific activity of the enzyme also was reduced a similar amount. The steady-state rate for the reduction of 0.5 mM AcacCoA (0.70 s^{-1}) or 2 mM CrotCoA (0.12 s^{-1}) remained unchanged in the presence of a CoA scavenging system (10 mM acetyl phosphate, 15 units/mL phosphotransacetylase) or when the enzyme was loaded with acetyl groups by AcCoA in the presence of the CoA scavenging system (10 mM acetyl phosphate, 15 units/mL phosphotransacetylase, 50 μM acetyl-CoA) prior to the measurement. The scavenging system effectively locks the acetyl groups on to the enzyme. This shows that neither CoA nor acetyl bound to the enzyme influences the reductions. In the presence of 100 or 13.5 μM MalCoA with 0.5 mM AcacCoA or 2 mM CrotCoA, respectively, the initial steady-state rates were 4.5 and 0.87 s^{-1}, respectively. Addition of the CoA scavenging system, or prior incubation of the enzyme with the CoA scavenging AcCoA system, reduces the steady-state rates to the reduction rates observed in the absence of MalCoA. This 6–7-fold increase in reduction rates indicates that AcacCoA and CrotCoA can serve as primers for the synthetic activity of the enzyme. Furthermore, CoA and the enzyme sites to which acetyl groups bind are required for this synthetic activity but not for the steady-state reduction of AcacCoA or CrotCoA.

Stopped-Flow Studies of AcacCoA, CrotCoA, and HBCoA Reduction. The single turnover of the fatty acid synthase β-ketoacyl reductase has been previously studied with the stopped-flow method (Cognet et al., 1983). The experiments involved the rapid mixing of MalCoA and AcCoA in various proportions with the enzyme, E, that had been premixed with NADPH. If [MalCoA], [AcCoA] ≫ [E] ≫ [NADPH], the disappearance of NADPH as a function of time, t, is given by

$$[NADPH] = [NADPH]_0 \exp[-k_2[t + (1/k_1)(e^{-k_1 t} - 1)]] \quad (2)$$

This experiment was repeated, with AcacCoA substituted for MalCoA and AcCoA, in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. The concentrations of reactants were E = 2.05 μM, NADPH = 0.35 μM, and purified AcacCoA = 100–800 μM. A typical kinetic trace (labeled 1) is shown in Figure 1. As with AcCoA and MalCoA, a lag period, characterized by the first-order rate constant k_1, is observed due to the formation of the acetoacetyl-enzyme intermediate; this is followed by the oxidation of NADPH characterized by the first-order rate constant k_2. The data conform to eq 2 very precisely, as indicated in Figure 1. The value of k_2 changed slightly as the concentration of AcacCoA was varied. In order to eliminate any covariance of k_1 and k_2, the data were reanalyzed with k_2 fixed at its average value, 4.9 (±0.7) s^{-1}. This procedure did not significantly alter the fit of the time course. The values of k_1 obtained are plotted vs. the concentration of AcacCoA in Figure 2. These data are consistent with a mechanism in which a rapid binding of AcacCoA is followed by the covalent binding of acetoacetyl to the enzyme:

$$E + AcacCoA \overset{K_d}{\rightleftharpoons} E{\cdot}AcacCoA \xrightarrow{k_1'} E\text{–}Acac + CoA \quad (3)$$

For this mechanism

$$k_1 = k_1'/(1 + K_d/[AcacCoA]) \quad (4)$$

A fit of the data in Figure 2 according to eq 4 gives k_1' = 10.2 (±0.8) s^{-1} and K_d = 654 (±74) μM. The curve in Figure 2 has been calculated with these parameters and eq 4.

The nature of the E–Acac intermediate was examined in three separate experiments. In the first experiment, the enzyme was premixed with AcacCoA and reacted as quickly as possible (1–3 min) with NADPH in the stopped-flow apparatus to final concentrations of 3.1, 200, and 0.125 μM, respectively. A typical kinetic trace is shown in Figure 1, curve 2. The time course no longer exhibits a lag phase (after the binding of NADPH to the enzyme is complete in ~20 ms) and can be well fit by a single exponential decay with a rate constant of 11.8 s^{-1}. In a second experiment, the enzyme was mixed with AcacCoA (final concentration 250μM) at room temperature. The mixture (1.7 mL) was passed through four

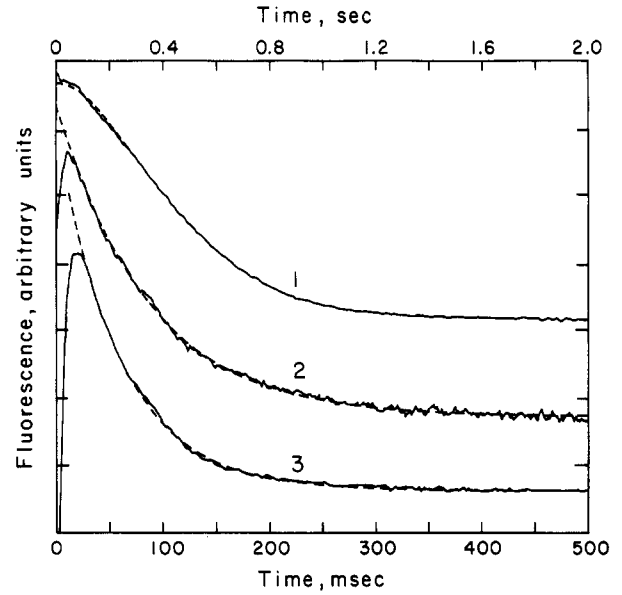

FIGURE 1: Typical stopped-flow kinetic traces of the change in fluorescence vs. time. (Curve 1) The enzyme (4.1 μM) and NADPH (0.7 μM) were mixed with purified AcacCoA (168 μM). The curve was fit to eq 2 with the best fit parameters, $k_1 = 2.2\ s^{-1}$, $k_2 = 4.95\ s^{-1}$, $A = 176$, and $F(\infty) = 48.8$ and the relationship [NADPH] = $F(t) - F(\infty)$ and $[NADPH]_0 = A$. (Curve 2) The enzyme (6.2 μM) and AcacCoA (400 μM) were incubated 3 min and mixed in the stopped-flow apparatus with NADPH (0.25 μM). The curve was fit to a single exponential decay, $F(t) - F(\infty) = A \exp(-kt)$, with the best fit parameters $A = 156$, $k = 11.8\ s^{-1}$, and $F(\infty) = 17.2$. (Curve 3) The enzyme (6.9 μM) and CrotCoA (260 μM) were incubated 3–12 min and mixed in the stopped-flow apparatus with NADPH (20 μM). The curve was fit to a single exponential decay, $F(t) - F(\infty) = A \exp(-kt)$, with the best fit parameters $A = 288.6$, $k = 17.0\ s^{-1}$, and $F(\infty) = 31.7$. The upper time scale applies to the upper trace and the lower time scale to the two lower traces. All concentrations are given before mixing, and all solutions were in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. The units of fluorescence, F, are arbitrary and different for all three curves. The experimental and calculated curves are essentially indistinguishable. Where differences exist, the calculated curve is shown as dashes.

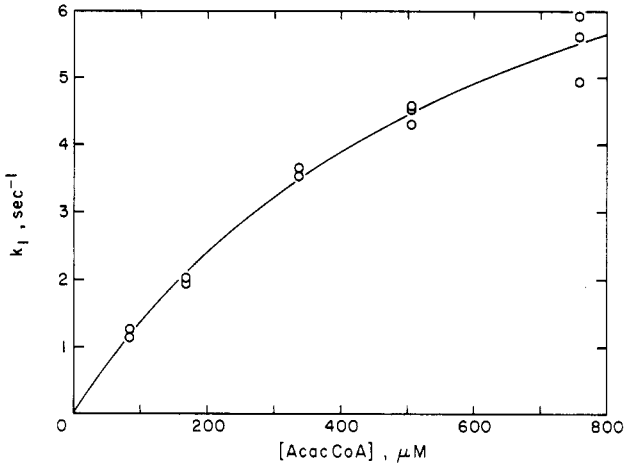

FIGURE 2: Plot of the pseudo-first-order rate constant for the formation of the acetoacetyl-enzyme, k_1, vs. the concentration of AcacCoA in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. Other experimental conditions are the same as the legend to Figure 1, curve 1. The curve was calculated with eq 4 and the best fit parameters $k_1 = 10.2$ (±0.8) s^{-1} and $K_d = 654$ (±74) μM.

centrifuge columns. The E–Acac intermediate (total enzyme concentration ≈2.5 μM, free of CoA and AcacCoA) was then reacted in the stopped-flow apparatus with NADPH (0.5 μM before mixing). The kinetic traces again conformed to a simple exponential decay. These results indicate that the lag period

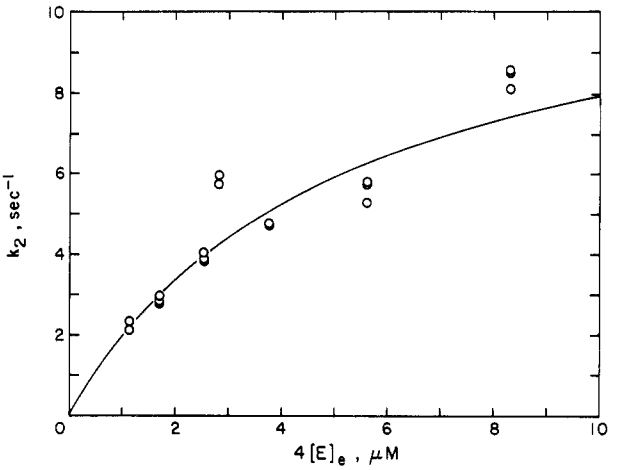

FIGURE 3: Plot of the first-order rate constant for the reduction of acetoacetyl-enzyme by NADPH, k_2, vs. the equilibrium concentration of unoccupied NADPH sites, 4[E]$_e$, in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. The molar ratio of total enzyme to total NADPH is constant (5.48) for all experiments, and the AcacCoA concentration is 400 μM after mixing. Other experimental conditions are as in Figure 1, curve 1. The curve was calculated with eq 6 and the best fit parameters $k_2' = 24.4$ (±3.2) s^{-1} and $K_d' = 5.3$ (±1.1) μM.

is due to the formation of E–Acac.

In a final experiment, the enzyme was mixed in the stopped-flow apparatus with AcacCoA and NADPH to final concentrations of 2.0, 200, and 0.35 μM, respectively, in 0.1 M MOPS–1 mM EDTA (pH 7.0), at 25 °C. The rate constant obtained by fitting the data to eq 2 was $k_1 = 2.2\ s^{-1}$ (with k_2 fixed at $4.9\ s^{-1}$). Prior incubation of the enzyme with AcCoA and the CoA scavenging system (200 μM AcCoA, 10 mM acetyl phosphate, and 72 units/mL phosphotransacetylase before mixing) completely alters the kinetic trace. A single exponential decay is observed, characterized by a first-order rate constant of $\approx 0.09\ s^{-1}$. Since the preincubation should have no effect on the oxidation–reduction reaction, the simplest interpretation of this result is that formation of E–Acac has become rate determining in the overall reaction, and the enzyme sites that are blocked by acetyl groups in the preincubation must be available for the formation of E–Acac.

The dependence of k_2 on the concentration of NADPH was determined in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. An NADPH–enzyme mixture was mixed with purified AcacCoA (400 μM after mixing). The molar ratio [enzyme]/[NADPH] was kept constant at 5.48 with concentrations of enzyme varying from 0.289 to 2.19 μM (after mixing). (The molar ratio of NADPH binding sites to NADPH is 21.9.) The value of k_1 varied slightly as the concentrations of enzyme and NADPH were changed. In order to eliminate any covariance of k_1 and k_2, the data were reanalyzed with k_1 fixed at its average value, 2.8 ± 0.9 s^{-1}. The values of k_2 obtained as a function of the equilibrium concentration of empty NADPH binding sites, 4[E]$_e$, are shown in Figure 3. As previously discussed (Cognet et al., 1983), these data suggest the reduction mechanism consists of a rapid binding of NADPH followed by a relatively slow reduction:

$$\text{EAcac} + \text{NADPH} \overset{K_d'}{\rightleftharpoons} \text{EAcac·NADPH} \xrightarrow{k_2'} \text{E-hydroxybutyryl} + \text{NADP}^+ \quad (5)$$

For this mechanism

$$k_2 = (k_2'/2)/[1 + K_d'/(4[E]_e)] \quad (6)$$

The factor of $^1/_2$ arises because only half of the NADPH is

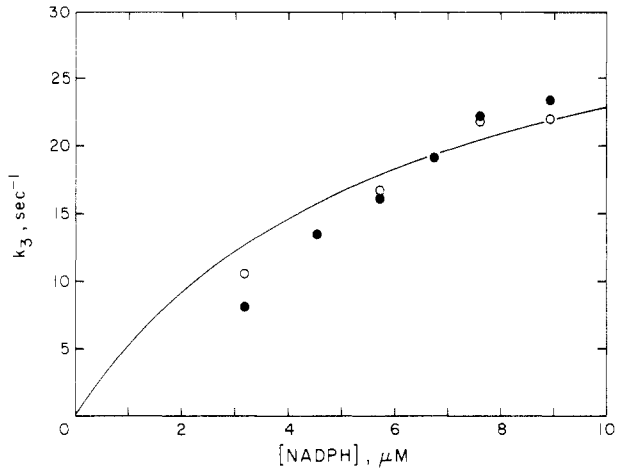

FIGURE 4: Plot of the first-order rate constant, k_3, for the reduction of crotonyl-enzyme or D-hydroxybutyryl-enzyme by NADPH vs. the equilibrium concentration of free NADPH in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at 25 °C. The enzyme (6.9 μM) and CrotCoA (260 μM) (○) or HBCoA (520 μM) (●) were incubated in the stopped-flow apparatus and mixed with variable concentrations of NADPH (10–40 μM). The time course of the fluorescence was fit to a simple exponential, $F(t) - F(\infty) = A \exp(-k_3 t)$. Each point is the average of at least three kinetic experiments. The curve was calculated with eq 8, $K_d' = 6.0$ μM, and the best fit parameter $k_3' = 36.6$ (±0.08) s^{-1}.

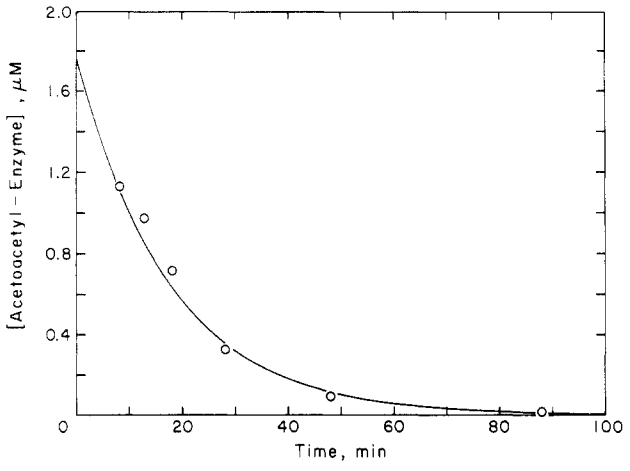

FIGURE 5: Plot of the concentration of the acetoacetyl-enzyme vs. time in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0) at room temperature. The curve is the fit to [acetoacetyl-enzyme] = $[A]_0 \exp(-kt)$ with the best fit parameters $[A]_0 = 1.77 \pm 0.22$ μM and $k = 9.5$ (±0.5) × 10^{-4} s^{-1}. In this experiment, the concentrations of enzyme and NADPH before mixing were 6.3 and 1.95 μM, respectively.

bound at the correct catalytic sites. The assumption is made that the final product is primarily hydroxybutyryl, rather than butyryl. This is supported by the analysis of products given below. A fit of the data to eq 6 by a nonlinear least-squares analysis gives $k_2' = 24.4$ (±3.2) s^{-1} and $K_d' = 5.3$ (±1.1) μM. The equilibrium concentrations of empty NADPH binding sites were obtained by successive approximations (Cognet et al., 1983). The curve in Figure 3 has been calculated with these parameters and eq 6.

When a solution of enzyme (3.85 μM) and NADPH (0.7 μM) was mixed in the stopped-flow apparatus with CrotCoA or HBCoA (400 μM) in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0), the reduction took over 20 s to go to completion. When the enzyme (5.65 μM) was premixed with CrotCoA (300 μM) or HBCoA (400 μM) and mixed in the stopped-flow apparatus with different concentrations of NADPH (0.23–1.02 μM), the time course of NADPH oxidation was independent of the NADPH concentration and dependent on the duration of incubation of the enzyme with CrotCoA or HBCoA. The reduction rate increased slightly with time for HBCoA but decreased for CrotCoA, again illustrating the inhibitory effect of CrotCoA on the enzyme. These two sets of experiments demonstrate the very slow formation and/or small amounts of crotonyl and D-hydroxybutyryl-enzyme intermediates that are competent for reduction by NADPH. Therefore, the method used for measurement of the enoyl reductase rates was to mix high concentrations of enzyme (6.9 μM) with relatively low concentrations of CrotCoA (260 μM) or HBCoA (520 μM) and to react this mixture with high concentrations of NADPH (10–40 μM) in the stopped-flow apparatus. The reaction then becomes pseudo first order with respect to the small amount of reactive crotonyl- and D-hydroxybutyryl-enzyme since [NADPH] ≫ [crotonyl- and D-hydroxybutyryl-enzyme]. The maximum NADPH concentration is limited by the very small signal change at high NADPH concentrations (≈1.5% at 40 μM before mixing). Two rate processes are observed following the rapid binding of NADPH: a relatively fast drop in fluorescence corresponding to the reduction of the crotonyl- and D-hydroxybutyryl-enzyme species and a very slow steady-state reduction. The faster rate process can be fit by a single exponential (Figure 1, curve 3). The intermediates represent less than 5% of the total enzyme, as judged by the ratio of the rapid signal change to the total NADPH fluorescence signal at high NADPH concentrations (40 μM). The values of the rate constant, k_3, are plotted vs. the equilibrium concentration of free NADPH in Figure 4. An equilibrium dissociation constant of 6.0 μM was assumed (Cognet et al., 1983). These data are consistent with a reduction mechanism in which the rapid binding of NADPH is followed by a relatively slow reduction:

$$\text{E–S} + \text{NADPH} \overset{K_d'}{\rightleftharpoons} \text{E–S·NADPH} \xrightarrow{k_3'} \text{E–butyryl} + \text{NADP}^+ \quad (7)$$

where S is crotonyl or D-hydroxybutyryl. For this mechanism

$$k_3 = k_3'/(1 + K_d'/[\text{NADPH}]) \quad (8)$$

A nonlinear least-squares fit of the data to eq 8, with $K_d' = 6.0$ μM, gives $k_3' = 36.6$ (±0.8) s^{-1}. The data are insufficient for an independent determination of K_d'. As expected, the data are indistinguishable for the two substrates since the dehydratase activity of the enzyme equilibrates them.

Hydrolysis of Enzyme Intermediates. The rate of spontaneous hydrolysis of the acetoacetyl-enzyme intermediate or of a reduced species containing D-hydroxybutyryl- and butyryl-enzyme was measured following the isolation of the acetoacetyl-enzyme freed of CoA and AcacCoA with centrifuge columns. This eliminates the possibility of the enzyme-bound acetoacetyl transferring to CoA. The amount of acetoacetyl-enzyme was determined at different times by complete reduction to butyryl-enzyme with an excess of tritiated NADPH (at least two-fold) in the quenched flow apparatus in 0.1 M potassium phosphate–1 mM EDTA (pH 7.0). The radioactivity retained on the enzyme was measured by filter assays. A typical time course is shown in Figure 5. Analysis of the reaction products by high-performance liquid chromatography showed that the enzyme intermediate was essentially fully reduced: [D-hydroxybutyryl]/[butyryl] = 0.11 ± 0.06 for all points but the first in the time course shown in Figure 5. The second time point (≈13 min) was used to calibrate the concentration of acetoacetyl-enzyme: since the product ratio reached its limiting value at this time, the initial concentration of NADPH (1.95 μM) was assumed to be equal to twice the

concentration of acetoacetyl-enzyme. The rate of hydrolysis follows first-order kinetics, and a nonlinear least-squares analysis of the data gives a first-order rate constant of 9.5 ($\pm$0.5) $\times$ 10^{-4} s^{-1} at room temperature ($\approx$23 °C). The initial stoichiometry of the acetoacetyl moiety, immediately after mixing AcacCoA with enzyme (t = 0 in Figure 5), was 0.28 per enzyme molecule.

In a separate set of experiments, the isolated acetoacetyl-enzyme (total enzyme concentration 3.85 μM) was reacted with an equal volume (0.15 mL) of 0.6 μM tritiated NADPH in the quenched-flow apparatus at room temperature. A mixture of hydroxybutyryl- and butyryl-enzyme was obtained with [D-hydroxybutyryl]/[butyryl] = 1.6 $\pm$ 0.4. This mixture was reacted with tritiated NADPH, and aliquots were quenched at various times (0–40 min). Filter assays then were carried out as described above. If the hydrolysis of hydroxybutyryl- and butyryl-enzyme is assumed to be first order, with the same rate constant for both species, a first-order rate constant of 2.8 ($\pm$1.5) $\times$ 10^{-4} s^{-1} can be estimated.

Reaction Product Analysis. When the acetoacetyl-enzyme (A) is mixed with tritiated NADPH (N), two reductions take place leading to D-hydroxybutyryl- and butyryl-enzyme. Under the experimental conditions used, the binding of NADPH is rapid relative to the oxidation–reduction reactions. Furthermore, since the total enzyme concentration, $[E]_0$, is much greater than the total concentration of NADPH, $[N]_0$, the amount of enzyme-bound NADPH, E·NADPH, is $[N]/[1 + K_d'/(4[E]_e)]$, with half of this amount being available for each reduction. The apparent first-order rate constant for each reduction then is $k_i = (k_i'/2)/(1 + K_d'/4[E]_e)$, with i = 2 or 3. In the experiments carried out $[E]_0 \gg [A]_0$ ($[E]_0/[A]_0 \approx$ 11 where $[A]_0$ is the initial concentration of acetoacetyl-enzyme) and $2[A]_0 > [N]_0$. Therefore, $[E]_0 \gg [N]_0$, and the apparent first-order rate constant is a true constant throughout the course of the reaction. The sequential reductions can then be written as

$$A + N \xrightarrow{k_A} E\text{–}HB \qquad E\text{–}HB + N \xrightarrow{k_B} E\text{–}B \tag{9}$$

where E–HB and E–B are the hydroxybutyryl- and butyryl-enzyme, respectively. The dehydratase reaction has been assumed to be relatively fast; this will be discussed later. The differential equations describing this mechanism are

$$\frac{d[N]}{dt} = -k_A[A][N] - k_B[E\text{–}HB][N] \tag{10}$$

$$\frac{d[E\text{–}HB]}{dt} = k_A[A][N] - k_B[E\text{–}HB][N] \tag{11}$$

with the conservation equations

$$[N]_0 = [N] + [E\text{–}HB] + 2[E\text{–}B]$$

$$[A]_0 = [A] + [E\text{–}HB] + [E\text{–}B]$$

Initially no D-hydroxybutyryl–E and butyryl–E are present. Although a simple analytical solution to these coupled rate equations is unavailable, the special scaling properties of the equations allow analytical solutions to be obtained for infinite time (Cognet, 1984), which in this case is 10–20 s. At infinite time

$$[E\text{–}HB]_\infty/[E\text{–}B]_\infty = [[X]_0/(1 - W^\gamma) - 1]^{-1} - 1 \tag{12}$$

where $[X]_0 = [N]_0/[A]_0$, $\gamma = k_A/k_B$, and W is the solution of the equation

$$(2 - \gamma)W^\gamma - \gamma W + (\gamma - 1)(2 - [X]_0) = 0 \tag{13}$$

for $\gamma \neq 1$ and $0 < W < 1$. In some cases a simple analytical solution can be obtained: for example for $\gamma = 2$, $[E\text{–}HB]_\infty/[E\text{–}B]_\infty = 4/[X]_0 - 2$. The practical application of this analysis is that if any two of the three variables $[X]_0$, γ or $[E\text{–}HB]_\infty/[E\text{–}B]_\infty$ are known, the third can be calculated. In Figure 6 the ratio $[E\text{–}HB]_\infty/[E\text{–}B]_\infty$ is plotted vs. $[X]_0$. The variation of $[X]_0$ was obtained by mixing known concentrations of NADPH, $[N]_0$, with the acetoacetyl-enzyme after different periods of hydrolysis of the latter species. Since the rate of hydrolysis is known, the concentration $[A]_0$ can be calculated at any time. A least-squares fit of the data to eq 12 gives $\gamma = k_A/k_B = 0.55 \pm 0.11$.

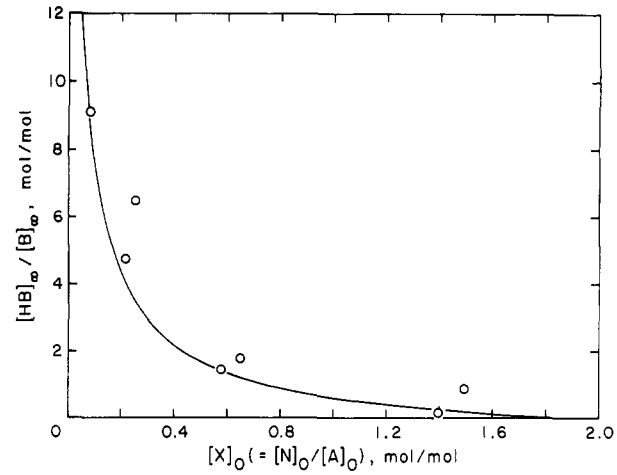

FIGURE 6: Plot of the ratio of the D-hydroxybutyryl-enzyme concentration $[HB]_\infty$ to the butyryl-enzyme concentration $[B]_\infty$ at infinite time formed by reduction of acetoacetyl-enzyme with tritiated NADPH vs. the ratio, $[X]_0$, of the initial concentration of tritiated NADPH, $[N]_0$, to the initial concentration of acetoacetyl-enzyme, $[A]_0$. The curve was calculated with eq 12 and the best-fit parameter $k_A/k_B = 0.55 \pm 0.11$.

A reaction product analysis also was carried out under typical conditions used for the kinetic studies of the reduction of AcacCoA by the enzyme in the stopped-flow apparatus: AcacCoA (200 μM) and tritiated NADPH (0.3 μM) were mixed with enzyme (2.0 μM) where the concentrations are those after mixing. Under these conditions $[E\text{–}HB]_\infty/[E\text{–}B]_\infty$ = 5.4.

Discussion

The steady-state kinetics of the reduction of AcacCoA and CrotCoA are characterized by large Michaelis constants and turnover numbers different from the rate constants observed for single turnover reductions in the stopped-flow studies. Careful purification of substrates (to eliminate AcCoA) and kinetic measurements have demonstrated that these reduction reactions are quite distinct from fatty acid synthesis. However, steady-state fatty acid synthesis can be initiated with each of these substrates as a primer and MalCoA. This conclusion is derived from the observed rates of reduction, from the effects of the elimination of CoA by phosphotransacetylase, and from the "locking" of acetyl groups onto the enzyme. The difference between the steady-state and stopped-flow reduction rates for AcacCoA and CrotCoA is that the steady-state reductions occur primarily with substrates not covalently bound to the enzyme, whereas the reductions observed in the stopped-flow studies and the steady-state priming of fatty acid synthesis occur through covalent intermediates. The reason for two different mechanisms is probably the relatively slow rates of formation and dissociation of covalent intermediates formed with acetoacetyl, D-hydroxybutyryl, and crotonyl which block rapid steady-state reduction via covalent intermediates. The steady-state turnover number (noncovalent reduction) of AcacCoA is similar to the rate constant for the reduction of

the covalent intermediate (33 vs. 20 s^{-1}), whereas a large difference exists for CrotCoA (0.9 vs. 37 s^{-1}). Apparently the turnover number for the steady-state reduction of CrotCoA is not determined by the reduction reaction. The Michaelis constant for acetoacetyl-CoA is larger than the dissociation constant found in the stopped-flow study.

The formation of the NADPH-reducible acetoacetyl-enzyme from enzyme-bound AcacCoA is considerably slower than formation of acetyl-enzyme from enzyme-bound AcCoA (k = 10.2 and 43.1 s^{-1}, respectively), and the rates with CrotCoA and HBCoA as substrates are even slower. Incubation times of 30 s or longer are required. Moreover, the amounts of reducible covalent intermediate are very small: ≈0.28 mol/mol of enzyme for AcacCoA and ≈0.05 mol/mol for CrotCoA and HBCoA. [For AcCoA, >1.4 mol/mol of enzyme can be isolated under similar conditions (Cognet & Hammes, 1983).] This is also reflected in the fact that AcacCoA and CrotCoA are relatively poor primers for fatty acid synthesis. The reason for these differences is not obvious. This is not due to the presence of CoA since the elimination of CoA by phosphotransacetylase does not increase the amounts of reducible enzyme-bound acetoacetyl and crotonyl significantly (unpublished results). Apparently this problem does not occur when the acetoacetyl-enzyme is formed during the normal catalytic cycle with AcCoA and MalCoA as substrates. The rate of formation of the acetoacetyl-enzyme is faster with AcCoA and MalCoA as substrates than with AcacCoA as substrate. The rate constant for the reduction of isolated acetoacetyl-enzyme [Figure 1, curve 2, 23.6 s^{-1} (twice the observed rate constant since only half of the NADPH is bound to the β-ketoacetyl reductase sites)] agrees well with that obtained with AcacCoA as substrate (Figure 3; 24.4 s^{-1}). These rate constants are overestimates of the actual values since the assumption has been made that the only product is hydroxybutyryl, whereas product analysis indicates ≈15% of the product is butyryl. Since this is primarily a stoichiometric effect that does not alter the kinetic equations significantly, the actual rate constant is ≈15% smaller than that observed, about 20 s^{-1}. With MalCoA and AcCoA, this rate constant was found to be 17.5 s^{-1} with the assumption that butyryl is the only product and the enoyl reductase reaction is much faster than the β-ketoacyl synthase reaction (Cognet et al., 1983). The more detailed analysis presented here indicates this is an underestimate of the true rate constant, consistent with the value of 20 s^{-1} reported above. The dissociation constant for NADPH agrees well with previous measurements (Cognet et al., 1983).

Determination of the rate of reduction of the hydroxybutyryl- and crotonyl-enzymes was difficult because high concentrations of NADPH had to be used to obtain first-order kinetics. The measured rate constant describes the reduction of a mixture of the hydrated and dehydrated intermediate. To obtain the rate constant for the reduction of the unsaturated intermediate, the observed rate constant should be multiplied by $(1 + K_{eq})$ where K_{eq} is the ratio of hydrated to dehydrated intermediate. This analysis assumes the dehydratase has a much larger turnover number than enoyl reductase, which is consistent with the observed dependence of k_3 on the concentration of NADPH. For a similar reaction, the hydration–dehydration catalyzed by fumarase, K_{eq}, is ≈4, and the turnover number is ≈10^3 s^{-1} (Frieden et al., 1957).

The use of product analyses to determine the relative rates of the β-ketoacyl reductase and enoyl reductase reactions is an alternative to direct rate measurements. Quantification of the product yields is difficult because of possible losses in the filter assays and the difficulty in converting the tritium incorporation into absolute concentrations; however, these potential errors are largely eliminated if only the ratio of products is required. In the present case, the benzylamine also is assumed to scavenge all of the intermediates equally well. Factors due to the rapid equilibration between enzyme and NADPH cancel when the ratio of second-order rate constants is taken so that this ratio is equal to that of the turnover numbers for β-ketoacyl reductase and enoyl reductase. The ratio of rate constants found from product analyses, 0.55, is in good agreement with the ratio of directly measured first-order rate constants, 20/37 = 0.54. Product analyses for the determination of the relative rate constants of sequential reactions with a common substrate may be applicable to other systems and in fact might permit determination of the dependence of the reaction rates on the chain length of the covalently bound intermediates. An important requirement for application of this procedure is that the concentration of the unbound common substrate be approximately proportional to the total concentration of the common substrate and be limiting in the amount of product formed. Figure 7 shows the product ratios expected for various values of γ and the initial ratio of substrates, $[X]_0$. Clearly this method has considerable sensitivity and can be used for a wide range of relative rates and initial conditions. Not that the maximum sensitivity for the method ocurs when $\gamma \approx 1$ and that a specific product can be obtained in high yield by suitable adjustment of the initial conditions.

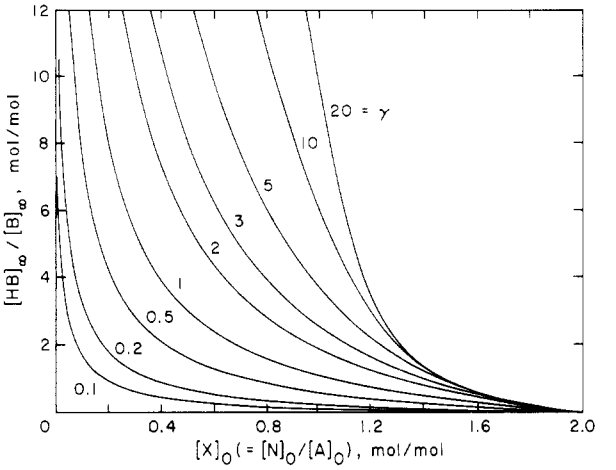

FIGURE 7: Plot of the ratio of the D-hydroxybutyryl-enzyme concentration, $[HB]_\infty$, to the butyryl-enzyme concentration, $[B]_\infty$, at infinite time formed by reduction of acetoacetyl-enzyme with tritiated NADPH vs. the ratio of the initial concentration of tritiated NADPH, $[N]_0$, to the initial concentration of acetoacetyl-enzyme, $[A]_0$. The curves were calculated with eq 13 for different values of the ratio of the reduction rate constants, $\gamma = k_A/k_B$.

Thus far, estimates of the turnover numbers for acetyl transacylase [43 s^{-1} (Cognet & Hammes, 1983)], β-ketoacyl synthase [≥30 s^{-1} (Cognet et al., 1983], β-ketoacyl reductase (20 s^{-1}), and enoyl reductase (37 s^{-1}) have been determined. (As previously discussed the dehydratase reaction complicates the interpretation of the last number.) If the turnover numbers are assumed to be independent of chain length, an upper bound to the steady-state turnover number can be estimated as $(1/43 + 7/30 + 7/20 + 7/37)^{-1}$ = 1.3 s^{-1}, which can be compared with the measured value of 0.8 s^{-1} (Cox & Hammes, 1983). Obviously the malonyl transacylase and thioesterase reactions can only make small contributions to the turnover number. [The turnover number for malonyl transacylase is >30 s^{-1} (Cognet et al., 1983).] Thus, many of the individual enzymes in fatty acid synthase appear to have comparable turnover

numbers, with no single step clearly having the smallest turnover number.

REFERENCES

Akiyama, S. K., & Hammes, G. G. (1981) *Biochemistry 20*, 1491–1497.

Anderson, V. E., & Hammes, G. G. (1984) *Biochemistry 23*, 2088–2094.

Bloch, K., & Vance, D. (1977) *Annu. Rev. Biochem. 46*, 263–298.

Cardon, J. W., & Hammes, G. G. (1982) *Biochemistry 21*, 2863–2870.

Cardon, J. W., & Hammes, G. G. (1983) *J. Biol. Chem. 258*, 4802–4807.

Cognet, J. A. H. (1984) Ph.D Thesis, Cornell University, Ithaca, NY.

Cognet, J. A. H., & Hammes, G. G. (1983) *Biochemistry 22*, 3002–3007.

Cognet, J. A. H., Cox, B. G., & Hammes, G. G. (1983) *Biochemistry 22*, 6281–6287.

Cox, B. G., & Hammes, G. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4233–4237.

Dugan, R. E., & Porter, J. W. (1970) *J. Biol. Chem. 245*, 2051–2059.

Frieden, C., Wolfe, R. G., Jr., and Alberty, R. A. (1957) *J. Am. Chem. Soc. 79*, 1523–1525.

Horecker, B. L., & Kornberg, A. (1948) *J. Biol. Chem. 175*, 385–390.

Hsu, R. Y., & Yun, S. L. (1970) *Biochemistry 9*, 239–245.

Katiyar, S. S., Cleland, W. W., & Porter, J. W. (1975) *J. Biol. Chem. 250*, 2709–2717.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Soulié, J. M., Sheplock, G. J., Tian, W., & Hsu, R. Y. (1984) *J. Biol. Chem. 259*, 134–140.

Stadtman, E. R. (1957) *Methods Enzymol. 3*, 931–941.

Stern, A., Sedgwick, B., & Smith, S. (1982) *J. Biol. Chem. 257*, 799–803.

Volpe, J. J., & Vagelos, P. R. (1973) *Annu. Rev. Biochem. 42*, 21–60.

Wakil, S. J., Stoops, J. K., & Joshi, V. (1983) *Annu. Rev. Biochem. 52*, 539–580.

Yuan, Z., & Hammes, G. G. (1984) *J. Biol. Chem. 259*, 6748–6751.

Calorimetric Studies of the Binding of *Streptomyces* Subtilisin Inhibitor to Subtilisin of *Bacillus subtilis* Strain N′ †

Katsutada Takahashi* and Harumi Fukada

Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

Received March 23, 1984

ABSTRACT: The binding of *Streptomyces* subtilisin inhibitor (SSI) to subtilisin of *Bacillus subtilis* strain N′ (subtilisin BPN′, EC 3.4.21.14) was studied by isothermal calorimetry at pH 7.0 and at various temperatures ranging from 5 to 30 °C. Thermodynamic quantities for the binding reaction were derived as a function of temperature by combining the data reported for the dissociation constant with the present calorimetric results. At 25 °C, the values are $\Delta G° = -57.9$ kJ mol^{-1}, $\Delta H = -19.8$ kJ mol^{-1}, $\Delta S° = 0.13$ kJ K^{-1} mol^{-1}, and $\Delta C_p = -1.02$ kJ K^{-1} mol^{-1}. The entropy and the heat capacity changes are discussed in terms of the contributions from the changes in vibrational modes and in hydrophobic interactions.

Many protein–ligand interactions are known to be accompanied by large changes in the entropy and heat capacity of the reacting species (Sturtevant, 1977; Hinz, 1983). For such systems, it seems probable that changes in hydrophobic interactions and in internal vibrational modes are major contributions to the large changes in thermodynamic quantities (Sturtevant, 1977). Hydrophobic interactions and vibrational modes are obviously very much dependent on protein conformation, and the process of specific interactions between protein molecules may thus be accompanied by large changes in them, leading to large entropy and heat capacity changes.

Streptomyces subtilisin inhibitor (Murao et al., 1972; Sato & Murao, 1973) is a unique protein with an M_r of 23 000 (dimer), which specifically and tightly binds to the alkaline proteases, especially subtilisin, to inhibit the catalytic activity, with a dissociation constant in the case of subtilisin of $K_d = 7.12 \times 10^{-11}$ mol dm^{-3} at pH 7.0 and 25 °C (Uehara et al., 1978). It has also been shown by a kinetic study (Uehara et al., 1980) that although the SSI[1] molecule exists as a dimer of identical subunits, the binding process can be interpreted by an independent site model. Thus, the dimerization state is not affected by the association with subtilisin.

In an earlier paper (Takahashi & Sturtevant, 1981), the thermal denaturation of the inhibitor and of the inhibitor–subtilisin complex was studied by differential scanning calorimetry, and it was found that the unfolding temperature of the enzyme is raised by about 20 K upon the binding of the SSI molecule at pH 7.00. The result was also obtained that the SSI molecule does not dissociate from the enzyme even after the complex is heated up to its denaturation temperature, 87 °C. These findings indicate that the binding of SSI to subtilisin BPN′ induces a large change in the molecular situation and that the process should thus be accompanied by large changes in the thermodynamic quantities. It would,

† Supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (No. 536005, 1980–1982).

[1] Abbreviations: SSI, *Streptomyces* subtilisin inhibitor; subtilisin BPN′, subtilisin of *Bacillus subtilis* strain N′.

0006-2960/85/0424-0297$01.50/0