Transient Kinetic Studies of Fatty Acid Synthetase

A KINETIC SELF-EDITING MECHANISM FOR THE LOADING OF ACETYL AND MALONYL RESIDUES AND THE ROLE OF COENZYME A*

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A kinetic self-editing mechanism for correcting errors in the loading of thioester substrates is described for the animal fatty acid synthetase reaction. In the catalyzed reaction, these substrates load competitively on a common phosphopantetheine site, and during each of the eight loading steps the enzyme sites are partitioned between competent and incompetent substrate molecules. The incompetently bound substrate is removed by CoA through reversal of the loading reaction and partitioning again occurs. The loading-unloading cycle is repeated until competent enzyme complex is formed and the reaction proceeds. Furthermore, at each step the loading of a malonyl residue is competitively favored as is the unloading of enzyme-bound acetyl groups.

This mechanism is entirely consistent with the recently postulated role (Stern, A., Sedgwick, B., and Smith, S. J. Biol. Chem. (1982) 257, 799–803) of CoA as a co-substrate. Supporting evidence is obtained by monitoring the progress curves of NADPH oxidation by chicken liver fatty acid synthetase in the stopped flow apparatus. At noninhibiting acetyl-CoA, the reaction shows an initial lag period as the result of preferential formation of malonyl-enzyme and time-dependent recycling of the loading step to obtain competent acetyl-enzyme. At a malonyl-CoA/acetyl-CoA ratio of 2:1, the induction time of the reaction is 1.02 ± 0.05 s at 6 °C. It decreases with increasing acetyl-CoA concentration or preincubation of the enzyme with acetyl-CoA which promotes acetyl-enzyme formation but is slightly increased upon preincubation with malonyl-CoA. Increasing acetyl-CoA causes a parallel decrease in steady state cycle time (i.e. the average time required to complete a single malonyl-CoA condensation cycle), suggesting that the latter is limited by the lag period. At inhibitory acetyl-CoA, the steady state cycle time is lengthened due to acetyl-enzyme formation at malonyl-CoA loading steps and to the recycling necessary to obtain competent malonyl-enzyme.

A requirement of CoA for the first condensation cycle is unequivocally demonstrated in conventional spectrophotometric assays and stopped flow experiments by using phosphotransacetylase and acetyl phosphate as a CoA trap. This requirement at each loading step is normally met by CoA generated through initial loading. At noninhibitory acetyl-CoA, added CoA inhibits the reaction and slightly increases the lag. At inhibitory acetyl-CoA, a low concentration of CoA increases the lag period by removing competently bound acetyl residues, while the steady state cycle time is decreased owing to the preferential removal of incompetently bound acetyl residues at each of the malonyl-CoA loading steps. At very high CoA, the lag period and steady state cycle time are increased by indiscriminate unloading and by competitive inhibition of noncovalent binding of both substrates.

In recent reports, the fatty acid synthetase reaction has been shown to require CoA as a co-substrate in catalytic amounts (1–5). The role of CoA was thought to be as the acyl acceptor in the termination step of the reaction (1, 2) or alternatively in substrate loading steps to remove inappropriately bound acetyl- or malonyl-residues in abortive complexes formed through competitive loading of these substrates (3, 4). In the present study, stopped flow experiments are undertaken to determine the transient behavior of this reaction and the effect of CoA. Progress curves of NADPH oxidation obtained at noninhibitory acetyl-CoA show an initial lag period due to preferential formation of malonyl-enzyme and time-dependent repetition of unloading-loading to acquire competent acetyl-enzyme. The lag period and steady state cycle time calculated from the steady state rate are affected by changing the acetyl-CoA/malonyl-CoA ratio and by preincubation of synthetase with either thioester substrate in a manner entirely consistent with the latter interpretation. A kinetic self-editing mechanism for correcting errors in the loading of substrates is delineated. A preliminary report of this work has been published (6).

EXPERIMENTAL PROCEDURES

Materials

DTT, HEPES, CoA (Calbiochem-Behring); acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, AcPO4, NADPH (P-L Biochemicals); phosphotransacetylase (EC 2.3.1.8, Boehringer Mannheim), blue Sepharose 6B, and Sephadex PD-10 columns (Pharmacia Fine Chemicals) were obtained from the above designated sources. S-Acetoacetyl-N-acetylcysteamine was synthesized according to Kumar et al. (7). Reagents for electrophoresis were from Bio-Rad. All other reagents were of analytical grade. Distilled water, further purified with a Millipore Milli-Q system, was used throughout. In some experiments, 1

1 The abbreviations used are: DTT, dithiothreitol; HEPES, 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid; AcPO4, acetyl phosphate.
commercial malonyl-CoA was purified chromatographically as described by Hsu et al. (8). As expected from the early data of Waki and Ganguly (9), acetyl-CoA was required for fatty acid synthesis with purified malonyl-CoA.

Purification of Chicken Liver Fatty Acid Synthetase

Fatty acid synthetase was purified from male Hubbard grandparent chickens, kindly provided by Agway Farms at 2 days of age, which were killed at 4–6 weeks of age following fasting and refeeding. The enzyme was isolated by modification of the original procedure of Hsu and Yun (10). All purification steps were carried out at 4 °C instead of room temperature to minimize proteolysis, and the buffers contained 1 mM DTT and 1 mM EDTA unless otherwise specified. The ammonium sulfate fractionation and calcium phosphate gel adsorption steps were performed as before, except the dialysis step prior to gel treatment was eliminated. During DEAE-cellulose chromatography, the column loaded with fatty acid synthetase was successively eluted with 0.04, 0.075, and 0.14 M potassium phosphate buffer, pH 7.0. A minor activity peak (peak I) was obtained in the 0.075 M buffer, whereas the bulk of enzyme (peak II) was eluted by 0.14 M buffer. Each peak was collected and concentrated by precipitation with 40% saturated ammonium sulfate. The pellet was dissolved in 40 ml of buffer containing 0.3 M NaCl. The purified enzyme was concentrated with ammonium sulfate as before and stored in 0.2 M potassium phosphate buffer containing 0.3 M NaCl. The purified enzyme was concentrated with ammonium sulfate as before and stored in 0.2 M potassium phosphate buffer, pH 7.0, containing 10 mM DTT, 3 mM EDTA, and 10% (v/v) glycerol at -70 °C. Under these conditions, the enzyme was completely stable for 1 year. The presence of two enzymatically active peaks confirmed earlier data from this laboratory (10). Peak II had a yield of 40% and a specific activity of 160 pmol of acetyl-CoA incorporated per min/mg of protein at 37 °C. It was homogenous in sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (Fig. 1) and is routinely used in the present study. Before each experiment, the enzyme was equilibrated with the appropriate buffer by passage through a Sephadex PD-10 column. The specific activity and kinetic parameters as well as fatty acid product pattern of peak I were comparable to those of peak II (i.e. 67% palmitic and 11% stearic acids). Furthermore, rechromatography of peak I on a DEAE-cellulose column yielded a bimodal elution pattern similar to that obtained for the unresolved enzyme, indicating interconvertibility between two forms. The reason for this behavior is not clear. However, the possibility of conformational isomers deserves consideration.

The concentration of purified fatty acid synthetase was determined at 279 nm using a dimer molecular weight of 500,000 (11).

Steady State Kinetic Assays of Fatty Acid Synthetase Activities

The kinetic parameters of fatty acid synthetase activities were obtained by initial velocity measurements of NADPH oxidation at 340 nm in a Gilford 250 or a Cary 16 spectrophotometer. The reaction mixtures contained 0.15 M potassium phosphate buffer, pH 7.0, 1 mM DTT, 3 mM EDTA, and appropriate substrates and enzyme as indicated below in a volume of 1.0 ml. The temperature was thermostatically regulated at 37 °C unless otherwise specified. In all cases, velocities were corrected for endogenous NADPH oxidation of a control without enzyme. Substrate and enzyme concentrations for the acetyl-CoA-primed reaction, and the ketoeductase reaction with acetoacetyl-CoA or S-acetoacetyl-N-acetylcysteamine were, respectively, acetyl-CoA, 0.2-14.4 μM, malonyl-CoA, 0.43-11.5 μM, NADPH, 140 μM, chicken liver fatty acid synthetase, 2-3 μg acetoacetyl-CoA, 3.8-91 μM, NADPH, 75 μM, chicken liver fatty acid synthetase, 100 μg; S-acetoacetyl-N-acetylcysteamine, 0.9-10 mM, NADPH, 75 μM, chicken liver fatty acid synthetase, 4-5 μg. Kinetic parameters of the acetyl-CoA-primed reaction were determined by fitting initial velocity data to an equation for a ping-pong mechanism with double competitive substrate inhibition

\[ V = \frac{V_m}{1 + \frac{K_s}{A} \left( 1 + \frac{M}{K_M} \right)} + \frac{K_M}{M} \left( 1 + \frac{A}{K_A} \right) \]

where \( V_m \) is the maximum velocity; \( A, K_A, K_M \) are the concentration, Michaelis constant, and inhibition constant for acetyl-CoA; and \( M, K_M, K_A \) are the corresponding values for malonyl-CoA. Parameters for the reductase partial reaction were determined by a weighted Lineweaver-Burk analysis. The concentrations of substrates used in steady state and stopped flow experiments were determined by absorption measurements according to the following molar extinction coefficients: acetyl-CoA and acetoacetyl-CoA, \( ε = 15,400 \text{ M}^{-1} \text{ cm}^{-1} \) at 259 nm, pH 7.0; malonyl-CoA and CoA, \( ε = 14,600 \text{ M}^{-1} \text{ cm}^{-1} \) at 260 nm, pH 2.0; S-acetoacetyl-N-acetylcysteamine, \( ε = 4,700 \text{ M}^{-1} \text{ cm}^{-1} \) at pH 7.0 (7); and NADPH, \( ε = 6,200 \text{ M}^{-1} \text{ cm}^{-1} \) at 346 nm, pH 9.0.

In experiments employing a CoA trap, phosphotransacetylase and 5 mM AcPO₄ were included in each assay. Since inorganic phosphate strongly inhibits the phosphotransacetylase reaction (12), potassium phosphate was replaced by a buffer containing 0.12 M HEPES, pH 7.0, and 20 mM activator KCl. Control experiments indicated that the enzyme was fully active in the presence of either phosphotransacetylase or AcPO₄ alone.

Stopped Flow Studies

Instrumentation—Stopped flow experiments were performed in a Durrum-Gibos Model D 110 apparatus equipped with a 20-mm light path cuvette. The output of this instrument (10 V for 1 absorbance unit) was fed through a Biomation Model 805 wavelength recorder (Gould-Biomation) and a I.C.S. Model 4880 instrument coupler to a Hewlett-Packard Model 85 computer provided with extended memory, floppy disc drive, model 7225 B plotter, and various enhancing software. The storage of data in a numeric form limits the time interval between acquisition of individual experiments to less than 1 min.

The instrumental dead time of 1 ms was determined by monitoring the reaction of 0.01 M ferric nitrate in 1 N sulfuric acid with 0.01 M
potassium thiocyanate at 650 nm. The initial absorbance data were fitted to the equation \( A = a(1 - e^{-b+c}) \) with a weighted nonlinear regression program, as described by Muenier et al. (13).

**Experimental**—The reaction mixtures contained 0.15 M potassium phosphate buffer, pH 7.0, 1 mM DTT, 3 mM EDTA, and appropriate amounts of substrates and fatty acid synthetase as indicated in legends. Assays of fatty acid synthetase activities were monitored in the stopped flow apparatus. The zero time absorption was corrected by appropriate reagent blanks obtained prior to each experiment. Endogenous oxidation of NADPH in the blank without enzyme was not detectable in these short term experiments. The progress curves of stopped flow experiments were highly reproducible. The results shown are averages of a minimum of five runs/experiment repeated at least once with different enzyme preparations.

**RESULTS**

**Initial Rate Profiles of Fatty Acid Synthesis from Acetyl- and Malonyl-CoA**—In these experiments, the fatty acid synthesis reaction was monitored in the stopped flow apparatus at 340 nm by mixing equal volumes of a solution containing enzyme and NADPH with one containing acetyl- and malonyl-CoA. At noninhibiting concentrations of CoA thioesters in the final reaction mixture, a lag period was observed before NADPH oxidation reached a steady state as shown in a typical experiment (Fig. 2, curve 1). A value of 1.02 ± 0.05 s for \( \tau \), the induction time (cf. Table I for definitions), was obtained which equals, within experimental error, 50% of the steady state cycle time (\( \tau \)) of 1.94 ± 0.02 s calculated from the steady state rate (Table I, Experiment 3). This lag of NADPH oxidation is presumably due to the presence of slow processes in the first malonyl-CoA condensation cycle. \( \tau \) decreases with increasing temperature to 100 ms at 27 °C and 60 ms at 37 °C, but is relatively unaffected by enzyme concentration between 35 nM and 0.7 \( \mu \)M. In order to maximize the lag period for precise quantitation, subsequent experiments were performed at 6 °C.

If an experiment is carried out with enzyme and thioester substrates placed in separate syringes as done in Fig. 2, curve 1, at final concentrations of 3.5 \( \mu \)M chicken liver fatty acid synthetase, 70 \( \mu \)M NADPH, 14 \( \mu \)M acetyl-CoA, and 28 \( \mu \)M malonyl-CoA, the progress curves (not shown) are similarly shaped. However, a burst of NADPH oxidation is not observed despite the high enzyme concentration employed, suggesting that the slow process occurs prior to the release of the oxidized nucleotide.

In other experiments employing constant malonyl-CoA at 13.5 \( \mu \)M, \( \tau \) is found to decrease with increasing acetyl-CoA (Table I, Experiments 1–3 and 5–7), accompanied by a parallel decrease in steady state cycle time in reactions containing up to 13.5 \( \mu \)M acetyl-CoA. The former result indicates that the slow process is facilitated by acetyl-CoA. The decrease in steady state cycle time obeys Michaelian kinetics and yields an apparent \( K_{m} \) of 1.6 \( \mu \)M. At high concentrations (27 and 100 \( \mu \)M) of acetyl-CoA, substrate inhibition occurs and steady state cycle time is markedly increased (Fig. 2, curve 2 versus curve 1; Table I, Experiments 5 and 6). The initial rate profile at 100 \( \mu \)M acetyl-CoA is complex (Fig. 2, curve 2) with a relatively slow burst. The burst size cannot be determined but is significantly smaller than the optical density change (0.0175) calculated for oxidation of two NADPH/enzyme dimers. The presence of this burst indicates that the slow process responsible for the observed lag is not the result of slow addition of substrates or subsequent rate-limiting step(s) in the reaction sequence, and that this process is limiting the rate of the first steady state cycle. The latter conclusion is supported by proportional decreases of \( \tau \) and \( c \) as indicated by a constant \( \tau/c \) ratio of 0.47–0.53 (Table I, Experiments 1, 2, 3, and 5) with a 9-fold increase of noninhibitory acetyl-CoA.

When the experiment in Fig. 2, curve 1, is repeated by preincubation of acetyl-CoA with fatty acid synthetase (Fig. 2, curve 3), a partial burst is again observed and is more prominent than that seen in Figure 2, curve 2, at high acetyl-CoA. A residual lag is also observed, but the steady state cycle time (Table I, Experiment 8 versus 3) is unchanged. In a separate experiment employing 100 \( \mu \)M acetyl-CoA and 200 \( \mu \)M malonyl-CoA, the effects of high acetyl-CoA are reversed by high malonyl-CoA concentration (Table I, Experiment 9 versus 7), and a lag period of 0.93 ± 0.01 s is regenerated with a decreased steady state cycle time. Preincubation with malonyl-CoA (Fig. 2, curve 4) slightly lengthens the lag period of 1.02 ± 0.05 s to 1.13 ± 0.05 s without an accompanying change in steady state cycle time (Table I, Experiment 10 versus 3). These effects of preincubation on initial rate of NADPH oxidation (i.e. acetyl-CoA shorts, and malonyl-CoA lengths the lag) but not on steady-state cycle time confirm the occurrence of a slow process as an early event in
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the reaction. Furthermore, this process may be identified with the time-dependent reordering of substrate binding for competency in catalysis. In the course of this study, it was found that commercial malonyl-CoA is contaminated by acetyl-CoA despite a claim to the contrary. Smaller amounts of CoA are also present. The acetyl-CoA content as analyzed by incubating the commercial product with phosphotransacylase (10 units/ml in 0.15 M K phosphate, 10 mM EDTA at pH 7.0) followed by determination of CoA release with 0.5 mM 5,5'-dithiobis(nitrobenzoic acid) is 7 mol%. Key experiments (Table I, Experiments 3 and 10) were therefore repeated with acetyl-CoA free malonyl-CoA obtained by paper chromatography (cf. see "Experimental Procedures" for details). The lag was determined by stopped flow experiments agree with those obtained the induction time. It should be noted that the steady state cycle time is used to facilitate comparison in this study, the steady state cycle time is used to facilitate comparison in conventional spectrophotometry at the same temperature within experimental error.

### Table I

| Experiment | Acetyl-CoA | Malonyl-CoA | CoA | t** | t*** |
|------------|------------|-------------|-----|-----|------|
| **a**      | 1.55 μM    | 13.5 μM     | 1.23 ± 0.06 | 2.58 ± 0.02 |
| **b**      | 3.35 μM    | 13.5 μM     | 1.08 ± 0.05 | 2.30 ± 0.04 |
| **c**      | 6.5 μM     | 13.5 μM     | 1.02 ± 0.05 | 1.94 ± 0.02 |
| **d**      | 6.5 μM     | 13.5 μM     | 1.05 ± 0.02 | 2.05 ± 0.02 |
| **e**      | 13.5 μM    | 13.5 μM     | 0.86 ± 0.04 | 1.74 ± 0.02 |
| **f**      | 27.0 μM    | 13.5 μM     | 0.66 ± 0.08 | 2.10 ± 0.04 |
| **g**      | 100 μM     | 13.5 μM     | 5.58 ± 0.40 |
| **h**      | 6.5 μM     | 13.5 μM     | 2.65 ± 0.02 |
| **i**      | 100 μM     | 200 μM      | 0.93 ± 0.01 | 1.74 ± 0.04 |
| **j**      | 6.5 μM     | 13.5 μM     | 1.13 ± 0.05 | 1.90 ± 0.04 |
| **k**      | 6.5 μM     | 13.5 μM     | 1.07 ± 0.04 | 2.02 ± 0.11 |
| **l**      | 6.5 μM     | 13.5 μM     | 1.07 ± 0.09 | 2.46 ± 0.02 |
| **m**      | 100 μM     | 13.5 μM     | 10 | 0.88 ± 0.05 | 3.72 ± 0.10 |
| **n**      | 100 μM     | 13.5 μM     | 100 | 2.65 ± 0.18 | 6.76 ± 0.18 |

* Average of the different runs inside the same experiment.

** Induction time which quantitates the lag period is determined by extrapolation of the linear steady state time course as shown in Fig. 2, curve 1.

** Steady state cycle time, the average time required to complete a single malonyl-CoA condensation cycle. This value is calculated from the steady state rate (i.e. the linear portion of progress curve) from the stopped flow instrument according to c = 2/(NADPH oxidized s⁻¹ chicken liver fatty acid synthetase subunit)⁻¹. For the purposes of this study, the steady state cycle time is used to facilitate comparison with the induction time. It should be noted that the steady state rates determined by stopped flow experiments agree with those obtained in conventional spectrophotometry at the same temperature within experimental error.

Experiments were carried out as in Fig. 2, curve 1, except acetyl-CoA and malonyl-CoA concentrations were varied as indicated.

Experiments were carried out as in Fig. 2, curve 1.

Same as in footnote e except purified malonyl-CoA (see "Experimental Procedures") was used.

Experiments were carried out as in Fig. 2, curve 3.

Experiments were carried out as in Fig. 2, curve 4.

Same as in footnote e except purified malonyl-CoA was used.

Experiments were carried out as in Fig. 2, curve 1, except CoA was included in syringe 2.

Experiments were carried out as in Fig. 6, curve 2.

Experiments were carried out as in Fig. 6, curve 3.

### Table II

| Variable substrates | kₐ | kᵦ | Vₙₐ/Ep |
|--------------------|----|----|--------|
| Acetyl-CoA + malonyl-CoA | 0.70 ± 0.22 × 10⁻⁴ (Kₐₐ) | 1.40 ± 0.43 × 10⁻⁴ (Kₐ) | 137 ± 20 |
| Acetoacetyl-CoA | 1.06 ± 0.31 × 10⁻⁴ (Kₐₐ) | 1.35 ± 0.45 × 10⁻⁴ (Kₐ) | |
| S-Acetoacetyl-N-acetylcysteamine | 30.5 ± 6.4 × 10⁻⁴ (Kₐₐₐ) | 31.4 ± 6.4 × 10⁻³ (Kₐₐₐₐₐ) | 1.99 ± 0.27 |

The reactions were carried out in a conventional Gilford spectrophotometer at 37 °C, pH 7.0, as described under "Experimental Procedures." FAS, fatty acid synthetase.
still present, and the parameters (Table I, Experiments 4 and 11) were identical with those employing impure malonyl-CoA within experimental error.

Acetoacetyl-CoA is reduced by NADPH via ketoreductase partial activity of the enzyme without de novo fatty acid synthesis. The steady state parameters of ketoreductase using this substrate or the alternate substrate S-acetoacetyl-N-acetylcysteamine and the acetyl-CoA-primed reaction as determined by conventional spectrophotometry are shown in Table II. The maximal rate for the S-acetoacetyl-N-acetylcysteamine reaction is ≈2 times that for the acetyl-CoA-primed reaction, suggesting that the hydride transfer step is the slow process, as expected. As shown in Fig. 5, progress curves of stopped flow experiments employing thiosters of acetocacetate as the only substrate are linear, with no detectable lag.

**Effects of CoA on Fatty Acid Synthesis from Acetyl- and Malonyl-CoA**—In recent reports, the fatty acid synthetase reaction has been shown to require CoA as a substrate in catalytic amounts (1-5). Since the observed lag results from slow reordering of enzyme-bound substrate and since the unloading reaction involves CoA, the effects of this coenzyme on the steady state and transient kinetic behavior of the enzyme are examined in the following experiments.

The CoA requirement for chicken liver fatty acid synthetase was established by inclusion of a CoA-trapping system containing phosphotransacetylase and AcP0₄ in the spectrophotometric assay for overall synthetase activity. NADPH oxidation is inhibited 90 ± 5% by this treatment (Fig. 4A). This inhibition is relieved 50% (to 45% of original activity) by addition of 100 μM potassium phosphate which inhibits the phosphotransacetylase reaction (12). It is also relieved transiently by addition of 10 mM CoA (Fig. 4B). Corresponding stopped flow experiments are shown in Fig. 5. The control curve containing no phosphotransacetylase (Fig. 5A, curve 1) is comparable to Fig. 2, curve 1. In the presence of phosphotransacetylase, (Fig. 5A, curve 2), the lag is infinitely lengthened by the CoA trap, as would be expected if CoA participates in the first malonyl-CoA condensation cycle prior to hydride transfer. Addition of 10 μM CoA (Fig. 5B, curve 2 versus curve 1) again relieves inhibition until the coenzyme is depleted by the trap.

Since CoA is a product of the reaction, it is expected to be also inhibitory. The steady state inhibition of fatty acid synthetase by CoA was shown in an early report by Katiyar et al. (14), and more recently by us2 at noninhibitory acetyl-CoA. At 100 μM acetyl-CoA and 13.5 μM CoA, if present, is included in S₅. Curve 1 is taken from Figure 2, curve 2, for comparison and has no added CoA. c = 2.93 s. Curve 2, 10 μM CoA. τ = 0.86 s; c = 1.79 s. Curve 3, 100 μM CoA. τ = 2.87 s; c = 6.52 s.

1) again relieves inhibition until the coenzyme is depleted by the trap.

Fig. 5. Effect of CoA depletion on the progress of NADPH oxidation with acetyl- and malonyl-CoA as co-substrates. A, curve 1 was obtained under conditions identical with those described in the legend to Fig. 2, curve 1, except that S₅ also contained AcP0₄. Final concentrations of chicken liver fatty acid synthetase and AcP0₄ were 0.5 μM and 5.0 mM, respectively. K phosphate buffer was replaced by HEPES buffer (see "Experimental Procedures"). Curve 2, conditions were the same as curve 1 except S₅ also contained phosphotransacetylase at a final concentration of 250 units/ml. B, curve 1 is the progress curve in A, curve 2, monitored for a longer time interval of 20 s. Curve 2, conditions were the same as in curve 1 except S₅ also contained CoA (final concentration, 10 μM).

Fig. 6. The effects of CoA on progress curve of NADPH oxidation at a high acetyl-CoA/malonyl-CoA ratio. Syringes in the stopped flow apparatus contained: S₅, chicken liver fatty acid synthetase plus NADPH; S₅, acetyl-CoA and malonyl-CoA at final concentrations of 0.7, 60, 100, 13.5 μM. CoA, if present, is included in S₅. Curve 1 is taken from Figure 2, curve 2, for comparison and has no added CoA. c = 2.93 s. Curve 2, 10 μM CoA. τ = 0.86 s; c = 1.79 s. Curve 3, 100 μM CoA. τ = 2.87 s; c = 6.52 s.

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Addition of 10 μM CoA (Fig. 6, curve 2) regenerates a lag period of \( \tau = 0.88 \pm 0.05 \) s and decreases the steady state cycle time from 5.58 ± 0.40 to 3.72 ± 0.10 s (Table I, Experiment 13 versus 7), indicating while CoA at this concentration activates the reaction, it lengthens the slow process. Addition of 100 μM CoA (Fig. 6, curve 3) is inhibitory and further increases \( \tau \) to 2.65 ± 0.18 s with a concomitant increase in steady state cycle time (6.76 ± 0.18 s; Table I, Experiment 14).

**DISCUSSION**

A simplified scheme for the reaction catalyzed by fatty acid synthetase is shown in Fig. 7. The reaction is primed by acetyl-CoA which condenses seven times with malonyl-CoA to yield palmitic acid as the final product. For the purpose of clarity, repetitive condensation cycles which differ only in the size of growing acyl chain are represented by a single, generalized cycle (enclosed area at right) and the hydroxyl acylation site is not shown. Following completion of chain elongation, palmitic acid is released from palmitoyl-enzyme by deacylation.

The catalytic reaction has an obligatorily ordered kinetic sequence for the covalent addition of substrates. During the first malonyl-CoA condensation cycle, acetylation occurs first (Step 1), which is followed by loading of a malonyl residue (Step 3), condensation (Step 4), and reduction of enzyme-bound acetoacetate to butyrate (Steps 5-7). In subsequent condensation cycles, only malonyl-CoA is required. Early studies (8, 14) established that the free, unliganded enzyme binds either substrate in a random manner. In the synthetase reaction when acetyl-CoA is added last to initiate NADPH oxidation, a time-dependent lag of initial rate was observed by Lynen (16) who suggested that this is due to malonyl-palmitic acid is released from palmityl-enzyme by deacylation.

The first step is noncovalent association of thioester substrate with the enzyme to yield the Michaelian complex, which is followed by acylation and dissociation of CoA. All steps are reversible. If an incompetent substrate molecule is loaded in a given step, it must be unloaded by CoA. Partitioning again

Recent discovery of a CoA requirement for the synthetase (1) led to the postulation by Sedgwick and Smith (3) and Stern et al. (4) of a role of this coenzyme for the removal of incorrectly bound acetyl or malonyl residues to allow access of the enzyme site to the competent substrate. This hypothesis is supported by data obtained in equilibrium binding studies which show rapid and competitive loading of both substrates and that CoA depletion enhances and CoA addition inhibits the extent of loading site occupancy. Critical evidence for such a role in the catalyzed reaction is provided by the transient kinetic experiments performed in this work.

On the basis of current knowledge, a kinetic self-editing mechanism for each of the eight loading reactions (Steps 1, 3, 9, etc.) in Fig. 7 is delineated as shown below.

In the loading process, acetyl-CoA and malonyl-CoA share the same phosphopantetheine (S1) site on the synthetase. The two substrates are partitioned on the basis of relative binding strength and concentration ratio, and for a given loading step, an abortive complex is also obtained. Initial loading of a malonyl residue on this site prevents loading of an acetyl residue (Step 1) and its transacylation to the cysteinyl (S2) site. Similarly, occupancy of this site by an acetyl residue during subsequent loading (Steps 3, 9, etc.) prevents loading of malonyl residue. Loading of either substrate may be described by a three step reaction (17) (where FAS is chicken liver fatty acid synthetase).

Acetyl-(malonyl-) CoA + FAS → acetyl-(malonyl-) CoA-FAS
→ acetyl-(malonyl-) FAS-CoA
→ acetyl-(malonyl-) FAS + CoA

The first step is noncovalent association of thioester substrate with the enzyme to yield the Michaelian complex, which is followed by acylation and dissociation of CoA. All steps are reversible. If an incompetent substrate molecule is loaded in a given step, it must be unloaded by CoA. Partitioning again

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**FIG. 7. A simplified scheme for the fatty acid synthetase reaction.** S1, phosphopantetheine-SH group; S2, cysteinyl-SH group on the ketoacyl synthetase active site domain; Ac, acyl group (C4, C6, etc. to C10); AcAc, ketoacyl group (C4, C6, etc. to C10); But, acyl group with carbon chain lengthened by 2C (C4, C6, etc. to C12); FAS, chicken liver fatty acid synthetase; Mal, malonyl. The heavy lines represent reversible loading steps.
occurs, and the loading-unloading sequence is repeated until a competent complex is obtained. While loading (Reaction 1 in forward direction) is not limiting the catalytic rate, repetitive partitioning is and is seen as a lag period in the two loading steps (Steps 1 and 3) during the first malonyl-CoA condensation cycle. Moreover, the competent complex derived from either initial or repeated loading is subjected to partitioning in the steady state between unloading by CoA and the forward reaction as determined by the relative magnitude of the rate constants and the CoA concentration. Since CoA activates unloading of both substrates, equilibrium between competent and the abortive complexes is accelerated. Increasing CoA has the effect of simultaneously facilitating competent loading by removing the abortive substrate and reducing it by removing the competent substrate, with the net result of either stimulating or hindering a given loading step depending on the relative sensitivity of the bound substrate toward CoA. Since acetyl-CoA is required for Step 1 and malonyl-CoA is required for subsequent loading, a stimulating (or inhibitory) effect on the former would be accompanied by an opposite effect on the latter. In addition, since noncovalent association of either acetyl- or malonyl-CoA (step 1; Reaction 1 in forward direction) for the enzyme owes, at least in part, to the affinity of the CoA group, free CoA would be a potent competitive inhibitor of this step.

In the following discussion, our experimental observations are analyzed on the basis of the kinetic self-editing mechanism. During the loading process, covalent incorporation of malonyl residues is competitively favored. When acetyl-CoA is in limited access as in Fig. 2, curve 1, malonyl-enzyme is formed predominantly and the lag period in NADPH oxidation is primarily due to slow re-equilibration to obtain competent acetyl-enzyme through Step 1. Reduction of the lag period under conditions (i.e., increasing acetyl-CoA concentration or preincubation with acetyl-CoA; Fig. 2, curves 2 and 3, and Table 1) predisposed to acetyl-enzyme formation, but not under conditions (i.e., preincubation with malonyl-CoA) inducing malonyl-enzyme formation, is in agreement with malonyl-CoA being the preferred substrate. Since synthesis of palmitic acid requires seven additions of malonyl-CoA each acetyl-CoA, this property permits the attainment of greater catalytic efficiency. The complex burst shown in experiments with acetyl-CoA more readily accessible (Fig. 2, curves 2 and 3) represents combined initial rate profiles of three separate enzyme species. Those molecules contain competently bound acetyl and malonyl residues which yield the burst, and those molecules contain incompetent malonyl (for Step 1) or acetyl (for Step 3) residues which require equilibration resulting in a lag period.

In addition to reducing the lag period, high acetyl-CoA induces acetyl-enzyme formation which prevents proper loading of malonyl residues (Steps 3, 9, etc.) and prolongs the steady state cycle time. The reversal of both effects by high malonyl-CoA (Table 1, Experiment 9) further supports the above analysis.

A CoA requirement for the acetyl-CoA-primed reaction of chicken liver fatty acid synthetase is unequivocally established in conventional assays (Fig. 4) and stopped flow experiments (Fig. 5) by the use of a CoA trap. The small increase (rather than decrease) of lag period by added CoA in the stopped flow experiment using limited acetyl-CoA (Table 1, Experiment 12 versus 3) indicates that this requirement is normally met by CoA released through loading of thioester substrates and that increased CoA preferentially stimulates unloading of enzyme-bound acetyl, rather than malonyl, residues as the result of differences in kinetic parameters of the two decarboxylation reactions. The larger increase in steady state cycle time and the inhibition of the steady state rate in conventional assays by CoA of "Effects of CoA on Fatty Acid Synthetase from Acetyl- and Malonyl-CoA" are due to this effect and more importantly to inhibition of noncovalent association (Step 1 and Reaction 1 in forward direction) of both substrates. At high acetyl-CoA (Fig. 6), this substrate is more extensively loaded. 10 μM CoA promotes its unloading, and this effect is manifested as a lag due to removal of competently bound acetyl residues obtained through Step 1 and a decrease in the steady state cycle time (Fig. 6, curve 2, and Table 1, Experiment 13 versus 7) due to removal of abortive acetyl residues obtained at steps (3, 9, etc.) where malonyl-CoA loads. At 100 μM CoA, the synthetase reaction is strongly inhibited (Fig. 6, curve 3, and Table 1, Experiment 14) by inhibition of nonvalent association and unloading of both substrates. Additional CoA effects, such as those on the termination reaction (1, 2) or reductase reaction, however, cannot be excluded.

The kinetic self-editing mechanism depicted in this study is similar to the proof-reading process in DNA polymerases and amino acyl-tRNA synthetases and provides fatty acid synthetase with the ability to correct errors in the loading of thioester substrates. For a more detailed understanding of this mechanism, equilibrium isotope exchange experiments are currently being carried out to determine the kinetic parameters of the loading reactions (Reaction 1) for acetyl- and malonyl-CoA.

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