On the Question of Half- or Full-site Reactivity of Animal Fatty Acid Synthetase*

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Our model of the animal fatty acid synthetase describes a head-to-tail arrangement of two identical subunits and predicts the presence of two centers for fatty acid synthesis. Current experiments which support this conclusion were conducted using the following approach. The thioesterase component of chicken liver fatty acid synthetase was either inhibited using phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate, or released from the synthetase by limited proteolysis with α-chymotrypsin, thus ensuring that the fatty acyl products remain bound to the enzyme. Employing such preparations, the amount of NADPH oxidized in the initial burst of fatty acid synthesis was determined by stopped flow techniques. Gas-liquid chromatography showed that C_{16:0} and C_{18:0} constituted 85% of the fatty acids formed de novo, a result that was confirmed using [1-14C]acetyl-CoA in the reaction. These data showed that 1.0 mol of fatty acyl enzyme product was formed per mol of phosphopantetheine; in addition, the measured stoichiometry of NADPH oxidation was sufficient to account for de novo fatty acid synthesis. Altogether, these results indicate that the two sites for fatty acid synthesis are active and function simultaneously. They also indicate that the thioesterase plays a key role in determining the chain specificity of fatty acid synthesis.

The animal fatty acid synthetase has been shown to be multifunctional in that a single peptide has the ability to catalyze a number of different reactions (1). The functional form of the synthetase is a homodimer and each subunit of this complex, although identical with the other, can by itself catalyze only six of the seven reactions involved in fatty acid synthesis (2). The missing β-ketoacyl synthetase catalytic activity is exhibited only when the two subunits are together in a complex. It has been shown by chemical modification studies that this condensation reaction requires cooperation involving actual sharing of catalytic residues between the subunits in the complex. Relying on this information, a structural model has been proposed for the homodimer in which the subunits are arranged in a head-to-tail manner (3).

In accordance with this model, the chicken liver fatty acid synthetase dimeric complex possesses two complements of each catalytic activity involved in fatty acid synthesis and, therefore, two potential fatty acyl synthesizing centers. There are also other enzyme systems known to have a similar structure (i.e. oligomers composed of an even number of identical subunits), a few of which are alkaline phosphatase, malate dehydrogenase, and lactate dehydrogenase (4). These enzymes are all thought to be half-sites active in that they exhibit strong negative cooperativity in substrate binding. Therefore, at any given time, only half of the available catalytic centers are active, indicating that the overall reaction is a flip-flop type (4). The evidence for this proposal is mostly derived from substrate-binding studies with few direct demonstrations of the stoichiometry of actual amount of product formed relative to the number of enzyme active sites available. One of the most convincing cases for half-site reactivity has been made for the alkaline phosphatase of Escherichia coli (4) which was found to exhibit negative cooperativity in formation of covalent enzyme-substrate intermediates.

For our continuing investigations into the mechanism of the fatty acid synthetase, it was important to test the applicability of the half-site reactivity hypothesis to this system. Toward this end, the stoichiometry of NADPH oxidized by thioesterase-deficient fatty acid synthetase and the type and amount of fatty acids synthesized in the process were determined. This stoichiometric relationship was then compared to determine if it corresponds more closely to that expected from a full-sites active synthetase or a half-sites active one.

**EXPERIMENTAL PROCEDURES**

Preparation and Assay of Enzyme—Chicken liver fatty acid synthetase was prepared and assayed as described previously (1) and had a specific activity of 1400~1800 nmol of NADPH oxidized min⁻¹ mg⁻¹ at 25 °C. The protein concentration was estimated using a Gilford 240 recording spectrophotometer and the relationship Aλ_{obs} = 9.56 at 280 nm (5). The wavelength and absorbance scales were calibrated as described previously (5).

Chemicals—Buffers were prepared from Baker reagent grade salts and pH was determined as described previously (6). NADPH and acetyl- and malonyl-CoA were purchased from P-L Biochemicals; [1-14C]acetyl-CoA obtained from New England Nuclear was determined to be essentially pure by high pressure liquid chromatography performed as described previously (3). Radioactivity was estimated by counting the samples in Triton-toluene (1). PMSF (Sigma) was recrystallized from benzene-hexane before use. DIFP (Sigma) was used without purification. α-Chymotrypsin (Worthington) was reported to have a specific activity of 50 units/mg.

Inhibition of Thioesterase—All inhibitions were carried out at 25 °C and were accomplished in the following three ways: (a) with DIFP, synthetase (4 mg/ml) in 0.1 M sodium phosphate, 1 mM EDTA, 1 mM

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1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DIFP, diisopropyl fluorophosphate; Tris base, tris(hydroxymethyl)aminomethane; FID, flame ionization detector.
dithiothreitol (pH 6.5) was made 12 mM in DIFP (added from solution in isopropanol; final isopropanol concentration 2%, v/v). When synthetase activity was 7% of control, the enzyme was subjected to dialysis against 1 and then 6 liters of the same buffer. After dialysis, the enzyme was diluted to 1.01 mg/ml, assayed (4% of control activity), and subjected to stopped-flow analysis as described below. (b) With PMSF, synthetase (4 mg/ml) in the same buffer as above, was treated with PMSF (final concentration of 2.5 mM, added from solution in acetone; final concentration of acetone in the enzyme solution (2.5%, v/v) until the overall fatty acid synthesizing activity was 4% of control value. The inhibited enzyme was dialyzed against buffer in the same manner as described above, to remove unreacted PMSF. After dialysis, the enzyme was diluted to 1.01 mg/ml, assayed (2% of control synthetase activity), and subjected to stopped-flow analysis. (c) With a-chymotrypsin, synthetase (10 mg/ml) in 0.05 M Tris base, 0.1 mM dithiothreitol, 1 mM EDTA (pH 7.5) was treated with a-chymotrypsin at a protease to enzyme ratio of 1:2500 (w/w). In 30 min, the synthetase activity was 0.2% of control after which the enzyme was diluted with 0.1 M sodium phosphate, 5 mM β-mercaptoethanol, 1 mM EDTA (pH 6.5) to 1.01 mg/ml, and subjected to stopped-flow analysis. Aliquots of native and proteolyzed synthetase were also treated for and subjected to Tris-glycine-sodium dodecyl sulfate-polycrylamide gel electrophoresis (5% polyacrylamide), as described previously (8), to confirm that no extra proteolysis had taken place. Under the conditions described, α-chymotrypsin proteolysis was shown to only result in the cleavage of a 33,000-D peptide containing the thioesterase activity from the native 250,000-D synthetase monomer (14).

4'-Phosphopantetheine Content of the Fatty Acid Synthetase—This was determined as described previously (5) by measuring the amount of taurine present in a known amount of the synthetase which had been subjected to performic acid oxidation and HCI hydrolysis.

Stopped-Flow Analysis—Stopped-flow analyses were performed using an Aminco DW-2 recording spectrophotometer with an Aminco stopped-flow attachment. The synthetase solution was loaded in one syringe and reaction substrates in the other, then the two were mixed (in a 1:1 volume ratio), and the burst of NADPH oxidation was observed at 340 nm. A standard absorbance solution (5) was used to calibrate the absorbance at 340 nm and determine the ratio in which solutions from the two loading syringes were mixed in the stopped-flow accessory; this ratio was found to be within 2% of the expected 1:1.

For purposes of collection and fatty acid extraction, 4.5 ml of enzyme solution and the same volume of substrate solution were loaded into the drive block. The two solutions were pushed through the mixing chamber (within 1–2 s) and collected in a tube where the reaction was allowed to proceed for 20–50 s (time required to reach the steady state of NADPH oxidation) before being quenched by making the solution 5% in trichloroacetic acid. At this stage, an aliquot containing a known amount (calculated to be in the same range as the expected amount of de novo synthesized fatty acids) of C17:0 and C18:0 fatty acids was added to each sample to serve as an internal standard to compensate for handling losses.

Extraction and Methylation of Fatty Acids—The samples collected above were centrifuged at 10,000 × g for 20 min and the supernatant was separated from the protein pellet. The supernatant; fluid was extracted separately and found to contain less than 10% of internal standard fatty acids and the synthesized C16:0–C20:0 fatty acids, respectively. The protein pellet was dissolved in 1 ml of the same enzyme buffer used before by raising the pH to 12–13. In order to release the fatty acyl product from its thioester linkage to the enzyme, the solution was heated in a boiling water bath for 30 min. The mixture was acidified to pH 1–2 and the fatty acids were extracted three times by the Bligh and Dyer procedure (7). Methyl esters of fatty acids were prepared by methylation with diazomethane as described previously (8, 9).

Chromatography of Fatty Acid Methyl Esters—The methyl esters prepared above were purified by thin layer chromatography on silica gel (HR) plates (20 × 6 cm), developed in chloroform. The spots containing methyl esters were scraped off and the esters were eluted from the gel with hexane/diethyl ether (90:10, v/v). The solvents were then evaporated and the esters were dissolved in carbon disulfide prior to analysis by gas-liquid chromatography on a Varian 3700 gas chromatograph equipped with a FID and a 180 cm × 2 mm (i.d.) glass column packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco). Carrier gas was helium with a flow rate of 40 ml/min and the column was programmed from 140 to 220 °C at 2 °C/min. Injector temperature was 230 °C and the FID was maintained at 240 °C. The fatty acid methyl ester peaks were identified by matching retention times with those of peaks from a known standard run under the same conditions. The area under each peak was integrated using a Columbia Scientific Industries Supergrator-3 programmable integrator, and converted to relative nanomoles by comparison with the peak areas of the internal standards.

Analyses of radioactive methyl ester samples were carried out using a Hewlett-Packard 57110 A gas chromatograph on a similar column as mentioned above. Carrier gas in this case was nitrogen at 40 ml/min. The instrument was equipped with a splitter device which channeled part (60%) of the effluent gas to the FID for determination of mass, and the remaining to an exit port where samples were
collected for measuring radioactivity. These samples were counted in toluene-Omnifluor (New England Nuclear) as described before.

RESULTS

Measurement of Burst of NADPH Oxidation by Thioesterase-deficient Fatty Acid Synthetase—The oxidation of NADPH by thioesterase-deficient fatty acid synthetase was monitored in a stopped-flow apparatus and a representative recording obtained is shown in Fig. 1A. The initial exponential decrease in absorbance, due to synthesis of bound fatty acyl moieties, is over within 20 s and is followed by a slow linear decrease resulting from turnover of product by a small amount of native (unmodified) synthetase present in the reaction. The change in absorbance (ΔA) measured at various times from the chart shown in Fig. 1A was plotted in semilogarithmic form (Fig. 1B). The fact that the plot is linear during the first two half-lives of the reaction allows an accurate estimation of the amount of NADPH oxidized in the burst by using the ordinate intercept value from this graph together with the rate constants for the oxidation of NADPH. The rate constants for the oxidation of NADPH were 0.95 × 10⁻³, 0.35 × 10⁻³, and 0.86 × 10⁻³ for PMSF-, DFP-, and chymotrypsin-treated synthetase, respectively. The correspondence between these values indicates that modification of the thioesterase has not resulted in any differential modification of the other component activities. It is interesting to note that the rate constant for the modified synthetase is approximately one-tenth that of the native enzyme (estimated from its specific activity). The reason for the difference is not known, but it could be related to activation of the native synthetase by the free fatty acids produced during its assay.

To further establish the validity of this analysis, the effect of different enzyme and substrate concentrations on the size of the NADPH burst was investigated and the data for PMSF-treated enzyme are summarized in Table I. The constancy of the stoichiometry of NADPH oxidized in the burst demonstrates that this value is independent of enzyme or substrate concentration. Similar results were obtained with DFP- and chymotrypsin-treated synthetase. We confirmed separately that the thioesterase-minus synthetase alone with NADPH, or with NADPH and acetyl-CoA, or NADPH and malonyl-CoA led to less than 10% of the NADPH oxidation observed over the time period of the burst (20-50 s) in the presence of all the substrates. These results satisfy the requirements for a successful active site titration, namely saturating substrate concentration, rate constant of the burst much greater than rate due to turnover of product and occurrence of reaction only at the active site of the enzyme (10). Also, in Table II are compared NADPH bursts obtained with synthetase treated in three different ways to inhibit the thioesterase. Two of the treatments involve derivatization with chemical reagents (DFP and PMSF), while the third entails limited proteolysis. In all cases, the stoichiometry of the NADPH oxidation is in good agreement, thus indicating that all three modification procedures affect only the thioesterase. This result is in agreement with previous studies which showed that PMSF and DFP specifically inhibit the thioesterase without affecting the other component activities of the animal fatty acid synthetase. Moreover, α-chymotrypsin is known to specifically cleave the thioesterase component of the synthetase under limited proteolysis conditions and the “core” peptide retains all the other six enzymatic activities.

**Table I**

| Treatment* | [Synthetase] | [NADPH] | NADPH consumed/ enzyme dimer |
|------------|-------------|---------|-----------------------------|
|            | µM          | µM      | mol/mol                      |
| PMSF       | 0.28        | 6.5     | 28                           |
| DFP        | 0.53        | 12.6    | 24                           |
| Chymotrypsin | 1.05      | 27.1    | 26                           |

*See "Experimental Procedures" for a detailed description of each treatment.
In order to relate the observed stoichiometry of 26 mol (on average) of NADPH oxidized/mol of enzyme dimer to the yield of fatty acyl moieties it was necessary to identify and determine the relative amounts of the fatty acyl products synthesized during the burst of NADPH oxidation. In addition, the maximum number of fatty acyl chains per dimer of thioesterase-minus synthetase is limited by (can only be less than or equal to) the amount of 4'-phosphopantetheine cofactor in the dimeric complex. Previous studies have shown that this value varies between 1.4 and 1.7 for different enzyme preparations (5). Therefore, it was important to determine the content of this residue (which carries the fatty acyl chain during synthesis) in the enzyme preparation used for NADPH and fatty acyl stoichiometry determinations. The 4'-phosphopantetheine content of the synthetase preparation used in this study was found to be 1.4 and 1.2 mol/mol of dimer in two separate determinations and the average value of 1.3 mol was used for stoichiometry calculations.

Identification and Quantitation of de Novo Synthesized Fatty Acids—The fatty acids synthesized were extracted, converted into methyl esters, and analyzed by gas-liquid chromatography. Profiles of endogenous fatty acids (noncovalently bound to the synthetase) and endogenous plus de novo synthesized fatty acids, are shown in Fig. 2. It is apparent that the fatty acids synthesized to the greatest extent are C16:0 and C18:0. This was confirmed by using [1-14C]acetate- CoA and Fig. 3 shows the distribution of radioactivity in de novo synthesized fatty acids; again C18:0 and C20:0 account for the majority (85%) of the labeled product.

The calculated relative amounts of the fatty acids synthesized de novo are shown in Table III. The total amount of fatty acids synthesized relative to 4'-phosphopantetheine was 1.0 mol of fatty acid/mol of 4'-phosphopantetheine.

The number of moles of NADPH required to synthesize fatty acids in the relative distribution observed can be calculated, and in Table IV these values are compared with the stoichiometry of NADPH oxidation (normalized to 4'-phosphopantetheine content) actually observed by the spectrophotometric determinations.

DISCUSSION

In our investigations, we have treated chicken liver fatty acid synthetase with either DIFP, PMSF, or α-chymotrypsin, thus inhibiting the thioesterase activity, and preventing the release of the product fatty acid. This modified synthetase can be used to determine the stoichiometry of substrate utilized, and product synthesized, relative to the number of enzyme active sites available.

The amount of NADPH oxidized by the proteolyzed enzyme in the presence of all of its substrates was determined by stopped-flow analysis and was found to be approximately 26 mol/mol of enzyme dimer. The size of this burst is very close to that obtained using synthetase inhibited with DIFP or PMSF indicating that all three treatments allow a valid comparison.
Fig. 4. Diagrammatic scheme of fatty acid synthesis by full-site active chicken liver fatty acid synthase.
measurement of NADPH oxidized during the burst period (Table II). Also, the burst is independent of enzyme or substrate concentration in the range used for our experiments (Table I).

The physiological, and in vitro, product of chicken liver fatty acid synthetase is mostly palmitate (C_{16}o), but the thioesterase-lacking enzyme synthesizes mainly C_{18}a and C_{20}a fatty acids with longer chain fatty acids being formed after prolonged incubation (Tables III and IV). This observation indicates that the formation of palmitate by the native synthetase is a function of the specificity of the thioesterase component to preferentially hydrolyze the palmitoyl-S-enzyme linkage. This result is in agreement with previous studies on the substrate specificity of thioesterase isolated from goose uropygial gland fatty acid synthetase (12) and chicken liver fatty acid synthetase (13).

On quantitation of the de novo synthesized fatty acids and calculation relative to the number of available active centers (i.e. number of 4'-phosphopantetheine residues per dimeric complex) it was determined that 1.0 fatty acyl chain was formed per 4'-phosphopantetheine of the enzyme (Table III). This indicates that all of the available sites for fatty acid synthesis are active together. Table IV provides supportive evidence in this regard by demonstrating that the amount of NADPH oxidized that is accounted for by synthesis of fatty acid and quantitation of product closely with the amount oxidized as determined spectrophotometrically.

Although the 4'-phosphopantetheine content of the synthetase is only 1.3 mol/mol of dimer compared with the theoretical maximum of 2.0, the question of half- or full-site reactivity can be resolved. The synthetase preparation used may be composed of a mixture of dimeric molecules that have 2, 1, or 0 (the apo-form) complements of the 4'-phosphopantetheine cofactor. If there is no apoenzyme in the mixture, then the expected stoichiometry for a half- or full-site active enzyme would be 1.0 and 1.3 mol of fatty acid/mol of enzyme dimer, respectively. On the other hand, for a mixture containing 35 mol % apoenzyme, the corresponding stoichiometry would be 0.65 and 1.30 mol/mol. This latter composition finds some experimental support in cross-linking studies with dibromopropanone. We have previously shown that dibromopropanone cross-links the two subunits by cross-linking a bromopropanone. We have previously shown that dibromopropanone cross-links the two subunits by cross-linking the bromo- moiety to the cysteine so that the palmitoyl-S-enzyme linkage. This result is in agreement with previous studies using reagents for investigation of this question requires a large degree of precision in measurement of the various parameters; and quantitation of product may be subject to error. It is also important to relate the 4'-phosphopantetheine content of the synthetase to the amount of product formed, which was not done in this study. Smith and Stern (18) have more recently reported a similar study in which they concluded that trypsinized synthetase, which has 0.85 mol of phosphopantetheine/mol of $M_r = 220,000$ subunit, synthesizes 1 mol of long chain fatty acyl product/mol of dimer and therefore it is half-sites active. In this regard, we feel that our data accurately represent the functional nature of the enzyme since the direct estimation of NADPH oxidation from the burst is quantitative and agrees closely with the amount of fatty acyl product made. Also, we have normalized the data relative to the 4'-phosphopantetheine content of the synthetase preparation, since this value ultimately limits the maximal amount of product that can be synthesized. The results from these studies using reagents for chemical modification as well as evidence from the present investigation have led us to propose a scheme for fatty acid synthesis by the animal synthetase (19), as depicted in Fig. 4. In this mechanism, the initial transacylase reactions lead to the acylation of the condensation site cysteine-SH and the acyl carrier site cysteamine-SH with an acetyl and malonyl group, respectively. The subsequent condensation reaction results in the formation of acetoacetyl-S-pantetheine and the cysteine-SH is set free. The processing of the four-carbon chain to the butyryl derivative occurs, followed by transfer back of the butyryl moiety to the cysteine so that the pantetheine is free to be acylated with another malonyl group. Condensation and subsequent reactions are then cyclically repeated, ultimately forming the palmitoyl derivative which is hydrolyzed off by the thioesterase component of the complex. The two centers for fatty acyl synthesis in each dimer are equally active in this process.

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