EVIDENCE FOR AN "ACTIVE SERINE" IN EACH FATTY ACID SYNTHETASE PEPTIDE

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Summary: Ultracentrifugally homogeneous fatty acid synthetase was isolated from the uropygial gland of goose by a one-step purification procedure. Formation of fatty acids from malonyl-CoA and hydrolysis of palmitoyl-CoA catalyzed by the synthetase were inhibited to an equal extent by diisopropylfluorophosphate. With labeled inhibitor, it was shown that one mole of the inhibitor was covalently attached per mole of the subunit of the enzyme. Sodium dodecyl sulphate electrophoresis showed that all of the label was contained in a 270,000 M.Wt peptide. That the active serine was not at the loading site was suggested by the observations that neither acetylation nor malonylation of the enzyme affected the reaction of the enzyme with the inhibitor and acetylation or malonylation of the enzyme was not affected by this inhibitor. Thus, each fatty acid synthetase peptide is shown to have one active serine which most probably is at the chain terminating active site of the peptide.

Fatty acid synthetase of bacteria can be readily separated into catalytically active component enzymes and a 4'-phosphopantetheine containing protein (acyl carrier protein) by classical protein fractionation techniques (1). On the other hand, it has not been possible to separate the component enzymes from the fatty acid synthetase of animals, and it has been assumed that the component enzymes are held together by noncovalent interaction (2-5). Isolation of a small protein containing the 4'-phosphopantetheine from animal synthetases (6,7) has been taken as evidence for the multipeptide nature of the animal synthetase. However, recent experimental evidence indicated that the synthetases from chicken liver and rat liver (8), yeast (9), and the uropygial gland of goose (10) consist of two peptides, each with a molecular weight of about 250,000. 4'-Phosphopantetheine was shown to be attached to this large peptide in both chicken liver (8) and the uropygial gland of the goose (10). In the goose, each peptide appeared to contain a covalently attached 4'-phosphopantetheine moiety (10). In this paper we present evidence that each of the two

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peptides (270,000 M.Wt) of the synthetase of the goose contains an "active serine," strongly suggesting that each peptide contains a segment which catalyzes hydrolysis of thioesters (chain terminating activity).

MATERIALS AND METHODS

[^{3}H]Diisopropylfluorophosphate (3.9 Ci/mmole) was purchased from Amersham/Searle Corp. [1-^{14}C]Palmitoyl-CoA (30 Ci/mole) was purchased from New England Nuclear Corp. [2-^{14}C]Malonyl-CoA was synthesized chemically from [2-^{14}C]malonic acid as described previously (10). All other reagents were purchased from Sigma Chemical Co.

Excised uropygial glands of geese were homogenized in 100 mM phosphate buffer, pH 7.6, containing 250 mM sucrose, 1 mM $MgCl_2$, and 1 mM dithioerythritol (DTE). From the 105,000 xg supernatant, ultracentrifugally homogeneous fatty acid synthetase was purified by a one-step gel filtration method described elsewhere (10).

Fatty acid synthetase activity was usually determined spectrophotometrically by measuring the initial rates of NADPH oxidation. Reaction mixtures contained 15 µmoles phosphate buffer, pH 7.6, 0.04 µmole NADPH, 0.04 µmole malonyl-CoA, 0.01 µmole acetyl-CoA, 0.1 µmole DTE, and enzyme in a total volume of 0.2 ml. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard. Fatty acid synthetase with a specific activity of 900-1300 nmoles NADPH oxidized/min/mg protein was used in all experiments. Thioesterase activity was measured with palmitoyl-CoA. Reaction mixtures containing 40 µmoles phosphate buffer, pH 7.6, 5 nmoles [1-^{14}C]palmitoyl-CoA (2.4 Ci/mole), enzyme, and 0.4 µmole DTE in a total volume of 0.5 ml were incubated for 10 min at 30°C. After acidification with HCl, [1-^{14}C]palmitic acid was extracted with chloroform, which was in turn extracted with water, evaporated to dryness, and assayed for ^{14}C.

Solutions containing ^{14}C or ^{3}H were assayed by liquid scintillation spectrometry with 15 ml of Aquasol as the counting fluid using a Packard Model 3003 Tri-Carb scintillation spectrometer. Internal standards of [^{3}H]toluene and [^{14}C]toluene were used to determine a counting efficiency of 75% and 25% for ^{14}C and ^{3}H, respectively. Radioactivity in sodium dodecyl sulfate (SDS) polyacrylamide gels was analyzed by liquid scintillation spectrometry after hydrogen peroxide treatment as described before (12).

Treatments of the fatty acid synthetase with [2-^{14}C]malonyl-CoA and [^{3}H]diisopropylfluorophosphate are described in the appropriate legends. For peracid oxidation, fatty acid synthetase (0.3-0.8 mg), which was labeled with [2-^{14}C]malonyl-CoA, was lyophilized, then dissolved in 0.3 ml of 90% formic acid, 0.05 ml of 30% H_2O_2 was added, and the resulting mixture was incubated at 0°C for 12 hr. After incubation, 0.35 ml of water was added and the protein was precipitated by the addition of trichloroacetic acid (TCA) to a concentration of 10%. The precipitated protein was centrifuged and the resulting pellet washed four times with 1% TCA. The washed pellet was dissolved in 1 ml of 0.1 N NaOH and assayed for specific radioactivity.

RESULTS AND DISCUSSION

In a previous communication, we showed that the fatty acid synthetase of the uropygial gland of the goose, which synthesizes mainly multimethyl branched fatty acids *in vivo*, is composed of two dissociable peptides, each with a molecular weight of 270,000 (10). With a one-step purification procedure, we obtained ultracentrifugally homogeneous synthetase of specific activity which ranged from 800-1300 nmoles NADPH oxidized/min/mg. A classical 'active serine' directed reagent, diisopropylfluoro-

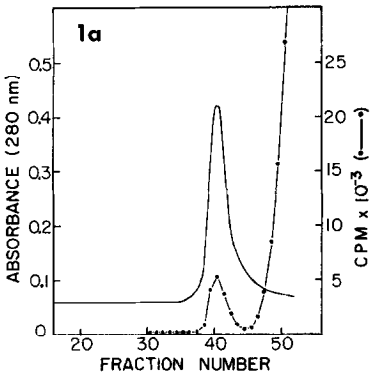
Table I. Effects of Dip-F treatments on formation of fatty acids and hydrolysis of palmitoyl-CoA catalyzed by fatty acid synthetase (FAS).

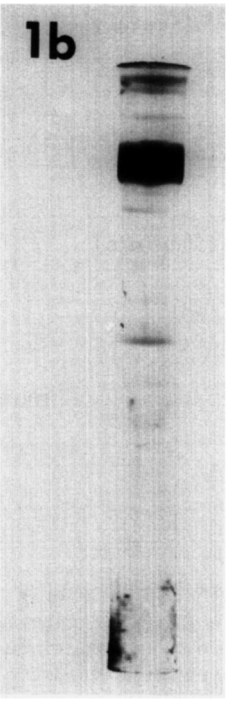
Experimental Conditions[a]	Activity of the Enzyme (% of Control)						
	Experiment I					Experiment II	
	FAS					FAS	Thioesterase
Incubation Period (min):	10	20	30	45	60	30	30
Dip-F (mM)							
1	--	--	--	--	--	89	74
2	100	94	91	88	85	68	69
5	74	63	51	45	43	45	32
10	34	17	8	6	4	13	8

[a]Fatty acid synthetase (0.3 mg in Expt. I and 0.54 mg in Expt. II) was incubated with Dip-F at room temperature at various concentrations and times as indicated above. In Experiment I, incubation mixtures containing 2, 5, and 10 mM Dip-F also contained 1, 2, 3, and 6% isopropanol, respectively, and in Expt. II all reaction mixtures contained 10% isopropanol. The highest concentration of isopropanol used had no effect on either the fatty acid synthetase activity or thioesterase activity, both of which were measured as indicated under the Experimental section.

phosphate (Dip-F), inhibited the fatty acid synthetase of the uropygial gland of the goose (Table I, Expt. I). This inhibition was dependent on the concentration of Dip-F and the time of preincubation. For example, a 10 min preincubation with 5 mM Dip-F resulted in only 26% inhibition, whereas a 60 min preincubation with 5 mM Dip-F and a 10 min incubation with 10 mM Dip-F showed 57% and 66% inhibition, respectively, and a 30 min incubation of the enzyme with 10 mM Dip-F resulted in a nearly complete inhibition of the synthetase. These results suggested that the inhibitor might be covalently attaching itself to the synthetase.

Since the fatty acyl thioesterase found in certain fatty acid synthetase preparations is known to be inhibited by phenylmethanesulphonylfluoride, the inhibition we observed with Dip-F was suspected to be by inactivation of the thioesterase involved in the chain termination process. A direct test of this hypothesis was made by determining the effect of Dip-F on palmitoyl-CoA hydrolysis (15-20 nmoles of





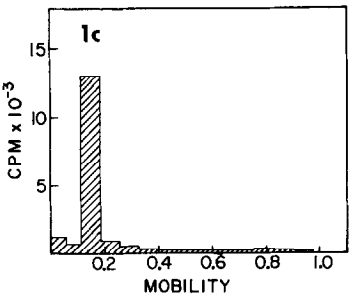


Figure 1a. Gel filtration of fatty acid synthetase after treatment with [^{3}H]Dip-F. The purified fatty acid synthetase (2 mg) was incubated with 20.5 nmoles [^{3}H]Dip-F (3.9 Ci/mmole) in 100 mM phosphate, pH 7.6, containing 1 mM DTE in a total volume of 0.5 ml for 60 min at 30°C. After incubation, the labeled fatty acid synthetase was filtered through a Bio-Gel P-2 column (1.4 × 45 cm), 1 ml fractions were collected, and ^{3}H in 0.1 ml aliquots are shown. Figure 1b. SDS-polyacrylamide gel electrophoresis of Dip-F treated fatty acid synthetase. The protein fractions from the experiment in Fig. 1a were pooled, concentrated, and heated at 100° for 1 min prior to the addition of SDS (1%) and DTE (0.1 M), followed by an additional 3 min incubation at 100°C. SDS-disc gel electrophoresis was performed as described before with 50 µg protein/gel (12). Figure 1c. Distribution of radioactivity in the SDS-polyacrylamide gel shown in Fig. 1b. Three gels were assayed for ^{3}H as indicated in the Experimental section.

palmitoyl-CoA/min/mg) catalyzed by the pure fatty acid synthetase (Table I, Expt. II). Dip-F inhibited the thioesterase and the degree of inhibition of the thioesterase was quite similar to that observed for the fatty acid synthetase reaction.

In order to further test the hypothesis that Dip-F reacts with the serine at the active site of the chain terminating enzyme, pure fatty acid synthetase was treated with radioactive Dip-F and the preparation was subjected to gel filtration with Bio-Gel P-2. The protein fraction eluted from the P-2 column contained the

Table II. Stoichiometry of the covalent binding of malonate and diisopropylphosphate to fatty acid synthetase.

Pretreatment	moles ligand bound / mole FAS subunit	
	Before Peracid	After Peracid
Experiment I[a]		
Malonate Bound		
No pretreatment	2.07	0.38
5% isopropanol	1.90	0.39
5% isopropanol, 5 mM Dip-F	1.76	0.39
Experiment II[b]		
Diisopropylphosphate Bound		
No pretreatment	0.78	--
1.25 mM acetyl-CoA	0.51	--
1.25 mM malonyl-CoA	0.80	

[a]Fatty acid synthetase (2 mg) was incubated with 3.2 μmoles [2-^{14}C] malonyl-CoA (0.45 mCi/mmole) in 100 mM phosphate, pH 6.8, containing 1 mM DTE in a total volume of 0.3 ml for 60 min at 30°C. Pretreatment with Dip-F was done by incubating the enzyme (in 100 mM phosphate, pH 6.8, containing 1 mM DTE) with 5 mM Dip-F for 60 min at 30° prior to the addition of [2-^{14}C]malonyl-CoA. After incubation, the labeled fatty acid synthetase was isolated using a Sephadex G-50 column (1.7 × 45 cm), followed by concentration with powdered Ficoll and the specific radioactivity of the protein was determined. [b]Fatty acid synthetase (2 mg) was incubated with 2 μmoles of [^{3}H]Dip-F (96 Ci/mole) in 100 mM phosphate buffer, pH 7.6, containing 1 mM DTE and 5% isopropanol in a total volume of 0.4 ml for 60 min at 30°C. For pretreatment, the enzyme (2 mg in 100 mM phosphate, pH 7.6, containing 1 mM DTE) was incubated with 1.25 mM acetyl-CoA or malonyl-CoA for 30 min at 30°C prior to the addition of [^{3}H]Dip-F. After incubation, the labeled synthetase was isolated with a Bio-Gel P-2 column (1.4 × 45 cm). The labeled protein fractions were pooled and concentrated with powdered Ficoll and the specific radioactivity of the protein was determined.

label (Figure 1a), showing that the labeled Dip-F was covalently attached to the enzyme.

Since a 'loading site' on the synthetase has been suggested to be a serine which is, in some unknown manner, "activated" (13,14), there is a possibility that such a serine was involved in the reaction with Dip-F, rather than the serine at the chain terminating active site. In order to test this possibility, we determined

the effect of Dip-F treatment on the formation of malonyl-enzyme from [2-^{14}C]malonyl-CoA (Table II, Expt. I). Each mole of the subunit of the fatty acid synthetase appeared to have about two moles of malonyl group attached to it, and prior Dip-F treatment did not substantially inhibit this malonylation. In addition, in all cases about 80% of the malonyl groups attached to the synthetase were presumably bound as thioesters as indicated by their susceptibility to peracid treatment (Table II, Expt. I). Thus, Dip-F treatment affected neither qualitatively nor quantitatively the attachment of malonyl group to the synthetase. Furthermore, as shown in Table II, Expt. II, neither acetyl-CoA nor malonyl-CoA inhibited the reaction of Dip-F with fatty acid synthetase. Thus, it is highly unlikely that the Dip-F reactive site is the loading site. This observation and the direct demonstration that the palmitoyl-CoA hydrolysis catalyzed by the synthetase is inhibited by Dip-F strongly suggest that the Dip-F reactive site is the active site of the chain terminating enzyme.

If gene fusion has resulted in the formation of a multifunctional polypeptide catalyzing all of the steps involved in fatty acid synthesis, the chain terminating enzyme might also be on this large peptide. In order to test this possibility, [^{3}H]Dip-F treated fatty acid synthetase was subjected to SDS treatment at 100°, followed by SDS-electrophoresis. Only one major Coomassie blue staining band was observed which was at about 270,000 molecular weight (Figure 1b), and this band contained essentially all of the radioactivity (Figure 1c). Therefore, it is clear that the Dip-F reacting site, which is most probably the active site of the chain terminating enzyme, is contained in this large peptide. The absence of significant amounts of label elsewhere in the gel also suggests that no serine protease was contained in this fatty acid synthetase preparation.

Our previous analyses indicated that each of the two peptides of the fatty acid synthetase contains a covalently attached 4'-phosphopantetheine. Since it is conceivable that each peptide may contain the active sites required for the catalysis of all of the steps involved in fatty acid synthesis, we attempted to determine the number of sites which reacted with Dip-F (Table II, Expt. II). In repeated experiments, the amount of labeled diisopropylphosphate attached to the protein

from 5 mM Dip-F ranged from 0.65 to 0.85 moles per mole of the subunit (270,000 M.Wt), and under these conditions only 60-90% of the fatty acid synthetase activity was lost. For example, a synthetase sample which had 0.83 moles of labeled inhibitor attached per mole of the subunit retained 8% of the fatty acid synthetase activity. If a correction for this unreacted portion is made, it is clear that more than 0.9 mole inhibitor was attached per mole of the inactivated subunit. Thus, it appears quite clear that each peptide contains one active serine which most probably is at the active site of the chain terminating enzyme.

The results which indicate that each peptide contains one 4'-phosphopantetheine and one chain terminating site raise the possibility that the primary structure required for the active sites involved in the catalysis of all of the steps of fatty acid synthesis are contained in each peptide. If such is the case, the interaction between the two peptides, which appears to be necessary to bring about catalysis of fatty acid synthesis, represents a regulatory feature. Such a regulatory feature is manifested by the observations that fatty acyl-CoA can inactivate the enzyme by dissociating it and NADPH can activate the enzyme by bringing about association (5,10,15).

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