FATTY ACID SYNTHETASE COMPLEX

Selective Inactivation by Phenylmethylsulphonyl Fluoride

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SUMMARY

Reaction of pigeon and rat liver fatty acid synthetases with phenylmethylsulphonyl fluoride at pH 7.0 results in rapid and complete loss of activity for fatty acid synthesis. Acetyl and malonyl transacylation, two reductions, dehydration and condensation-CO_2 exchange reactions are not appreciably altered in the modified enzyme. However, the deacylation of palmityl CoA is completely inhibited. Complete inactivation results in the incorporation of about 1.9 moles of ^{14}C-phenylmethylsulphonyl groups/mole of the enzyme complex. These results suggest that either two moles of a fatty acyl deacylase or two deacylases with different fatty acyl chain length specificities may be functional in the enzyme complex.

Fatty acid synthesizing systems from avian and mammalian species have been isolated in the form of tightly held arrays of enzymes (1, 2). Pigeon and rat liver enzyme complexes consist of at least seven functional enzyme units and have molecular weights of about 500,000 daltons (3, 4). These enzymes have resisted attempts at complete separation into individual functional units. Thus, the stoichiometry of the component enzymes in the multienzyme complexes is not yet established, unlike the bacterial (5) and plant enzyme systems (6) which have been resolved into independent active entities.

Studies on the elucidation of the mechanism of fatty acid synthesis have suggested that the acetyl and malonyl groups of the CoA esters are first transacylated to a "hydroxyl site" on the complex (7, 8). In yeast enzyme, separate initial transacylation sites for acetyl and malonyl groups have been identified (9). Such information is as yet unavailable for avian and mammalian enzyme systems. Previous studies by Wakil and co-workers (10) have indicated that the E. coli palmityl CoA deacylase may also be a "serine" type enzyme.

We have recently started studies on the mechanism of initiation of fatty acid synthesis and the control of the fatty acyl chain length specificity. As a first step, we have attempted to identify the nature and number of the "hydroxyl" type enzymes that are catalytically functional in the complex. In this communication, we wish to report selective inactivation of pigeon and rat liver enzymes by phenylmethylsulphonyl fluoride (PMSF), a well characterized specific inhibitor of chymotrypsin and trypsin (11).

MATERIALS AND METHODS

Methods of purification and assay conditions for pigeon and rat liver enzymes have been described previously (3, 4). Specific activities measured at 30^{o} and expressed as μmoles of NADPH oxidized/min/mg protein were 1.0-1.2 for pigeon liver enzyme and 0.8 for rat liver enzyme (3). Benzyl chloride (7-^{14}C) was purchased from New England Nuclear and ^{14}C-PMSF was synthesized according to Gold and Fahrney (12). Synthesis of N-acetyl cysteamine derivatives, conditions for the assays of partial reactions of fatty acid synthesis, and the sources of other radioactive and non-radioactive materials have been described previously (3).

RESULTS AND DISCUSSION

When pigeon and rat liver enzyme complexes are incubated with low concentrations of PMSF at 30^{o} in 0.25 M K-phosphate containing 1 mM EDTA and 1 mM dithiothreitol (DTT), the activity for fatty acid synthesis is rapidly lost. The inactivation rate profile appears to suggest biphasic kinetics for this reaction (Fig. 1). However, PMSF is not very stable under these conditions of ionic strength and temperature and has been found to hydrolyze with a half life of 10 minutes (13). The slowing down of the rate of inactivation with time could, therefore, be partially attributed to the continuous decrease in PMSF concentration. Under the above conditions, the time for 50% loss of activity calculated from the extrapolation of initial rate is 3 min at 1.0 mM PMSF concentration.

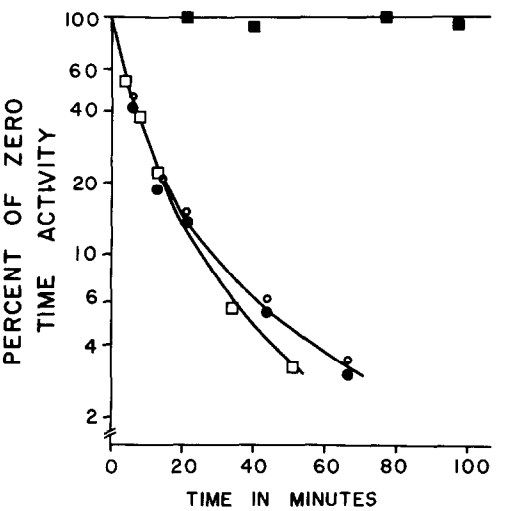

Fig. 1. Rate of inactivation of pigeon and rat liver fatty acid synthetases in the presence of phenylmethylsulfonyl fluoride. Enzymes stored in 0.25 M K-phosphate, 1 mM EDTA and 10 mM DTT, pH 7.0 were diluted into K-phosphate buffer to give a final concentration of 1.0 mg/ml. An aliquot (25 µl) of 40 mM phenylmethylsulfonyl fluoride in dry isopropanol was added/ml of the enzyme solution. Aliquots (50 µl) were removed at various time intervals and assayed for the activity for fatty acid synthesis under these conditions: Acetyl CoA, 30 µM; Malonyl CoA, 100 µM; NADPH, 100 µM and Enzyme, 10 µg/ml. Oxidation of NADPH was measured at 340 nm in 0.2 M K-phosphate, 1 mM EDTA, pH 7.0 at 30°. (■), Control enzyme incubated with isopropanol; (●) and (o), two different preparations of pigeon liver enzyme; (□), rat liver enzyme.

Since long chain fatty acid synthesis is the result of sequential coordination of at least seven reactions (3), further studies were carried out to identify the site or sites of PMSF reaction. Covalent binding of ^{14}C-acetyl and ^{14}C-malonyl groups of acetyl and malonyl CoA derivatives to native and PMSF modified enzymes were carried out. Electrophoretic patterns of ^{14}C-acetyl peptides obtained by peptic digestion of two protein species are shown in Fig. 2. Peptide fragments marked A_2, B_2, and B_1, have previously been identified as belonging to regions of the 4'-phosphopantetheine-SH, cysteine-SH and hydroxyl site, respectively (13). Identical peptide patterns for the native and PMSF modified enzyme were obtained. Additionally, no qualitative or quantitative differences in the binding of ^{14}C-acetyl groups

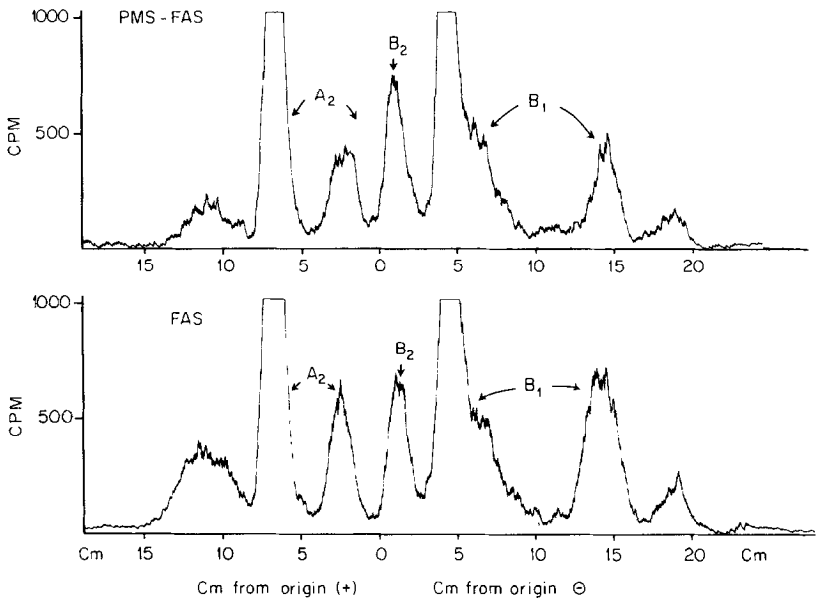

Fig. 2. Electrophoretic patterns of ^{14}C-acetyl peptides obtained from native (FAS) and PMSF modified pigeon liver fatty acid synthetase (PMS-FAS). Fatty acid synthetase (5 mg/ml) in 0.25 M K-phosphate, 1 mM EDTA, 1 mM DTT, pH 7.0 was treated with 1.0 mM PMSF. Rate of loss of activity was followed to about 1% of original activity. Inactive enzyme was dialyzed at room temperature against 2 1. of 0.25 M phosphate, 1 mM EDTA, pH 7.0 with buffer changes every 3 hours to a total of 12 hours. Activity for fatty acid synthesis was rechecked after dialysis and was found to be < 1% of the original. Fatty acid synthetase or the PMSF modified enzyme (1 ml of 5 mg/ml) was incubated at 0° for 30 seconds with 10 fold molar excess of ^{14}C-acetyl CoA (11,027 dpm/mμm). The reaction was then stopped with 0.03 ml of 60% $HClO_4$. Rest of the experimental procedure has been described previously (3). Electrophoresis was performed with an instrument from Savant Instruments, Inc. New York. Radioactive scanning was performed with Nuclear Chicago Actigraph III.

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to the hydroxyl, cysteine and 4'-phosphopantetheine sites were observed for the two enzyme species. Evidently, the enzymes associated with the transacylation of the acetyl group to the three sites are functional in the PMSF enzyme. The covalent binding of the ^{14}C-malonyl group of malonyl CoA to active and inactive enzyme species also gave identical patterns which were similar to those obtained previously (3). These studies were further confirmed by measuring the enzyme catalyzed transacylation of acetyl and malonyl groups from CoA esters to D-pantetheine in solution. The rates

of acyl pantetheine formation with active and with PMSF inactivated enzymes were identical (Table I). Thus, unlike E. coli malonyl transacylase, pigeon

TABLE I

Partial Reactions of Fatty Acid Synthesis

All partial reactions were carreid out in 0.2 M K-phosphate pH 7.0 containing 1 mM EDTA and 1 mM DTT.

No.	Reaction	Activity (mμm/min/mg protein)	
		Fatty Acid Synthetase	PMSF Inactivated Fatty Acid Synthetase[a]
1	Acetyl Transacylase[b]	157	148
2	Malonyl Transacetylase[b]	149	152
3	Condensation-CO_2 Exchange[c]	3.46	2.75
4	Palmityl CoA Deacylase[d]	36.4	~1

[a] PMSF inactivated fatty acid synthetase was prepared as described in the legend to Fig. 1.

[b] The transacylase reactions were carried out in a total volume of 0.1 ml at 0°. Assay conditions were: Acyl CoA, 0.1 mM; D-pantetheine, 3.4 mM and Enzyme 1 μg/0.1 ml. Specific radioactivities of acyl CoA compounds were 1-^{14}C Acetyl CoA, 11,027 dpm/mμm; 1,3^{14}C-malonyl CoA, 5472 dpm/mμm. Reactions were stopped after 20 min. Separation and quantitation of acyl CoA and acyl pantetheine has been described before (3).

[c] Reaction conditions were: malonyl CoA, 0.3 mM; hexanoyl CoA, 0.2 mM; CoA, 1.0 mM; $H^{14}CO_3^-$, 32.5 mM (0.9 μCi/μmole) and protein, 1.25 mg/ml. Reactions were carried out at 30° in 0.2 ml volume, stopped after 15 min with 50 λ of 5% $HClO_4$. Radioactivity incorporated into malonyl CoA was quantitated as described previously (3).

[d] Reaction was carried out at 30°. Palmityl CoA (4 μM; Specific activity 1.7×10^4 dpm/mμm); protein, 10 μg/ml.

liver transacylases are not inactivated by PMSF (14).

Other model partial reactions of fatty acid synthesis, for example, enzyme catalyzed reduction of acetoacetyl-N-acetyl cysteamine and crotonyl-N-acetyl cysteamine in the presence of NADPH and the dehydration of β-hydroxybutyryl-N-acetyl cysteamine, were also carried out. Unpublished results (15) show that within the errors of experimental measurements, the rates of reaction catalyzed by the native and PMSF modified enzymes are identical. Condensation-CO_2 exchange, a critical step in the elongation of fatty acyl chain, was carried out under the conditions described by Kumar et al (3). The rate of CO_2 exchange was about 20% lower in the PMSF inactivated enzyme (Table I). This lowering of rate is, however, insufficient to account for the complete loss of activity for fatty acid synthesis.

One striking difference between native and PMSF-inactivated fatty acid synthetases is in their relative ability to catalyze the hydrolysis of palmityl CoA. The hydrolysis rate measured in the presence of PMSF-inactivated enzyme is less than 3% of the rate obtained with the native enzyme (Table I). It is apparent that the loss of activity for fatty acid synthesis can be completely accounted for by the loss of palmityl CoA deacylase activity.

Since PMSF selectively inhibits one step in the sequence of partial reactions of fatty acid synthesis, it was of interest to estimate quantitatively the number of sites which react with PMSF. For these experiments, 7-^{14}C-PMSF was synthesized and the stoichiometry of its reaction with chymotrypsin was established. Chymotrypsin was used as a reference enzyme for two reasons. First, the chymotrypsin-PMSF reaction exhibits 1:1 stoichiometry even at high inhibitor/enzyme ratios (11), conditions which exist in the fatty acid synthetase-PMSF system. And, secondly, chymotrypsin is obtainable in a highly pure crystalline form and the active site titration correlates well with the protein material in solution. Based on a molecular weight of 25,000 and an extinction coefficient at 280 nm of 5×10^4 M^{-1} cm^{-1} (16), specific radioactivity

for the binding of a mole of PMSF per mole of enzyme was calculated. Results of the studies carried out with fatty acid synthetase are shown in Table II.

TABLE II

Covalent Binding of PMSF (7-^{14}C) to Fatty Acid Synthetase Complex

Enzyme	%, Original Activity	dpm/mg Protein	Moles Inhibitor/ Mole of Enzyme
Chymotrypsin[a]	< 1	1.21×10^5	1.0
Fatty Acid Synthetase[b]	~50	3.32×10^3	1.09
Fatty Acid Synthetase[c]	< 1	6.27×10^3	1.9

[a] Chymotrypsin (10 mg/ml) was treated at pH 7.0 with two fold molar excess of PMSF. pH was maintained with a pH stat. Concentration of active sites was monitored at pH 8.5 with p-nitrophenylacetate as substrate (15). After complete inactivation, the enzyme was dialyzed at 4° against 2 1. 0.001 N HCl. Dialysis solution was changed four times in a 12-hour period. Complete removal of ^{14}C-PMSF during dialysis was also checked in a control experiment.

[b] Fatty acid synthetase (2 mg/ml) in 0.05 M K-phosphate, 1 mM EDTA, pH 7.0 was treated with 0.2 mM ^{14}C-PMSF. After about 50% inactivation, the enzyme was precipitated with $(NH_4)_2SO_4$ (33% saturation), centrifuged and freed from excess inhibitor on G-25 Sephadex column. Radioactivity associated with protein was completely separated from the inhibitor. Moles of inhibitor were then calculated from the knowledge of dpm/mg of fatty acid synthetase and the specific radioactivity for mole/mole binding of PMSF with chymotrypsin.

[c] Procedure similar to b above except that 1.0 mM ^{14}C-PMSF was used.

Based on an average molecular weight of 500,000 daltons, complete inactivation of the enzyme complex in 1.0 mM ^{14}C-PMSF results in the incorporation of 1.9 moles of phenylmethylsulphonyl groups per mole of the complex. Partial inactivation (50% loss of activity) gives a value of 1.09 moles PMSF bound/ mole enzyme, showing thereby that the PMSF interaction is selective and specific.

From these observations, it is evident that PMSF, a specific inhibitor of "serine" esterases, selectively inhibits the fatty acyl deacylase activity

but does not affect the transacylase activities of pigeon liver fatty acid synthetase. Incorporation of 1.9 moles PMSF/mole of the enzyme complex appears to suggest the presence of two moles of fatty acyl deacylase/mole of the complex. Since pigeon liver fatty acid synthetase can be dissociated into subunits of nearly equal molecular weight (17), it may be that one mole of a deacylase is present on each of these subunits. However, it is not certain whether there is one "deacylase" with specificity for a whole range of fatty acyl side chains or whether there are two fatty acyl deacylases - one having higher specificity for shorter chain fatty acyl residues and the second having greater sepcificity for long fatty acyl chains. Further studies may lead to an understanding as to why short and long chain fatty acids are synthesized under different sets of experimental conditions (18).

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