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Biochemistry

Specific release of the thioesterase component of the fatty acid synthetase multienzyme complex by limited trypsinization

(lactating rat mammary gland/fatty acid synthetase partial reactions/immunological cross-reactivity)

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ABSTRACT **Limited trypsinization of the fatty acid synthetase multienzyme complex from rat mammary gland results in the release of a protein, molecular weight 32,000, with thioesterase activity. The other components of the multienzyme complex—the acyl carrier protein, acetyl and malonyl transferases, condensing enzyme, keto reductase, dehydrase and enoyl reductase—are not affected and remain associated with the complex. The thioesterase can be isolated by ammonium sulfate precipitation and gel filtration. Extensive trypsinization of fatty acid synthetase multienzyme complex results in a loss of thioesterase activity, probably due to cleavage of the thioesterase component into inactive peptides. However, the molecular weight and specific activity of the thioesterase isolated after limited trypsinization is relatively unaffected by the severity of the conditions of proteolysis. Both the thioesterase and the residual trypsinized complex react with antibodies produced against the native multienzyme. The results demonstrate that mild trypsinization can be used to release the thioesterase component of the multienzyme with little perturbation of either the thioesterase or the other components of the complex.**

In plants and primitive microorganisms, the seven enzymes involved in the synthesis of fatty acids from acetyl- and malonyl-CoA exist as discrete monofunctional units which can be readily separated by conventional methods of protein fractionation (1). In animals and more advanced microorganisms, the enzymes are held together tightly in a multienzyme complex, fatty acid synthetase (FAS), which behaves as a single unit (2). Until recently, attempts to separate these FAS multienzymes into their individual functional units have succeeded only in resolving the complexes into two subunits (3) and an acyl carrier protein (ACP) (4, 5).

We have recently found that the thioesterase component of the rat mammary gland FAS can be cleaved from the complex, in a catalytically active form, by limited trypsinization (6). In this communication we report the purification of the thioesterase. We present evidence that the action of trypsin on the complex is highly selective and the structure of the thioesterase is not greatly altered by the conditions employed.

EXPERIMENTAL

Fatty Acid Synthetase Purification. FAS was isolated from lactating rat mammary gland according to the procedure of Smith and Abraham (7) except that a column (35 × 2.5 cm) of Biogel A0.5m was used in place of Sephadex G-200. FAS, labeled in the 4'-phospho-[^{14}C]pantetheine moiety was isolated from lactating rats injected with 15 μCi D-[*1*-^{14}C]pantothenate (6.03 mCi/mmol) 20 hr prior to killing the animals. The purified enzyme had a specific radioactivity of 600 dpm/mg of protein.

Substrates. Acetyl-CoA (labeled and unlabeled), hexanoyl-CoA and malonyl-CoA were synthesized and purified as described previously (7). Palmityl-CoA was prepared by Dr. C. Y. Lin via the *N*-hydroxysuccinamide derivative (8). The *N*-acetylcysteamine was prepared according to the procedure of Martin *et al.* (9) and converted to *S*-crotonyl *N*-acetyl cysteamine by the method of Kumar *et al.* (2). The *S*-β-hydroxybutyryl *N*-acetyl cysteamine was synthesized by the method of Kass *et al.* (10) and *S*-acetoacetyl *N*-acetylcysteamine was purchased from Sigma Chemical Co. Pantetheine was prepared by reduction of pantethine (Sigma) with sodium amalgam (11). All radiochemicals were obtained from New England Nuclear Corp.

Enzyme Assays. FAS was assayed spectrophotometrically (7). The partial reactions of the fatty acid synthetase were assayed with model substrates based on the procedures described by Kumar *et al.* (2).

Acetyl- and malonyl-CoA: pantetheine transacylase incubation systems contained 0.2 M potassium phosphate buffer at pH 6.6, 4 mM D-pantetheine, 0.5 mM [2-^{3}H]acetyl-CoA (3 μCi) or 0.5 mM [2-^{14}C]malonyl-CoA (10 nCi), and enzyme in 0.05 ml. Reactions, at 0°, were started by addition of enzyme and stopped after 0.5 min by addition of 2 M HCl (0.02 ml). Acetyl- and malonyl-pantetheine were separated from their respective CoA esters by paper chromatography (2). Condensation-CO_2 exchange reactions contained 0.2 M potassium phosphate buffer at pH 6.6, 0.05 mM hexanoyl-CoA, 1.5 mM CoA, 0.13 mM malonyl-CoA, 0.02 M $NaH^{14}CO_3$ (9 μCi), and enzyme in 0.1 ml. Reactions, at 30°, were started with enzyme and stopped after 10 min with 6% $HClO_4$ (0.02 ml). The amount of $^{14}CO_2$ fixed was determined by liquid scintillation spectrometry (2). Incubation systems for *S*-acetoacetyl *N*-acetylcysteamine reductase contained 0.2 M potassium phosphate buffer at pH 6.6, 0.1 mM NADPH, 3 mM *S*-acetoacetyl *N*-acetyl cysteamine, and enzyme in 0.5 ml. Reactions, at 30°, were started with enzyme and monitored spectrophotometrically (2). Assay systems for *S*-DL-β-hydroxybutyryl *N*-acetylcysteamine dehydrase contained 0.2 M potassium phosphate buffer at pH 6.6, 5 mM *S*-DL-β-hydroxybutyryl *N*-acetylcysteamine, and enzyme in 0.5 ml. Reactions, at 30°, were started with substrate and monitored spectrophotometrically (2). Incubation systems for *S*-crotonyl *N*-acetylcysteamine reductase contained 0.2 M potassium phosphate buffer at pH 6.6, 10.0 mM *S*-crotonyl *N*-acetylcysteamine, 0.1 mM NADPH, and enzyme in 0.5 ml. Reactions, at 30°, were started with substrate and monitored spectrophotometrically (2). Assay systems for thioesterase activity contained 0.2 M potassium phosphate buffer at pH 6.6, 50 μg of bovine serum albumin, 5 μM [*1*-^{14}C]palmityl-CoA (10 nCi), and enzyme in a final volume of 1.0 ml.

Abbreviations: FAS, fatty acid synthetase multienzyme complex; ACP, acyl carrier protein; V_e, elution volume; V_o, void volume.

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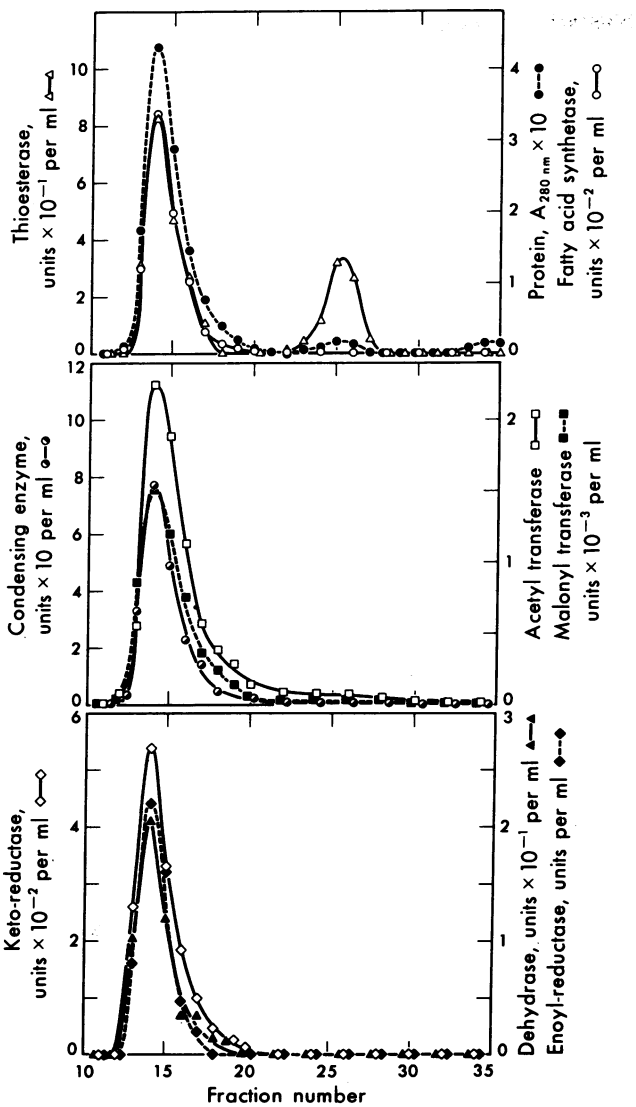

FIG. 1. Chromatography on Biogel A0.5m of the products formed from trypsinization of rat mammary gland FAS. Rat mammary gland FAS (10,500 units, 10 mg of protein) was incubated at 30° with 5 μg of trypsin in 4.0 ml of 0.1 M potassium phosphate buffer at pH 7.0 containing 7 mM dithiothreitol and 1 mM EDTA. After 1 hr, 15 μg of trypsin inhibitor was added and a 1.0 ml portion was removed for determination of the activities of the partial reactions. The activities, expressed as units/mg of original FAS, compared with a control preparation of FAS which was incubated in the absence of trypsin were: FAS, control 1010, trypsinized 611, thioesterase, control 696, trypsinized 422; acetyl transferase, control 6600, trypsinized 6600; malonyl transferase, control 3600, trypsinized 3600; condensing enzyme, control 3.6, trypsinized 3.4; keto reductase, control 1240, trypsinized 1210; dehydrase, control 60.1, trypsinized 57.4; and enoyl reductase, control 6.8, trypsinized 7.6. The remaining 3.0 ml was applied to a column (35 × 2.5 cm) of Biogel A0.5m equilibrated with 0.25 M potassium phosphate buffer at pH 7.0, 1 mM dithiothreitol, 1 mM EDTA at 21°. Fractions of 5.5 ml were collected for determination of protein and enzyme activities.

Reaction mixtures were preincubated at 30° for 2 min, substrate was added, and 1 min later the reaction was stopped with 0.1 ml of 21% perchloric acid. The [1-^{14}C]palmitic acid released was extracted with petroleum ether (2 × 3 ml), the solvent evaporated, and the radioactivity determined by liquid scintillation spectrometry (12). The units of enzyme activities are defined as the number of nanomoles of substrate metabolized per minute. All reaction rates were linear functions of protein concentration and time.

Protein Determination. The procedure of Warburg and Christian (13) was used.

Immunological Studies. Anti-(lactating rat mammary gland) FAS γ-globulin was prepared as described previously (14). Double diffusion analyses were carried out in 1% agarose gels (14) which were subsequently stained with Ponceau S (15).

Sucrose Density Gradient Centrifugation. Samples were centrifuged at 0–4° on 5–20% sucrose gradients for 15 hr at 39,000 rpm in a Beckman SW 39 rotor (16). Pyruvate kinase was used as a 10 S marker (17) and its activity determined spectrophotometrically (18).

Chromatography on Sephadex G-75. A column of Sephadex G-75 (93 × 2.5 cm) was equilibrated at 21° with 0.25 M potassium phosphate buffer at pH 7.0, 1 mM dithiothreitol, and 1 mM EDTA. Samples were applied in a volume of 1.0 ml and the column was eluted with the same buffer. The void volume (V_0) was determined with blue dextran 2000. Chicken ovalbumin, 45,000 molecular weight (19), bovine β-lactoglobulin, 36,000 molecular weight (20), soybean trypsin inhibitor, 21,500 molecular weight (21), and horse heart cytochrome *c*, 12,400 molecular weight (22) were used as standards.

Other Materials. Bovine pancreatic trypsin (210 units/mg) was obtained from Worthington and trypsin inhibitor from Calbiochem. Chicken ovalbumin and horse heart cytochrome *c* were purchased from Mann and β-lactoglobulin was a gift from Dr. R. Townend.

RESULTS

Preliminary experiments had shown that trypsinization of FAS under mild conditions released the thioesterase component of the complex (6). To determine whether only the thioesterase component was cleaved from the multienzyme complex by proteolysis, the products of mild trypsinization were subjected to gel filtration on a column of Biogel A0.5m. The activities of acetyl transferase, malonyl transferase, condensing enzyme, keto reductase, dehydrase, and enoyl reductase cochromatographed with the residual FAS activity (Fig. 1). Of the thioesterase activity eluted from the column, 70% emerged with the fatty acid synthetase (V_e/V_0 = 1.0) and 30% was eluted as a separate component (V_e/V_0 = 1.8). Based on the A_{280nm} measurements, 97% of the protein eluted was associated with the void volume and 3% with the thioesterase peak. The increase in A_{280nm} around fraction 35 (corresponding to 1 bed volume) could be completely accounted for by the presence of dithiothreitol (determined with Ellman's reagent, ref. 23) originating from the enzyme preparation. It thus appears that no significant polypeptides other than the thioesterase were released by limited trypsinization.

The ACP of the FAS appears to be a labile component of the complex. It can be removed by dialysis of the subunits at 0° (4) or by treatment of the native enzyme with sodium dodecyl sulfate (5). The condensing activity of the complex requires the participation by ACP (2) and since its activity was unaffected by trypsinization, it seemed likely that the ACP was not released from the multienzyme during proteolysis. This was confirmed by an experiment in which fatty acid synthetase labeled in the ACP with [^{14}C]pantotheine was subjected to trypsinization followed by sucrose density gradient centrifugation (Fig. 2). Even though the conditions for

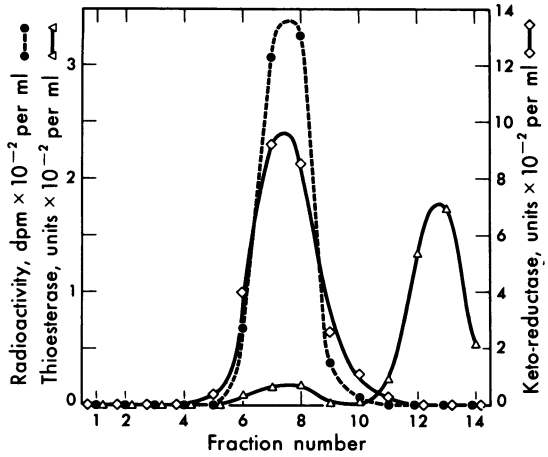

FIG. 2. Sucrose density gradient centrifugation of trypsinized 4′-phospho-[^{14}C]pantetheine-FAS. Rat mammary gland FAS, labeled in the pantetheine moiety (1500 FAS units, 273 thioesterase units, 1.6 mg of protein), was incubated at 30° for 1 hr with 5 μg of trypsin in 0.2 ml of 0.1 M potassium phosphate buffer at pH 7.0, 10 mM dithiothreitol, and 1 mM EDTA. The reaction was stopped with 10 μg of trypsin inhibitor (0.01 ml) and the mixture layered onto a sucrose density gradient. Trypsinization resulted in the loss of 91% of the FAS activity and 70% of the thioesterase activity.

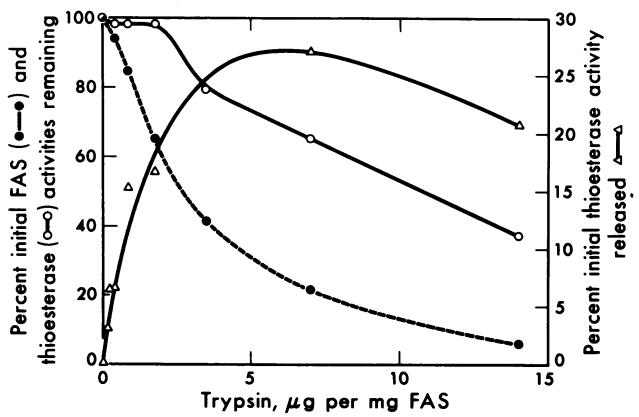

FIG. 3. The effect of trypsin concentration on the release of the thioesterase component. Rat mammary gland FAS (1130 FAS units, 330 thioesterase units, 1.0 mg of protein) was incubated at 30° for 10 min with various amounts of trypsin in 1.0 ml of 0.1 M potassium phosphate buffer at pH 7.0, 3 mM dithiothreitol, and 1 mM EDTA. Trypsin inhibitor (2 μg/μg of trypsin) was added and the FAS and thioesterase activities determined. The thioesterase activity remaining in the supernatant at 40% ammonium sulfate saturation was taken to represent released thioesterase.

trypsinization were more severe than in the experiments shown in Fig. 1, all of the pantotheine label sedimented as a 13S species. Keto reductase activity, which remains attached to the multienzyme on trypsinization, coincided with the radioactive label indicating that the ACP had not been released from the complex. Thioesterase activity, on the other hand, was associated mainly with a slower sedimenting component verifying that it had become detached from the FAS.

These results suggested that the initial attack of trypsin on FAS is in the region linking the thioesterase to the complex. Further support for this hypothesis was obtained from a study of the effect of trypsin concentration on the release of the thioesterase component (Fig. 3). At low trypsin concentrations, up to 15% of the thioesterase activity was released from the complex without any loss in the total (i.e., multienzyme-bound plus free thioesterase) activity. This release of thioesterase resulted in a parallel decrease in capacity for catalysis of the overall FAS reaction sequence. At higher trypsin concentrations there was a progressive loss in total thioesterase activity so that the maximum activity recovered in the free form was limited to 27%. This experiment suggested that once a sizeable pool of free thioesterase species had accumulated, it became the object of a secondary attack by trypsin, which resulted in a loss of thioesterase activity. We were concerned, therefore, that the molecular size and catalytic activity of the released thioesterase might be influenced by the severity of the trypsinization procedure. With this question in mind, we treated FAS with trypsin under various conditions which resulted in the loss of up to 99% of its capacity to catalyze the overall FAS reaction (Table 1). The specific activity of the free thioesterase, separated from the residual multienzyme complex by ammonium sulfate fractionation, did indeed reflect the severity of the trypsinization process, being lowest in the sample trypsinized for the longest time. When these samples were chromatographed on Sephadex G-75, the thioesterase activity always emerged from the column with the same relative elution volume. This showed that the molecular size of the catalytically active thioesterase was always the same no matter how rigorous the conditions of proteolysis. Furthermore, the specific activities of the thioesterase samples eluted from Sephadex G-75 were now much closer, indicating that the inactive peptides produced by extensive trypsinization of the thioesterase had been separated from the catalytically active enzyme. In recent experiments where larger quantities of thioesterase were purified and the gel column was run at 0–4° rather than room temperature, somewhat higher specific activities were obtained.

The thioesterase cleaved from the multienzyme complex by trypsinization was found to precipitate between 50 and 60% ammonium sulfate saturation. Gel permeation chromatography, on Sephadex G-75, of this ammonium sulfate frac-

Table 1. The effect of extent of trypsinization on the molecular size and specific activity of the thioesterase

Trypsinization time (min)	FAS activity remaining (%)	Thioesterase activity in 40–70% $(NH_4)_2SO_4$ fraction (units/mg)	Thioesterase after Sephadex G-75 chromatography	
			V_e/V_0	Units/mg
2	43	947	1.43	1340
10	2.4	291	1.43	1130
20	1.2	143	1.43	940

Rat mammary gland FAS (86,000 units FAS activity, 26,000 units thioesterase activity, 76 mg of protein) was incubated at 30° in 48 ml of 0.16 M potassium phosphate buffer at pH 7.0 containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mg of trypsin. At the times shown, portions were removed and trypsin inhibitor (2 μg/μg of trypsin) was added. The thioesterase was purified successively by ammonium sulfate precipitation (40–70% saturation) and chromatography on Sephadex G-75.

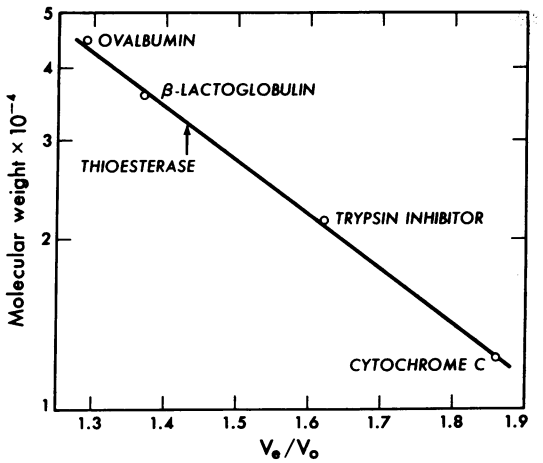

FIG. 4. Estimation of molecular weight of thioesterase by gel filtration. The relative elution volume (V_e/V_0 = 1.43) of the thioesterase corresponded to a molecular weight of 32,000.

tion resulted in the elution of a thioesterase with constant specific activity throughout the peak. Assuming the enzyme to be a typical globular protein, the molecular weight was estimated to be about 32,000 (Fig. 4). The thioesterase component isolated by trypsinization of the rat liver fatty acid synthetase is also eluted from a column of Sephadex G-75 with a relative elution volume of 1.43, corresponding to a molecular weight of 32,000 (E. Agradi, K. N. Dileepan, and S. Smith, unpublished observations).

The retention of palmityl-CoA thioesterase activity by the polypeptide cleaved from the synthetase complex is in itself a good indication that the tertiary structure of the free thioesterase is not greatly different from that of the thioesterase bound to the multienzyme. Further evidence was obtained from immunodiffusion studies. The immunological cross-reactivity of proteins is believed to be closely related to the native three dimensional structure of the proteins. Thus, immunochemical methods have been used to study conformational changes in ribonuclease (24), pepsinogen (25), α-amylase (26), and L-amino acid oxidase (27). To determine whether the thioesterase component released by trypsinization was still recognizable by antibodies against the native multienzyme complex, we carried out a double diffusion study. Approximate antigen-antibody equivalence points were established using both native FAS and the thioesterase, and these proportions were used in the experiment shown on Fig. 5. The thioesterase gave a strong reaction against the anti-FAS γ-globulin; the precipitin line always formed closer to the antibody well than when FAS was used as antigen indicating that the diffusion coefficient for the thioesterase was greater than that of the native FAS. Extensively trypsinized FAS also reacted with the antibodies, although the intensity of the precipitin line appeared rather weaker than that observed with the native enzyme. The anti-FAS γ-globulin also gave a single precipitin line when reacted against the mammary gland cytosol; in other experiments using a wide range of cytosol concentrations, we have always observed a single precipitin line which gives a reaction of complete identity with the purified FAS. When cytosol and thioesterase were included in the same antigen well, two distinct precipitin lines were formed (Fig. 5D). These results suggest that the thioesterase is not found in the free form in the cytosol as the result of proteolysis before or after homogenization of the tissue. Although we have found a free thioesterase in the cytosol of lactating rat mammary gland,

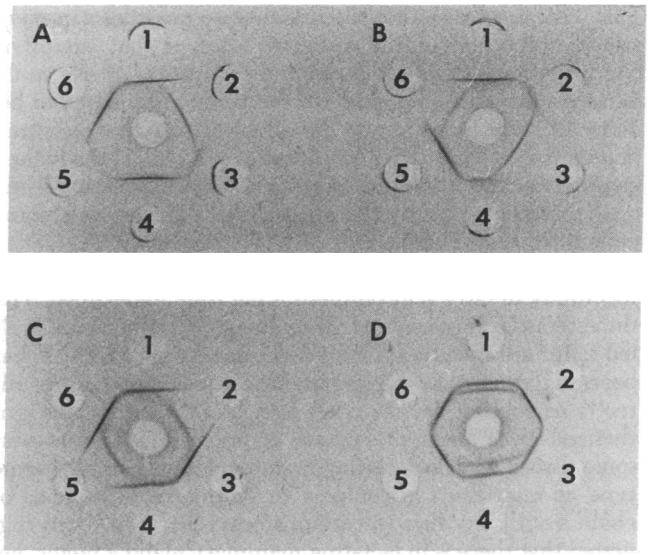

FIG. 5. Double diffusion studies. Center wells contained 0.24 mg of anti-FAS γ-globulin. Outer wells A1, A4, B1, C1, and C4 contained 7 μg of FAS; wells A3, A5, B2, and B4 contained 5 μg of extensively trypsinized FAS (97% FAS activity lost); wells A2, B3, B6, C2, and C5 contained 7 μg of thioesterase; and wells A6, B5, C3, C6, D2, D3, D5, and D6 contained cytosol from lactating rat mammary gland equivalent to 7 μg of FAS. Outer wells D1 and D4 contained both cytosol and thioesterase.

this enzyme is immunologically distinct from the FAS-related thioesterase (L. Libertini and S. Smith, unpublished results).

DISCUSSION

The inability of trypsin and trypsin inhibitor to hydrolyze palmityl-CoA (6), and the immunological relationship between the thioesterase and the fatty acid synthetase multienzyme, rule out the possibility that the thioesterase activity could have originated from any source other than the multienzyme complex. The action of trypsin on the FAS multienzyme complex is highly specific. The initial site of attack is probably a single peptide bond which links the thioesterase component to the complex (Figs. 1 and 3). Once released, the thioesterase appears to be susceptible to further tryptic action which cleaves the enzyme into inactive fragments. This secondary attack by trypsin limits the yield of thioesterase which can be obtained by the procedure but does not appear to raise any serious questions as to whether the native configuration of the thioesterase might be affected, since the inactive peptides can be readily removed and the remaining thioesterase can be isolated with high specific activity. It is difficult to assess unequivocally whether any alteration of the native thioesterase structure occurs on removal from the complex. The immunological evidence—while admittedly of a qualitative rather than a quantitative nature—seems to suggest that at least there is no gross perturbation of the three dimensional organization of the enzyme. That the procedure for removal of the thioesterase component is indeed only mildly disruptive is further supported by the fact that the trypsinized complex retains full activity of all other partial reactions and is still recognizable by antibodies against the native FAS multienzyme. However, the intensity of the precipitin line formed between anti-FAS γ-globulin and extensively trypsinized FAS is somewhat weaker than that obtained with the native FAS.

This indicates that either the thioesterase provides a considerable proportion of the antigenic sites or that extensive trypsinization causes some other modification of the multienzyme structure. Support for the latter argument can be deduced from the study on the effect of trypsin concentration on thioesterase and FAS activities (Fig. 3). At the higher trypsin concentrations, the loss of FAS activity cannot be entirely accounted for by the release of the thioesterase component from the complex.

The exact role of thioesterase in determination of product specificity in fatty acid synthesis is not fully understood. The thioesterase components of all multienzyme complexes studied so far are specific for long chain thioesters (28, 29). It has been suggested that the specificity of the thioesterase is directly responsible for the chain length of the fatty acids synthesized by FAS multienzyme complexes (28). However, some tissues, such as lactating mammary gland, use the same type of multienzyme complex to synthesize fatty acids of chain length less than 16 carbon atoms (29). It has been suggested that there is in lactating mammary gland a factor, not part of the multienzyme complex (30, 31), perhaps a thioesterase (32), which modifies the product specificity of the FAS. We have recently isolated a thioesterase from lactating rat mammary gland which will hydrolyze both long and medium chain thioesters (L. Libertini and S. Smith, unpublished results). In this respect, it resembles the type II thioesterase found in E. coli (33) whereas the thioesterase released from the multienzyme complex resembles the *Escherichia coli* type I enzyme (28) in that it is specific for long chain thioesters (S. Smith, C. Lin, and L. Libertini, unpublished results). A detailed study of the properties of these two thioesterases is now possible and the results may clarify their relationship to the *E. coli* enzymes.

We have shown that the thioesterase component of the FAS multienzyme can be removed without altering the activity of the other constituent enzymes. If the overall reaction of fatty acid synthesis can be reconstituted from a combination of trypsinized FAS and free thioesterase, the system may provide a useful model for study of the advantage in catalytic efficiency inherent in a multienzyme complex compared to a dissociated enzyme system.

Recent publications by Alberts *et al.* (34) and Stoops *et al.* (35) have suggested that the mammalian fatty acid synthetases may be composed of only two polypeptides. If this is so, then the technique of dissecting the multienzymes with specific proteolytic enzymes may prove to be invaluable in unraveling the structure of the complexes.

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