Release of Two Thioesterase Domains from Fatty Acid Synthetase by Limited Digestion with Trypsin

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Limited digestion, with trypsin, of the fatty acid synthetase from rat mammary gland releases an enzymically active thioesterase component that, under denaturing conditions, consists of two major species of mol.wts. 35000 and 17500 and a minor species, mol.wt. 15000. The 17500- and 15000-mol.wt. species are shown to originate from the 35000-mol.wt. species as a result of nicking by trypsin. The nicked polypeptides are enzymically active. The fatty acid synthetase is inhibited by [1,3-¹⁴C]di-isopropyl phosphorofluoridate, which is shown to bind to, and inactivate, two thioesterase active sites. When the [1,3-¹⁴C]di-isopropyl phosphate-labelled fatty acid synthetase is subjected to limited digestion with trypsin, all of the radioactivity is recovered in the isolated thioesterase component, i.e. in the 35000-mol.wt. polypeptide and its nicked products. Since the isolated thioesterase is shown to bind only one di-isopropyl phosphate residue per 35000-mol.wt. polypeptide, we conclude that the fatty acid synthetase has two thioesterase domains, both of which are removed by limited trypsin treatment.

In animals and some micro-organisms, the enzymes involved in the synthesis of fatty acids *de novo* from acetyl-CoA and malonyl-CoA are covalently linked on two polyfunctional polypeptides (for review, see Bloch & Vance, 1977). We have demonstrated that the thioesterase component of the fatty acid synthetase can be released, in active form, by limited trypsin treatment (Smith *et al.*, 1976). Thioesterase preparations obtained in this way appear homogeneous under non-denaturing conditions, but under denaturing conditions consist of a mixture of intact 35000-mol.wt. polypeptides and 17500-mol.wt. nicked polypeptides (Dileepan *et al.*, 1977). In the present paper we report a procedure for the separation of the intact and nicked polypeptides with partial retention of enzyme activity.

Materials and Methods

Materials

Bio-Gel A 0.5m, SDS, ammonium persulphate and NNN'-tetramethylethylenediamine were purchased

Abbreviation used: SDS, sodium dodecyl sulphate.

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from Bio-Rad Laboratories, Richmond, CA, U.S.A. Agarose, NADPH, EDTA and trypsin inhibitor were products of Sigma Chemical Co., St. Louis, MO, U.S.A. [1-¹⁴C]Palmitoyl-CoA, [1,3-¹⁴C]di-isopropyl phosphorofluoridate, and Omnifluor were obtained from New England Corp., Boston, MA, U.S.A.; di-isopropyl [³²P]phosphorofluoridate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled di-isopropyl phosphorofluoridate was purchased from both Sigma and Koch-Light Laboratories, Colnbrook, Bucks., U.K. Sephadex products were obtained from Pharmacia, Uppsala, Sweden, and bovine pancreatic trypsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Phosphorylase and glycogen-debranching enzyme were gifts from Dr. P. Cohen, Department of Biochemistry, University of Dundee, Scotland.

Isolation of fatty acid synthetase and thioesterase

Fatty acid synthetase was purified from lactating rat mammary gland and treated with trypsin to release the thioesterase component (Smith *et al.*, 1976).

Assay of thioesterase activity

Activity was measured by a radiochemical procedure. Assay systems contained 0.05 M-potassium phosphate buffer, pH 8, 5 μ M-[1-¹⁴C]palmitoyl-CoA (10 nCi) and enzyme (usually 0.1–0.5 μ g of purified thioesterase) in a final volume of 0.5 ml. Reactions were started by the addition of substrate, continued at 30°C for 1–2 min and terminated by the addition of

0.1 ml of 21% (v/v) HClO_4 . The liberated $[1\text{-}^{14}\text{C}]$ -palmitic acid was extracted with light petroleum (b.p. 35–60°C) and the solvent evaporated under a stream of air. Radioactivity was assayed by liquid-scintillation spectrometry. A unit of thioesterase activity is defined as the amount of enzyme catalysing the hydrolysis of 1 nmol of palmitoyl-CoA/min.

Protein concentration

Protein concentration was determined from the A_{280} by using specific absorption coefficients ($A_{1\text{cm}}^{1\%}$) of 10.0 and 7.14 for fatty acid synthetase and thioesterase respectively (Lin & Smith, 1978).

Determination of radioactivity in polyacrylamide gels

Frozen gels were cut into slices with a razor blade, transferred to scintillation vials and dried for 3 h at 80°C. The dried gel slices were digested by heating in 30% (v/v) H_2O_2 for 3–4 h at 80°C. Scintillation fluid was added and radioactivity determined by liquid-scintillation spectrometry.

Radioautography of polyacrylamide gels

Polyacrylamide gels were dried flat on Whatman 3mm paper and exposed to Kodak X-ray film for 4 weeks.

Densitometric measurements

Stained gels were scanned in either a Beckman DU spectrophotometer equipped with a Gilford gel-scanning adaptor or a Joyce-Loebl Chromoscan microdensitometer. The area under the peaks was determined by making a Xerox copy of the scan and cutting out and weighing the peaks.

Binding of radioactively labelled di-isopropyl phosphorofluoridate to fatty acid synthetase

Fatty acid synthetase (1 mg/ml) was incubated for various time intervals at room temperature (20–22°C) with 1 mM-di-isopropyl phosphorofluoridate [either $1,3\text{-}^{14}\text{C}$ -labelled (sp. radioactivity 8.3 Ci/mol) or ^{32}P -labelled (sp. radioactivity 79 Ci/mol)] in 0.05 M-sodium phosphate buffer, pH 7, containing 5% (v/v) propylene glycol. Unbound radioactivity was removed either by gel filtration on a column (6 cm $\times$ 1 cm) of Sephadex G-25 or by exhaustive dialysis against 0.1 M-sodium phosphate buffer (pH 7)/1 mM-EDTA/1 mM-dithiothreitol.

Binding of $[1,3\text{-}^{14}\text{C}]$ di-isopropyl phosphorofluoridate to thioesterase

Thioesterase (0.5 mg/ml) was incubated for various time intervals at room temperature with 1 mM- $[1,3\text{-}^{14}\text{C}]$ di-isopropyl phosphorofluoridate (sp. radioactivity 8.3 Ci/mol) and 1 mM-EDTA in 0.05 M-sodium phosphate buffer, pH 7. Unbound radioactivity was removed by gel filtration on a column

(6 cm $\times$ 1 cm) of Sephadex G-25 equilibrated with 0.05 M-sodium phosphate buffer (pH 7)/1 mM-EDTA.

Liquid-scintillation spectrometry

The radioactive palmitic acid, formed in the thioesterase assay, was assayed by using 3 ml of toluene/2-ethoxyethanol (2:1, v/v), containing 15 mg of Omnifluor.

Radioactively labelled proteins (0.1 ml portions) were mixed with 0.1 ml of formic acid before addition of 15 ml of scintillation fluid (55% 2-ethoxyethanol, 45% toluene, containing 6.7 mg of Omnifluor/ml.) The same scintillation mixture, without formic acid, was used for assaying radioactivity in digested slices of polyacrylamide gels.

Efficiency of counting was determined by the internal-standard or channels-ratio methods.

Immunochemical procedures

Rabbit anti-(fatty acid synthetase) γ -globulin was prepared as described previously (Smith, 1973). Antiserum against thioesterase was raised by injecting a rabbit intraperitoneally with 0.5 mg of thioesterase in 1 ml of 0.9% NaCl twice weekly, for 5 weeks. Booster doses of 0.1 mg of thioesterase, in 0.9% NaCl, were administered intramuscularly at 4-week intervals to maintain the titre of antiserum. A partially purified γ -globulin fraction was prepared by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Smith, 1973). Ouchterlony double-diffusion analyses were carried out as described previously (Smith, 1973); immunoprecipitates were stained with Ponceau S (Ringle & Herndon, 1965).

Results

Evidence that the 17500-mol.wt. species is derived through nicking of the 35000-mol.wt. species by trypsin

The isolated thioesterase was digested with trypsin for various lengths of time and the products identified by SDS/polyacrylamide-gel electrophoresis (Fig. 1). Trypsin treatment resulted in a loss of thioesterase activity and a parallel disappearance of the 35000-mol.wt. species. After 20 min, 4.7 (S.E.M. $\pm$ 0.3)% of the thioesterase activity remained and the amount of 35000-mol.wt. species present had fallen to $2.4 \pm 0.1\%$ of the initial level. The amount of 17500-mol.wt. species present increased significantly ($P < 0.001$), reaching a maximum value after 5 min. A third component (mol.wt. 15000), present in the starting material in only trace amounts, also increase in amount as digestion with trypsin progressed. Traces of a component of mol.wt. 33000 were also detected during the course of digestion with trypsin. The mobility of this component was too close to that of the 35000-mol.wt. component to allow resolution by scanning and its contribution to

Fig. 1. Trypsin treatment of thioesterase: correlation between thioesterase activity and the amount of each polypeptide present at different stages of digestion

Thioesterase (0.62 mg/ml) and trypsin (18 μ g/ml) were incubated in 0.1 M-sodium phosphate buffer, pH 7, containing 1 mM-EDTA and 1 mM-dithiothreitol at 30°C. Portions of the reaction mixture were removed at intervals and treated with trypsin inhibitor (3 times the weight of trypsin). As a control, a sample of thioesterase was incubated, without trypsin, for 60 min at 30°C. A second control, lacking only thioesterase, served to identify those minor electrophoretic components not attributable to thioesterase. SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) was carried out in duplicate on two samples equivalent to 8.5 and 11.4 μ g of thioesterase. For each polypeptide component, the amount of Coomassie Blue-stained material detected by scanning was directly proportional to the amount of protein applied to the gel. The molecular weights of the polypeptides were determined by comparison of their mobilities with those of bovine serum albumin (67000), chymotrypsin (25200), apoferritin (24000), trypsin inhibitor (22000) and haemoglobin (16000). Controls, incubated in the absence of trypsin, showed no change in thioesterase activity (3520 ± 270 units/mg of protein) compared with the starting material (3470 ± 100 units/mg of protein) and no change in the polypeptide composition. The total amount of Coomassie Blue stain detected on the polyacrylamide gels decreased from 122 ± 5 to $95 \pm 2 A_{570}$ unit/cm of gel per mg of applied protein after treatment of the thioesterase with trypsin for 40 min. All values are mean $\pm$ S.E.M. for four determinations; where no S.E.M. is shown, the value lies within the area of the symbol. $\square$, Thioesterase activity; $\blacksquare$, polypeptide 35000-mol.wt. species; $\bullet$, 17500-mol.wt. species; $\blacktriangle$, 15000-mol.wt. species.

Coomassie Blue binding has been included with that of the 35000-mol.wt. species.

Separation of the nicked 17500-mol.wt. polypeptides and the intact 35000-mol.wt. polypeptides by gel filtration in the presence of SDS

Usually 2 mg of thioesterase was incubated for 1 h at 20–22°C in 1.0 ml of 50 mM-sodium phosphate

Fig. 2. Separation of intact and nicked polypeptides of the thioesterase by gel filtration in the presence of SDS

Sample was applied, at 20–22°C, to a column (112 cm $\times$ 1 cm) of Sephadex G-100 (superfine grade), equilibrated and eluted with 50 mM-sodium phosphate/1 mM-dithiothreitol/1 mM-EDTA/0.1% (w/v) SDS; fraction volume was 0.6 ml. The two elution zone maxima were characterized by relative elution volumes (V_e/V_0) of 1.119 and 1.262. Recoveries of protein and enzyme activity after gel filtration were both 90%. The insert shows SDS/polyacrylamide-gel electrophoretic profiles (Weber & Osborn, 1969) for the material applied to the column (a) and material from fractions 46 (b) and 53 (c). The molecular weights of the two polypeptides were 35000 and 17500. $\square$, Thioesterase activity; $\bullet$, A_{280} .

buffer, pH 7, and 1% SDS. In a typical experiment, the thioesterase (initial specific activity 3440 units/mg of protein) retained 7% of its activity after SDS treatment. Control experiments indicated that the amount of SDS carried over into the assay system (0.00025% final concentration) was insufficient to cause significant inhibition of enzyme activity. Activity in the presence of 0.00025% SDS was 92% of that observed in the absence of SDS. Although this is not strictly a valid control (the activities of the intact and nicked forms of the enzyme may have different susceptibilities to inhibition by SDS) it is the best available. The solution was then applied, at 20–22°C, to a column (112 cm $\times$ 1 cm) of Sephadex G-100 (superfine grade) equilibrated with 50 mM-sodium phosphate (pH 7)/1 mM-dithiothreitol/1 mM-EDTA/0.1% (w/v) SDS. Gel filtration resolved the thioesterase into two components that were identified by SDS/polyacrylamide-gel electrophoresis as the intact and nicked polypeptides of the enzyme (Fig. 2). Both the intact and nicked polypeptides of the thioesterase had enzyme activity; the mean specific activity in the fractions containing the 35000-mol.wt.

species was 327 units/mg of protein and that in those containing the 17 500-mol. wt. species was 29 units/mg.

Stoichiometry of di-isopropyl phosphorofluoridate binding to the thioesterase

The isolated thioesterase is inhibited by the serine esterase inhibitor di-isopropyl phosphorofluoridate. To determine the number of active sites, the enzyme was treated with $[1,3-^{14}\text{C}]$ di-isopropyl phosphorofluoridate for various lengths of time and the amount of radioactivity bound covalently to the enzyme was measured (Table 1 and Fig. 3). Binding of $[1,3-^{14}\text{C}]$ di-isopropyl phosphorofluoridate to the thioesterase increased only slightly after 6.5 h. Since the thioesterase preparation consisted of a mixture of intact and nicked polypeptides, it was necessary to separate these species by SDS/polyacrylamide-gel electrophoresis and determine the extent of binding of di-isopropyl phosphorofluoridate to each species.

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of $[1,3-^{14}\text{C}]$ -di-isopropyl phosphate-labelled thioesterase*
Thioesterase was treated with labelled inhibitor for 23.25 h as described in Table 1. The Weber & Osborn (1969) procedure was used for SDS/polyacrylamide-gel electrophoresis. The densitometer tracing (A_{570}) is indicated by a continuous line and the radioactivity distribution by the histogram. Gel-slice size was 3 mm.

Table 1. *Stoichiometry of binding of $[1,3-^{14}\text{C}]$ di-isopropyl phosphorofluoridate to thioesterase*

Conditions for binding of di-isopropyl phosphorofluoridate are given in the Methods section. The proportion of radioactivity covalently bound to the 17 500- and 35 000-mol. wt. species was determined by SDS/polyacrylamide-gel electrophoresis and the proportion of protein present in each species was measured by densitometry (Fig. 3). From these data and the amount of radioactivity bound to the 35 000- plus 17 500-mol. wt. species, the extent of di-isopropyl phosphate binding to each species was calculated on a molar basis.

Time (h)	Di-isopropyl phosphate bound to 35 000 + 17 500-mol. wt. species		Radioactivity (%)		Protein (%)		Di-isopropyl phosphate bound (mol/35 000-mol. wt. species)		Inhibition of thioesterase activity (%)
	(mol/35 000-mol. wt. thioesterase)		species		species		species		
			17 500-mol. wt. species	35 000-mol. wt. species	17 500-mol. wt. species	35 000-mol. wt. species	17 500-mol. wt. species	35 000-mol. wt. species	
2	0.27		9	91	45	46	0.05	0.53	61
4	0.41		12	88	47	45	0.10	0.80	82
6.5	0.55		16	84	44	47	0.20	0.97	92
23.3	0.58		25	75	47	44	0.31	0.98	100

In determining relative amounts of 35000- and 17500-mol.wt. species by densitometry, we have assumed that the intact and nicked thioesterase polypeptides bind the Coomassie Blue stain to similar extents. This may be a reasonable assumption since conversion of the 35000-mol.wt. species into the 17500- and 15000-mol.wt. species by digestion with trypsin leads to only a small decrease in the capacity for binding of Coomassie Blue stain (Fig. 1). As shown in Fig. 3, binding of [1,3- 14 C]di-isopropyl phosphorofluoridate was not limited to the intact 35000-mol.wt. species. The nicked thioesterase polypeptides also bound di-isopropyl phosphorofluoridate, but at a much slower rate; binding of di-isopropyl phosphorofluoridate to the nicked polypeptides was incomplete after 6.5h. Binding of di-isopropyl phosphorofluoridate to the 35000-mol.wt. species reached a maximum value, after 6.5h, of 1 mol of di-isopropyl phosphate bound per 35000-mol.wt. species, indicating a single active site per 35000-mol.wt. polypeptide. The extent of di-isopropyl phosphorofluoridate binding correlated well with the loss of thioesterase activity.

Fig. 4. Fractionation of products from trypsin digestion of [1,3- 14 C]di-isopropyl phosphate-labelled fatty acid synthesis by gel filtration

Fatty acid synthetase (190 μ g) labelled with [1,3- 14 C]di-isopropyl phosphate (1.8 mol/mol of enzyme) was treated with trypsin (20 μ g) in 0.4 ml of 50mM-sodium phosphate buffer (pH 7)/1mM-EDTA/10mM-dithiothreitol, for 15 min at 30°C. Trypsin inhibitor (40 μ g) was added followed by 1 mg of trypsin-treated fatty acid synthetase (not labelled with inhibitor, but digested with trypsin as described above) and 0.5 mg of thioesterase as carrier proteins. A column (25cm $\times$ 0.5cm) of Bio-Gel A 0.5m was used with 50mM-sodium phosphate buffer, pH 7, as eluent; fraction volume was 0.6 ml. ●, A_{280} ; ○, radioactivity.

Fig. 5. Isolation by SDS/urea/polyacrylamide-gel electrophoresis of [1,3- 14 C]di-isopropyl phosphate-labelled fatty acid synthetase

Fatty acid synthetase (4.3 mg/ml) labelled with [1,3- 14 C]di-isopropyl phosphorofluoridate [2.1 mol (21 Ci)/mol of enzyme] was treated with trypsin (2.1 μ g/ml) in 0.05M-sodium phosphate buffer (pH 7)/1mM-EDTA/1mM-dithiothreitol for 50 min at 30°C. Trypsin inhibitor (three times the weight of trypsin) was added to stop the reaction. Proteins were denatured by heating for 5 min in 8M-urea/2% SDS/10% 2-mercaptoethanol, before electrophoresis in 5% polyacrylamide gels containing 8M-urea (Schweizer *et al.*, 1973). The molecular weights of the polypeptides were determined by comparison of their mobilities with those of myosin (200000), *Escherichia coli* RNA polymerase subunits (α = 39000, β = 155000, β' = 165000), glycogen-debranching enzyme (160000), phosphorylase (100000) and bovine serum albumin (67000). Stained gels (a) and radioautograms (b) show the distribution of protein and radioactivity before and after trypsin treatment. The molecular weights corresponded to: before trypsin treatment, fatty acid synthetase subunit, 220000–240000; after trypsin treatment, trypsin-treated fatty acid synthetase core, consisting of three peptides of mol.wts. 100000, 110000 and 128000, and thioesterase, 15000-, 17500- and 35000-mol.wt. species.

Stoichiometry of di-isopropyl phosphorofluoridate binding to fatty acid synthetase

When fatty acid synthetase was treated with 1,3- 14 C- or 32 P-labelled di-isopropyl phosphorofluoridate, maximum binding of the inhibitor was found to correspond to 2.0 ± 0.07 (s.e.m. for four independent experiments) mol of di-isopropyl phosphate bound/mol of enzyme. The fatty acid synthetase treated in this way lacked both thioesterase and fatty acid synthetase activity. Trypsin-treated fatty acid synthetase, which retains all its partial activities except the thioesterase (Smith *et al.*, 1976), bound a maximum of only 0.08 mol of di-isopropyl phosphate/mol of enzyme.

Trypsin treatment of [1,3- 14 C]di-isopropyl phosphate-labelled fatty acid synthetase

The [1,3- 14 C]di-isopropyl phosphate-labelled fatty acid synthetase was digested with trypsin and the products were fractionated by gel filtration. All the radioactivity co-chromatographed with thioesterase (Fig. 4).

Fig. 6. Immunodiffusion studies with anti-(fatty acid synthetase) and anti-thioesterase antibodies

(a) Centre well contained 0.37 mg of anti-(fatty acid synthetase) γ -globulin. (b) Centre well contained 0.36 mg of anti-thioesterase γ -globulin. In both (a) and (b), the following antigens were present in the outer wells; FAS, 5 μ g of fatty acid synthetase; TE, 0.7 μ g of thioesterase; FAS-T, 5 μ g of trypsin-treated fatty acid synthetase. The latter preparation was freed from thioesterase by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration; it contained no residual thioesterase activity.

In a separate experiment, the ^{14}C -labelled fatty acid synthetase and its tryptic digestion products were analysed by SDS/urea/polyacrylamide-gel electrophoresis. The distribution of polypeptide species on the electrophoretogram was identical with that obtained after trypsin digestion of native (i.e. not labelled with di-isopropyl phosphate) fatty acid synthetase; this indicated that the presence of the two di-isopropyl phosphate groups on the fatty acid synthetase did not alter the course of trypsin digestion. Radioautography confirmed that initially the radioactivity was covalently bound to a polypeptide of mol.wt. 220000–240000, which corresponded to the subunit of the multienzyme complex (Fig. 5). After trypsin treatment, radioactivity was associated predominantly with the thioesterase 35000-mol.wt. polypeptide and its nicked 17500- and 15000-mol.wt. products. Two similar experiments were performed in which the polyacrylamide gels were cut into slices, digested, assayed for radioactivity and recoveries determined. Of the radioactivity applied to the gel, 94–96% was recovered in the polypeptides identified by radioautography, 91–94% being associated with the thioesterase and its nicked polypeptides.

Immunochemical studies

When native and trypsin-treated fatty acid synthetases were diffused against antibodies raised against the native multienzyme complex, a reaction of partial identity was observed; a weak spur pointing to the well containing the trypsin-treated fatty acid synthetase indicated the absence of certain antigenic sites on the trypsin-treated enzyme (Fig. 6). The thioesterase also cross-reacted with the anti-(fatty acid synthetase) γ -globulin; the precipitin line did not cross the line formed by the native fatty acid synthetase, but did cross the line formed by the trypsin-treated fatty acid synthetase. The same samples were also diffused against antibodies raised against the isolated thioesterase. The concentric circles around the antibody well are apparently due to non-specific precipitation of γ -globulins, since they are observed in the absence of antigen. The precipitin lines formed by the native fatty acid synthetase and the thioesterase showed no spurring, and were therefore indicative of a reaction of complete identity. No precipitin line was formed with the trypsin-treated fatty acid synthetase and anti-thioesterase antibodies, confirming that this preparation had been stripped of its thioesterase component.

Discussion

Our studies on the binding of di-isopropyl phosphorofluoridate to the fatty acid synthetase are consistent with the results of Kumar (1975) and Lornitzo

et al. (1975), who have proposed that the animal fatty acid synthetases contain two thioesterases. Our experiments indicate that di-isopropyl phosphorofluoridate binds specifically to the thioesterase domains of the fatty acid synthetase; thus trypsin-treated fatty acid synthetase, which lacks thioesterase activity and does not react with anti-thioesterase antibodies (Fig. 6), but retains all other partial activities (Smith *et al.*, 1976), binds di-isopropyl phosphorofluoridate to only 4% of the extent with the native fatty acid synthetase. Furthermore, the core of the multienzyme remaining after removal of the thioesterase from [1,3-¹⁴C]di-isopropyl phosphorofluoridate-labelled fatty acid synthetase consists of three polypeptides (or three groups of polypeptides of similar size), which contain less than 3% of the original radioactivity (Fig. 5). The release of all the di-isopropyl phosphate label and recovery in the thioesterase indicates that our procedure of limited digestion with trypsin results in the release and recovery of both thioesterase domains. This conclusion is supported by the observation that the fatty acid synthetase core remaining after trypsin treatment contains no thioesterase activity.

Thus the fatty acid synthetase appears to be composed of two subunits of equal, or near equal, molecular weight and contains two thioesterase domains, both of which are released by limited digestion with trypsin. Although these data seem to be compatible with a homodimer model for the multienzyme complex, such a conclusion may be premature at this time, particularly in view of the apparent separation of the fatty acid synthetases into two non-identical components by Lornitzo *et al.* (1975).

The line of identity formed between fatty acid synthetase and thioesterase on diffusion against anti-thioesterase antibodies indicates that all the antigenic sites on the isolated thioesterase are also present on the fatty acid synthetase. This is a good indication that no extensive changes in three-dimensional structure of the thioesterase domains have taken place during trypsin treatment. Nevertheless, there is evidence to show that the thioesterase is nicked by trypsin (Fig. 1). Electrophoresis of the denatured thioesterase reveals polypeptides of mol.wts. 35000 and 17500 (and a trace of a 15000-mol.wt. polypeptide). It seems probable therefore that the site of nicking lies near the middle of the 35000-mol.wt. polypeptide. Thus two polypeptides of 17500 mol.wt. are formed, which are not resolved by SDS/polyacrylamide-gel electrophoresis. The nicked halves of the thioesterase are apparently held together by non-covalent forces and copurify with the intact 35000-mol.wt. polypeptides. Extensive treatment with trypsin can cause nicking at a second point, resulting in the formation of a 15000-mol.wt. polypeptide. Only a trace of this 15000-mol.wt. species is found in the thioesterase isolated from the fatty acid synthetase,

presumably because we have intentionally selected mild conditions for trypsin treatment, to isolate thioesterase of high specific activity. A second minor component of mol.wt. 33000 was also detected on digestion of the thioesterase. It seems likely that this species results from a nick in the 35000-mol.wt. polypeptide at the same point as that which results in the formation of the 15000-mol.wt. species from the 17500-mol.wt. species.

There is strong evidence to suggest that the nicked halves of the thioesterase are enzymically active, but have a lower specific activity than the intact polypeptides.

(i) On destruction of the 35000-mol.wt. polypeptide by trypsin, a small amount of thioesterase activity remains (Fig. 1).

(ii) The 17500-mol.wt. species bind di-isopropyl phosphate covalently, but at a much lower rate than do the 35000-mol.wt. species (Table 2).

(iii) The nicked halves of the thioesterase isolated by gel filtration in the presence of SDS have approx. 9% of the activity of the intact thioesterase polypeptides isolated by the same procedure (Fig. 2).

The intact thioesterase polypeptides isolated by gel filtration in the presence of SDS are homogeneous as judged by SDS/polyacrylamide-gel electrophoresis and would appear to be suitable for structural analysis and primary sequencing. The SDS can be removed from the preparation by ion-exchange chromatography in the presence of 8M-urea. Although this procedure results in complete loss of thioesterase activity, in preliminary attempts to renature the enzyme by gradually diluting out the urea we have been successful in recovering about 10% of the original enzyme activity. This result suggests that the thioesterase domain is capable of refolding independently of the remainder of the polyfunctional polypeptide of which it is a part in the native multienzyme complex.

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