Pigeon liver fatty acid synthetase which contains two subunits of 240,000 daltons each has been treated with elastase. This treatment yields four protein fragments which can be separated on sodium dodecyl sulfate (SDS)-gel electrophoresis. After the subunit protein has been treated with elastase, all of the partial enzyme activities catalyzed by the complex are present, but enzyme activity for fatty acid synthesis is lost.

The formation of protein fragments during proteolysis has been followed by densitometric scanning of the SDS gels. The results of these scans have suggested that (a) there are two peptide components present in the highest molecular weight band, (b) both are rapidly digested to yield the second and third largest peptides, and (c) a further cleavage of the third largest peptide gives rise to the smallest of the four major peptides. Crossed-rocket immunoelectrophoretic analysis of the four protein fragments has confirmed these conclusions and established also that the three smallest peptides are homogeneous.

Each of the four peptides has been isolated by preparative SDS-gel electrophoresis, and antibody to one has been prepared. This antibody fraction immunoprecipitates the native enzyme. Immunoelectrophoresis of the four elastase-digested synthetase products against this antibody showed some cross-reactivity with a peptide that was neither the precursor nor the product of the immunogen. This cross-reacting antibody was removed by reaction with the nonrelated protein to yield antibody specific for one region of the fatty acid synthetase complex.

Fatty acid synthetase is a multienzyme complex of about 500,000 daltons which catalyzes the partial activities necessary for the de novo synthesis of fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. In Escherichia coli the individual partial activities are located on separate polypeptide chains (for a review see Ref. 1). Early work suggested that the complex from eukaryotic sources was also composed of several individual proteins. However, recent work has established that this enzyme complex has the following properties which are inconsistent with this conclusion: (a) rat liver fatty acid synthetase subunits are coded for by a 33 S mRNA (2), (b) the yeast genome contains only two fatty acid synthetase alleles (3), (c) after denaturation, the yeast enzyme complex yields only two proteins on electrophoresis (3, 4), (d) there are copurifying proteases in preparations of enzyme obtained from chicken and rat liver (5) and yeast (6), and (e) enzyme isolated from pigeon liver is proteolytically cleaved in the presence of other proteins which are recalcitrant to this digestion (7). These results have led to the conclusion that the eukaryotic multienzyme complex is composed of two large multifunctional subunits. The mechanism for fatty acid synthesis proposed by Lynen (6) for yeast and Phillips et al. (8) for pigeon liver fatty acid synthetase is consistent with the concept of a multifunctional enzyme complex.

After the initial condensation of acetyl and malonyl moieties in fatty acid synthesis, each two-carbon unit goes through two reductions and a dehydration before the next condensation without the appearance of any free intermediates (8). In the sequence of these reactions the growing acyl chain remains covalently bound to the prosthetic group, 4'-phosphopantetheine, until the product fatty acid is formed (8). Seven cycles of the above sequence are repeated until enzyme-bound palmitoylpantetheine is formed. The postulation that the growing acyl chain is carried from active site to active site by 4'-phosphopantetheine is supported by the rigid and stable orientation that is afforded by a multifunctional protein.

It is to be expected that the seven active sites of the synthetase would be located within autonomous domains of the two multifunctional proteins. Often the peptide sequences between the domains of such a multifunctional protein have less secondary and tertiary structure, and are more susceptible to proteolysis (9). Hence, limited proteolysis has often been employed to unequivocally identify multifunctional proteins (for a review see Ref. 10).

Limited digestion of fatty acid synthetase complexes in the absence of added proteases has been reported for yeast (6), pigeon (11), chicken (12), human, and rat enzymes (13). Most of these studies involved the release of an 8,000 to 12,000 molecular weight peptide containing 4'-phosphopantetheine. Procedures for the isolation of this peptide from rat (14) and pigeon liver (15) using limited trypsin digestion have also been reported. In addition, limited digestion by trypsin (15) and elastase (17) of rat mammary gland fatty acid synthetase have yielded the release of the thioesterase domain. This activity has been found on a 35,000-dalton peptide which readily dissociates from the core of the enzyme (18).

In the present paper, it is shown that all of the partial enzyme activities remain after the pigeon liver fatty acid synthetase complex has been subjected to elastase treatment, 1

1 In this paper the term "domain" specifically refers to an independent region of a multifunctional protein which contains a single catalytic or binding activity. Multifunctional proteins contain a minimum of two autonomous domains.

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Limited Elastase Digestion of Pigeon Liver Fatty Acid Synthetase with Retention of All Partial Enzyme Activities*

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even though fatty acid synthesizing activity is lost. Elastase
treatment of the pigeon liver enzyme yields four major protein
fragments which are separated by SDS-gel electrophoresis.
The relationship between these proteins is also studied in this
paper.
Recent work has demonstrated the presence of antigenic
determinants for several of the partial activities in the avian liver fatty acid synthetase complex (19, 20). Previous work by
Ommen et al. (21) using staphylococcal nuclease as a model
protein, suggested that there exist two classes of antigenic
determinants—conformational and linear. The latter is found in
denatured proteins and depends only on the amino acid se-quence. These workers also combined limited proteolysis
in denatured proteins and depends only on the amino acid
sequence. These workers also combined limited proteolysis
and immunoaffinity chromatography to localize antigenic de-
terminants on the nuclease sequence (22). In this paper we
also report on the application of this approach to the study of
the pigeon liver fatty acid synthetase. This work has been
greatly aided by a technique in which SDS-gel electrophoresis
is coupled to crossed-rodder immunoelectrophoresis as a two-
dimensional analytical probe (23, 24). Each of the four syn-
thesase protein fragments generated by elastase treatment
and resolved by SDS-gel electrophoresis cross-reacts with
antibody raised to the native undigested enzyme. Conversely,
antibody raised in response to one of these denatured proteins
immunoprecipitates fatty acid synthetase activity and immuno-
precipitates the native enzyme. Finally, evidence that anti-
odies to these proteins can be used to analyze distinct regions
of the fatty acid synthetase is also presented in this paper.

EXPERIMENTAL PROCEDURES

Materials—SDS, acrylamide, TEMED, Coomassie blue R-250, and
ammonium persulfate (electrophoresis grade) were purchased from
Bio-Rad; EDTA and N,N'-methylenebisacrylamide (electrophoresis
grade) from Mallinkrodt; ammonium sulfate (enzyme grade) from
ICN; acetyl-CoA, malonyl-CoA, and palmityl-CoA from P-L Bi-
chemicals; [14C]acetyl-CoA, [14C]malonyl-CoA, and [14C]palmitoyl-
CoA from New England Nuclear; diithiothreitol from Calbiochem;
potassium phosphate (analytical grade) from Mallinkrodt; porcine
pancreas elastase (type 1), Triton X-100 (type 1), low EEO agarose,
and ammonium persulfate (electrophoresis grade) were purchased from
Sigma; and Freund's complete and incomplete adjuvant from
Iscove's modification of a procedure employed for goose
fat synthesis activity (41, 42). Enoylreductase was as-
tilated by ammonium sulfate, pH 7.8, at room temperature at a final
concentration of 40% of saturation. After precipitation of the core
protein, thiostreotype assays were immediately carried out on the
 supernatant solution. Residual ammonium sulfate was removed from
the solubilized core protein by overnight dialysis at 4°C in 0.2 M
potassium phosphate, 1 mM EDTA, and 1 mM diithiothreitol. After a
1.5-h analysis in fresh buffer at room temperature, assays for enzyme
activity were carried out.

Quantitative Densitometric Scanning of SDS Gels—Scanning
was carried out at 570 nm on a Gilford 240 Spectrophotometer, using a
Gilford linear transport.

Immunoprecipitation of Proteins from SDS Gels—A number of different tech-
niques for this procedure have appeared in the literature (24, 34, 35).
In early experiments, modifications of two of these procedures were
used (24, 36). Briefly, 1.5-mm slabs of 5 to 10% acrylamide-SDS
gradient gels were constructed, and 150- to 300-pg aliquots of elastase-
treated synthetase protein were loaded into each well. The buffer and
gels were those described by Laemmli (40), and the modification
of Knowland (32) was introduced into the running gel. After electropho-
resis, two approaches were employed. In the first, the complete slab
was stained and destained to localize the protein digestion products.
After destaining, the slabs were vacuum dried onto Whatman No.
PM-10 membranes. Nonspecific adsorption sites on the membranes
were masked by a sodium deoxycholate wash. The membranes
were sectioned from the membranes was excised from the dried acrylamide slab with a razor blade. These sections
were cut into disc gel tubes over glass wool or 5% Laemmli acrylamide
plugs (40) with dialysis bags attached to the bottoms of the tubes
with paraffin.

Electrolysis was performed overnight in 2.5 mM Tris-glycine,
PH 8.3, buffer containing 0.1% SDS, 1 mM EDTA, and 1 mM 2-
mercaptoethanol at a constant voltage of 300. The lower chamber
of the unit was cooled by running tap water. The proteins were con-
centrated from the electrodialysis by acetone precipitation (9 parts
acetone to 1 part buffer) at 1°C for 30 min. The precipitated protein
was excised from the refrigerated gel after

SDS-Gel Electrophoresis—Samples were prepared for electropho-
resis by removing 20 pg of fatty acid synthetase protein, unless
otherwise indicated, from the incubation mixture, adding SDS and
diithiothreitol to concentrations of 2% and 20 mM, respectively,
and heating in a boiling water bath for 1 to 3 min. The proteins
were separated on 12% SDS-polyacrylamide gels, and the separa-
tion was carried out by the procedure of Knowland (32) on 8% acrylamide gels.
Molecular weight determinations were performed by the method of
Weber and Osborn (33) on 5% acrylamide gels. The marker proteins
used were fibronectin, urease, bovine serum albumin, glutamate de-
hydrogenase, and phosphorylase. Gels were stained in 0.25% Cooma-
sie blue in a solution of 30% ethanol, 10% acetic acid, 60% H2O,
and destained by diffusion.

Removal of Thioesterase from Elastase-Treated Fatty Acid Syn-
thetase—At the indicated times elastase-treated enzyme was precipi-
tated by ammonium sulfate, pH 7.8, at room temperature at a final
concentration of 40% of saturation. After precipitation of the core
protein, thioesterase assays were immediately carried out on the
supernatant solution. Residual ammonium sulfate was removed from
the solubilized core protein by overnight dialysis at 4°C in 0.2 M
potassium phosphate, 1 mM EDTA, and 1 mM diithiothreitol. After a
1.5-h analysis in fresh buffer at room temperature, assays for enzyme
activity were carried out.

Limited Proteolysis—Limited elastase digestion was performed by
incubating the fatty acid synthetase, 1.0 mg of protein/ml, with
porcine elastase, 1.0 or 5.0 pg of protein/ml, in 0.2 M potassium
phosphate, pH 7.0, 10 mM diithiothreitol, and 1 mM EDTA at 30°C
under nogen.

The abbreviations used are: SDS, sodium dodecyl sulfate;
TEMED, N,N,N'-tetramethylmethylenediamine; and EEO, elec-
tronephosmosis.
Elastase Digestion of Pigeon Liver Fatty Acid Synthetase

Elastase digestion in the presence of 1% Triton X-100 was carried out as described by Chua and Blomberg (24) and cross-rocket immunoelectrophoresis was performed by the method of Converse and Papamaster (23) as modified by Chua and Blomberg (24), with the following additional changes. To conserve antibody, the portion of the plate containing polyethylene glycol, antibody, and agarose was decreased by 33%; to establish a more stable pH gradient between the anode and cathode buffer chambers during electrophoresis, the amount of buffer or the ionic strength was increased 25%; and the processing of all immunoprecipitates was then carried out by the following procedure. Plates, or slides, were dried with Whatman No. 1 MM filter paper (45) for 15 min, with two changes of paper. The plates were then soaked for 20 min in saline with a change of solution at 10 min and again in distilled water for 15 min. After a further 15 min drying time (total time, 65 min), the plates were stained for 10 min as described for SDS gels. Background destaining was completed in 5 to 10 min.

Preparation of Antiserum to Peptide 3—A male albino rabbit of the New Zealand strain weighing 2 kg was used to raise antibody to peptide 3 that was isolated as described above. Prior to immunization, the protein was tested for homogeneity by SDS-gel electrophoresis and then dialyzed in electrodialysis buffer if it had been isolated in solubilization buffer. Initial injections contained 80 to 160 µg of peptide in 1 ml of electrodialysis buffer mixed with Freund’s complete adjuvant, plus 0.5 ml of Freund’s incomplete adjuvant. These were administered subcutaneously into multiple (12 to 20) sites of the upper dorsal region at 2-week intervals. The rabbit was then bled once or twice a week, 30 to 70 ml, until the titer began to fall. Titer was routinely measured by the ability of the rabbit serum to inhibit overall fatty acid synthetase activity. Booster injections were given as necessary to elevate the titer. These injections differed from the initial ones by the inclusion of 1 ml of Freund’s incomplete adjuvant and no complete adjuvant. The serum obtained from a number of bleedings was pooled and partially purified by two ammonium sulfate precipitations (46 to 40% of saturation), and a DEAE-cellulose chromatographic separation (46).

Absorption of Contaminating Antibody—Immunoprecipitation was carried out in immunoelectrophoresis buffer (24) containing 1.5% Triton X-100. A constant amount of DEAE-cellulose-purified antibody to peptide 3 and increasing amounts of homogeneous peptide 2 were incubated for 45 min at 37 °C and 48 h at 4 °C. After centrifugation, the precipitated immunoprecipitates, supernatant solutions were tested for antibody to peptide 2 by immunodiffusion (24).

RESULTS

Electrophoresis on Tris-glycine SDS gels (32) of pigeon liver fatty acid synthetase digested by elastase at a ratio of 1000:1 (weight:weight) yields four major peptide bands (Figs. 1b and 2b). The amounts of these peptides were quantitated by densitometric scanning of the stained gels at various times of elastase digestion. Initially, a standard plot was prepared by scanning gels containing 2 to 20 µg of undegraded fatty acid synthetase protein, as determined by the method of Lowry et al. (41). The amount of Coomassie blue stain binding to the synthetase protein was linear throughout this range of protein (data not shown). Therefore, all time course studies were performed by digesting 20 µg of protein.

SDS-gel electrophoresis patterns of fatty acid synthetase that was subjected to short or extended incubations with elastase and the results of the densitometric scans of these gels are presented in Figs. 1 and 2. The 5- and 7-h time points (Fig. 1b) show the four major elastase-derived synthetase peptides. During the initial 7 h of digestion by elastase these peptides account for virtually all of the starting material (Fig. 2b). It is apparent from Figs. 1a and 2a that peptide 1 appears first. After 20 min, about 25% of the synthetase subunit protein (Mw = 240,000) is converted into this protein fragment, and by 1 h, half of the protein is this component (Fig. 2a and 2b). The concentration of this protein fragment increases for 2 h and then gradually decreases until 24 h, when it has almost disappeared. At a 200:1 synthetase-elastase ratio, this peptide is almost completely digested in 2.25 h (data not shown).

After 2 h of elastase digestion there is less than 5% of the original 240,000-dalton protein remaining. Between 2 and 7 h, peptides 2 and 3 increase and peptide 1 decreases (Fig. 2b). Hence, both 2 and 3 appear to be derived from peptide 1. An alternative explanation would be that peptide 3 arises from peptide 2. However, two factors argue against this possibility. Peptide 2 is degraded very slowly. After 72 h at this enzyme:protease ratio, or after a 24-h incubation with a 10-fold higher ratio of protease to enzyme only peptides 2 and 4 remain (data not shown). Secondly, the immunological data presented later in this paper demonstrate the presence of antigenic determinants on peptide 3 that are not found on peptide 2. Since all of the 240,000-dalton subunit is degraded to the band 1 protein fragment, this component must be composed of two distinct peptides which are derived from different regions of the fatty acid synthetase subunits. This conclusion has been confirmed by immunological studies.

One of the two protein fragments of band 1 undergoes a second proteolytic cleavage and forms peptide 2, which is resistant to further digestion. The other peptide is cleaved to yield peptide 3. Then as peptide 3 decreases, peptide 4 increases (Figs. 1b and 2b). Thus, peptide 4 is derived from peptide 3, peptide 1, and the 260,000-dalton protein subunit. Further evidence for the conversion of peptide 3 to peptide 4 comes from the studies which demonstrated that peptides 2 and 4 are the ultimate products derived from elastase-treated fatty acid synthetase, the immunological cross-reactivity between peptides 3 and 4 (reported later in this paper), and the results of SDS-gel electrophoresis on elastase-treated [14C] pantetheine-labeled fatty acid synthetase (47). Radiochemical analysis of the gel slices has shown that the prosthetic group is found on peptides 1, 3, and 4 during elastase digestion.

As the fatty acid synthetase is cleaved by elastase, there is a loss in activity for fatty acid synthetase (Fig. 3). When the ratio of synthetase to elastase is 1000:1, overnight incubation is required for complete loss of activity. At this time, all four peptides are present, although peptide 1 is barely detectable by SDS-gel electrophoresis (data not shown). However, none of the partial activities of the complex are inhibited by the proteolysis, indicating that the hydrolysis of peptide bonds does not occur at the catalytically active sites of the complex. Two activities which may be rate-limiting in the complex (the thioesterase and condensing activities) are both increased by elastase treatment. Trypsinization of the pigeon liver fatty acid synthetase also results in an increase in thioesterase activity and a decrease in fatty acid synthesis. This is caused by the liberation of a peptide containing the thioesterase activity from the complex. The loss of fatty acid synthesizing activity during digestion of the rat mammary gland enzyme also results from the dissociation of the thioesterase domain from the complex (17).

Pigeon liver fatty acid synthetase was incubated with elastase as reported under “Experimental Procedures,” and at the indicated times the core protein was precipitated with ammonium sulfate and the supernatant solutions were assayed for thioesterase activity. In addition, ammonium sulfate was removed from the precipitated core protein by dialysis, and assays were performed for the partial enzyme activities. The loss of thioesterase activity from the precipitated core protein closely paralleled the loss of fatty acid synthesizing activity (Fig. 3). In addition, none of the other partial activities was significantly reduced in the core protein when 85% of the overall fatty acid synthesizing activity was lost (Table 1, Fig. 3). Hence, elastase treatment of pigeon liver fatty acid synthetase causes a specific cleavage of the thioesterase domain.

S. Rabinowitz, M. LaForté, and J. W. Porter, unpublished observations.
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Behavior on SDS-Gel Electrophoresis of Pigeon Liver Fatty Acid Synthetase Not Treated with Elastase—Gel electrophoresis of the fatty acid synthetase not treated with elastase occasionally shows the presence of additional minor proteins (Fig. 1a). These proteins are observed with older preparations of enzyme which have been thawed a number of times but still retain high specific activity (90 to 140 nmol of palmitate formed/min/mg of protein), or with preparations from the complex, which results in the loss of fatty acid-synthesizing activity.

Confirmation by SDS-Gel Electrophoresis of Elastase Liberation of Thioesterase Activity—The results of Figs. 3 and 4 suggest that the loss of fatty acid synthesizing activity on elastase digestion of the complex is due to the dissociation of a peptide containing thioesterase activity. Previously, it had been demonstrated that treatment of 1 mol of pigeon liver fatty acid synthetase with 2 mol of the serine modifying reagent, phenylmethylsulfonyl fluoride (48), results in the loss of thioesterase activity. Similar results were obtained by treating the pigeon liver enzyme with $[^{14}C]$disopropylfluorophos-
phate. Gel electrophoresis of this labeled enzyme after elastase digestion yielded a peak of radioactivity in the region indicated by the arrow in Fig. 1a. This radioactivity moved with the same RF as the subunit of lactate dehydrogenase, a protein of 35,000 molecular weight (49). The loss of overall enzyme activity resulting from tryptic digestion of the rat mammary gland fatty acid synthetase is also accompanied by the liberation of a peptide of 32,000 daltons that contains thioesterase activity or the radioactivity from [14C]diisopropylfluorophosphate (16, 50).

Trypsinization of the pigeon liver enzyme until no residual fatty acid-synthesizing activity remained, followed by ammonium sulfate precipitation, 0 to 40% of saturation, separated the core enzyme from the dissociated peptide. After desalting by gel filtration and concentration by ultrafiltration, the liberated peptide was subjected to polyacrylamide gel electrophoresis in the absence of detergents (43). A single peptide containing thioesterase activity migrated in the gel at an RF corresponding to a molecular weight of approximately 35,000 (42). These data are in agreement with the conclusion reached in the studies on time course of elastase inactivation of the enzyme.

The loss of the 35,000-dalton peptide from the 240,000 dalton subunit generates a 205,000-dalton peptide, which is further degraded by elastase (Fig. 1a). An interesting comparison between the actions of elastase and trypsin on the pigeon liver fatty acid synthetase can be drawn from these and other data. Elastase digestion results in the loss of fatty acid-synthesizing activity in approximately 24 h under the conditions reported in Fig. 1b and yields four major peptides. However, if trypsinization of the same enzyme is terminated as soon as there is no longer any overall fatty acid synthetase activity, high concentrations of the 205,000-dalton peptide still remain.3

Isolation of the Elastase-Derived Peptides—Elastase treatment of the pigeon liver fatty acid synthetase in the absence of detergents leads to the dissociation of the thioesterase domain. The remainder of the complex, referred to as the core, contains the four peptides observed on SDS-gel electrophoresis. However, strong noncovalent forces between the core peptides have made their resolution difficult.

Elastase digestion was performed under conditions designed to yield the four peptides, and then the core protein was concentrated by ammonium sulfate precipitation. The precipitated protein was dissociated by the method of Kumar et al. (51) and added to a Sephadex G-200 column, previously equilibrated at 0°C in dissociation buffer, 5 M Tris, 35 mM glycine, pH 8.3, 1 mM EDTA, and 3 mM 2-mercaptoethanol.

Table I

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Ketonoreduction</th>
<th>Condensing</th>
<th>Malonyl-CoA transacylase</th>
<th>Acetyl-CoA transacylase</th>
<th>Crotolyl-CoA reduction</th>
<th>Dehydration</th>
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<td>1478</td>
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<td>96</td>
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<tr>
<td>35</td>
<td>1029</td>
<td>11</td>
<td>319</td>
<td>84</td>
<td>5.34</td>
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<td>11</td>
<td>1449</td>
<td>295</td>
<td>80</td>
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<td>116</td>
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<td>1032</td>
<td>8.3</td>
<td>1615</td>
<td>341</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

Retention of partial activities by core protein

Fatty acid synthetase (1.0 mg of protein/ml) was incubated with elastase (1.0 µg of protein/ml) and aliquots were removed at the indicated times. The core of the synthetase was then precipitated between 0 and 40% of saturation with ammonium sulfate. The supernatant solutions were immediately assayed for thioesterase activity.

All of the protein eluted in the void volume. The core protein from an elastase digestion that was terminated by the addition of SDS was also loaded onto a 5% polyacrylamide gel, pH 8.8, containing 0.1% Triton X-100 (44) or 8 M urea (52). There was incomplete dissociation of the core protein and poor resolution of the four peptides on both gels (data not shown).

Since SDS-denatured rat liver fatty acid synthetase is immunogenic (53), the four products of elastase digestion were isolated by preparative SDS-gel electrophoresis, as described under "Experimental Procedures." Their homogeneity was initially analyzed by a second gel electrophoresis (Fig. 5). The five gels on the left were purposely overloaded to detect any possible contaminants that could serve as potent immunogens. Unexpectedly, the four peptides had been partially degraded by the time they were re-electrophoresed.4 However, each of the peptides was substantially enriched with respect to the other three (see gels on right, Fig. 5). In addition, any antibody raised to a minor contaminant could be removed by absorption of the antiserum with the contaminating peptide as described under "Experimental Procedures." In this way, antibody specific for each of the peptides is prepared.

Immunoreactivity of Native and Denatured Fatty Acid Synthetase—The investigators who had previously employed SDS-denatured rat liver fatty acid synthetase as an immunogen reported in the presence of Triton X-100 which forms mixed micelles with SDS (55). Presumably, in the wells containing antigen and during diffusion, SDS is stripped from the antigen, and immunoprecipitation then occurs. The SDS-denatured pigeon liver fatty acid synthetase cross-reacts with antibody raised to the native complex, Ouchterlony double diffusion experiments (54) were performed in the presence of Triton X-100 which forms mixed micelles with SDS (55).

4 The unusual lability of the polypeptide backbone of pigeon liver fatty acid synthetase has been investigated in detail (S. Rabinowitz and J. W. Porter, unpublished results).
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FIG. 5. Separation on SDS-gel electrophoresis of the four major peptides produced by elastase treatment of the fatty acid synthetase. From left to right, 100 μg of fatty acid synthetase treated for 6 h with elastase, 100 μg of peptides 1, 2, 3, and 4, and 15 μg of peptides 2, 3, and 4.

The native enzyme established that inhibition of the various partial activities of the enzyme requires different quantities of antibody (19, 20). Only the condensing activity inhibition was as sensitive as the loss of overall fatty acid synthetase activity (19). The similarity in the immunotitration end points for antibody to native enzyme and to peptide 3 suggest that the antibody to peptide 3 also inhibits the condensing activity. Subsequent immunotitration experiments have established this point (data not shown).

High titer preparations of anti-peptide 3 serum were combined and purified by ammonium sulfate precipitation, 0 to 40% of saturation, and DEAE-cellulose chromatography (46). Immunodiffusion of native enzyme with antibody to peptide 3 and to the native enzyme in the absence of detergents showed that the antibody to the fatty acid synthetase was more active than the antibody to peptide 3 in the precipitation of the fatty acid synthetase (Fig. 7). Previous studies on the immunogenicity of another denatured multifunctional protein demonstrated that it had a class of antigenic determinants that was unique to the denatured protein (58). However, the results shown in Figs. 6 and 7 prove that peptide 3 antibody must be at least partially directed against determinants accessible in the native complex. Hence, immunotitration experiments of the individual partial activities of the native fatty acid synthetase by this antibody should establish which partial activities are found on peptide 3.

Immunochemical Properties of the Four Elastase-derived Peptides—Preliminary data from gel scanning (Fig. 2) indicated that band 1 is composed of two peptides which arise from different regions of the complex. There were no data, though, prior to the present work to evaluate the homogeneity of peptides 2, 3, and 4 in Fig. 1. a and b. If any of these peptide bands contained more than one component, there would be two or more immune reactions when they were challenged with antibody to the synthetase. This would result in two or more distinct rockets which would form close to each other and cross on crossed-rocket immunoelectrophoresis; otherwise, a single homogeneous rocket would appear.

SDS-gel electrophoresis of the peptides generated during a 20-min incubation with elastase showed the presence of substantial amounts of undigested subunit and peptide 1 and smaller concentrations of peptides 2 and 3 (Fig. 8a). After a 40-min incubation the subunit protein had nearly disappeared, while peptides 2 and 3 had increased (Fig. 8b). Unstained strips with the same material were employed as the antigen for immunoelectrophoresis, and separate profiles of the immunoprecipitation pattern were obtained with each of two
Elastase Digestion of Pigeon Liver Fatty Acid Synthetase

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reactions (Fig. 8, a and c) or are crossed (Fig. 8b), indicating partial or complete nonidentity as was suggested by the gel scanning data. Peptides 3 and 4 are part of the same immune reaction (Fig. 8, a and d) and the overlap between their rockets is always smooth, with no evidence of spurring. Hence, the preliminary data from gel scanning, which indicated that cleavage of peptide 3 yields peptide 4, is confirmed by the

different concentrations of antibody. Fig. 8a illustrates that two basic immunoreactions, in the background and foreground, are occurring. In both figures there are two rockets and hence, two peptides, associated with band 1. In Fig. 8b the two rockets are clearly crossed at their junction, indicating partial or complete nonidentity (59) between the antigens. Hence, the two peptides contain unique determinants. The two rockets were resolved by differences in their respective immunoprecipitation end points at different concentrations of antibody, Fig. 8a. The higher peptide 1 rocket in the background is the precursor of peptide 3, and the lower peptide 1 rocket in the foreground is the precursor of peptide 2. An elastase incubation for 20 min yielded complete subunit, complete subunit minus thioesterase peptide, and a peptide which is a transient precursor of peptide 1 (three highest molecular weight proteins on gel), Fig. 8a. The position of the rocket formed from the transient peptide indicates that it is also a precursor to peptide 3.

The enzyme was then incubated with elastase for 5.5 h to yield the four major digestion products, Fig. 8c. None of the rockets for peptides 2, 3, and 4 seen in Fig. 8, nor the rockets seen using gel slices from other time points (data not shown), contained nonidentical rockets as observed for peptide 1 in Fig. 8, a–c. Hence, peptides 2, 3, and 4 are essentially homogeneous by the criteria of size and immunological properties. These immunoelectrophoresis plates also establish that each of the four SDS-denatured peptides contain antigenic determinants which cross-react with antibody raised to the native complex in the presence of Lubrol.

It is also possible to evaluate the immunological identity among the four peptides from the above data. The rockets to peptides 2 and 3 are either portions of separate immune

FIG. 7. Immunoprecipitation of fatty acid synthetase by DEAE-cellulose-purified antibody. The center wells contained 200 µg of antibody that was obtained from rabbits injected with native fatty acid synthetase (lower) or purified peptide 3 (upper). The peripheral wells contained native fatty acid synthetase: 1, 50 µg; 2, 5 µg; 3, 1 µg; 4, 200 ng; 5, 40 ng, and 6, 5 ng. Ten µl of serum from the rabbit injected with peptide 3 also precipitated the native enzyme, while 10 µl of serum from the same rabbit prior to immunization showed no cross-reactivity.

FIG. 8. Cross-reactivity of fatty acid synthetase treated with elastase and antibody raised to native enzyme. Fatty acid synthetase was treated with elastase as in Fig. 2 for (a) 20 min, (b) 40 min, and (c) 5.5 h. SDS-gel electrophoresis (8% acrylamide) resolved the four major peptides for crossed-rocket immunoelectrophoretic analysis. DEAE-cellulose-purified antibody raised to the native fatty acid synthetase (0.66 mg/cm² in b and 0.38 mg/cm² in a and c) was employed to form the immunoprecipitate in the crossed-rocket immunoelectrophoresis.
immunological identity of the peptides.

**Immunological Properties of Peptide 3**—The above conclusions were further confirmed by immuno-electrophoresis experiments employing antibody to peptide 3. Peptide 1 now forms only one homogeneous rocket (Fig. 8a), as do peptides 3 and 4 (Fig. 8b). Thus, antibody to peptide 3 cross-reacts with the immunogen, peptide 3, as well as the precursor and product of peptide 3, peptides 1 and 4. There is also a small arc present in the position where peptide 2 showed a rocket against the antibody to the fatty acid synthetase. There are three possible explanations for this. It could be due to (a) a slight contamination of the peptide 3 immunogen preparation with peptide 2, (b) peptides 2 and 3 arising from overlapping cleavage of the synthetase subunit, or (c) that different regions of the subunits share sequence homology. Two candidates for the last possibility are the acetyl-, malonyl-, and palmitoyl-CoA transferase domains or the multiple NADPH binding sites.

The cross-reactivity of antibody to peptide 3 with a number of antigens in 1% Triton X-100 has also been investigated (Fig. 10a). The antibody to denatured peptide 3 shows strong immunoprecipitation lines against native and SDS-denatured fatty acid synthetase (wells 1, 4, and 6). The line of identity between these antigens (wells 1 and 6) indicates that antibody raised in response to the denatured fragment cannot distinguish between denatured and non-denatured synthetase.

**Fig. 9. Cross-reactivity of fatty acid synthetase treated with elastase**—The antibody raised to peptide 3. Fatty acid synthetase was treated with elastase as in Fig. 2 for (a) 1 h and (b) 6 h. SDS-gel electrophoresis (6% acrylamide in a and 8% acrylamide in b) resolved the four major peptides for crossed-rocket immuno-electrophoretic analysis. DEAE-cellulose-purified antibody raised to peptide 3 (0.33 mg/cm²) in (a), and 0.29 mg/cm² in (b) was employed to form the immunoprecipitate.

**Fig. 10. Immunodiffusion in the presence of 1% Triton X-100.** A, center well, 200 µg of DEAE-cellulose-purified peptide 3 antibody. Peripheral wells: 1, 4 µg of fatty acid synthetase in 0.23% SDS + 0.3% Triton X-100; 2, 3 µg of peptide 2 in 0.05% SDS + 0.6% Triton X-100; 3, 4 µg of peptide 4 in 0.05% SDS + 0.6% Triton X-100; 4, 8 µg of fatty acid synthetase in 0.05% SDS + 0.6% Triton X-100; 5, 0.1% SDS + 1% Triton X-100; and 6, 4 µg of fatty acid synthetase. B, center well, 3 µg of peptide 2 in 0.1% SDS + 1% Triton X-100. Peripheral wells: 5% of the supernatant recovered after immunoprecipitation of 125 µg of DEAE-cellulose-purified peptide 3 antibody by 1, 0 µg, 2, 5.7 µg, 3, 11.4 µg, 4, 17.1 µg, 5, 22.8 µg, and 6, 28.5 µg of peptide 2.

There is also a strong reaction between denatured peptide 4 and antibody to denatured peptide 3 (well 3). This is consistent with the cleavage of a small fragment of peptide 3 to yield peptide 4. Further evidence is the line of identity between denatured peptide 4 and the native enzyme (wells 3 and 4) indicating that the antigenic determinants on the fatty acid synthetase which are recognized by the antibody to peptide 3 are found on peptide 4. Denatured peptide 2 also cross-reacts with antibody to peptide 3 (well 2). The precipitin line is not as sharp, and considerable spurting is observed with the anti-peptide 3 SDS-denatured fatty acid synthetase immune reaction (wells 2 and 3). Finally, the inclusion of both detergents in well 5 without any antigen as a control proves that the observed precipitin lines are the result of immunoprecipitation.

Specific antibody to peptide 3 was prepared by absorbing the contaminating antibody to peptide 2 with homogeneous peptide 2 (Fig. 10b), as described under "Experimental Procedures." The supernatant solutions remaining after constant amounts of antibody to peptide 3 were absorbed with increasing concentrations of peptide 2 were tested for antibody monospecificity in Ouchterlony immunodiffusion plates containing 1% Triton X-100. The absorbed antibody solutions were added to the peripheral wells and peptide 2 was placed in the central well. All of the cross-reacting antibody was removed from those absorptions that contained the higher concentrations of peptide 2. The remaining antibody which still cross-reacts with peptide 3 is now specific for this region of the synthetase.

**DISCUSSION**

Work on fatty acid synthetases from a variety of animals and tissues has indicated that the eukaryotic complex consists of two multifunctional proteins. These proteins would be expected, therefore, to contain multiple domains which individually catalyze the partial enzyme activities required for fatty acid synthesis. Limited trypsinization of the rat mammary gland enzyme has already yielded one native domain which has been isolated and characterized (18). An extension of this approach would be expected to lead to a greater insight into the structural organization and catalytic mechanism of pigeon liver fatty acid synthetase, and possibly contribute to our understanding of the mode of regulation of this complex.
After elastase treatment of the pigeon liver fatty acid synthetase, all of the protein and partial enzyme activities remain in a nondissociated core except for the thioesterase. Dissociation of this core into four individual peptides can be effected by SDS-gel electrophoresis. Since elastase cleaves proteins at a number of peptide bonds, the specificity of this proteolysis is determined by the synthetase conformation which exposes only a limited number of residues to hydrolysis. Because none of the partial activities are lost in the time course experiment, the nicks in the complex probably occur in the interdomain regions of the protein backbone, leaving all of the catalytically active sites functional. The liberation of the thioesterase domain from the complex enhances its capacity to hydrolyze the model substrate. This result is also observed with the rat mammary gland enzyme (17). The slight activation of the condensing enzyme is more enigmatic, since dissociation of the complex into its subunits is accomplished by a complete loss of this activity. Presumably, the nicking of the synthetase results in a slight change in its conformation, which makes this reaction more favorable.

It has recently been suggested (61) that one difference between the pigeon liver and bovine lactating mammary gland fatty acid synthetases is the inability of the former to reduce crotonyl-CoA (62). However, when the dehydrolase and the enoyl reductase activities of the pigeon liver fatty acid synthetase were measured by the procedure reported under "Experimental Procedures" in which crotonyl-CoA is used as the substrate, both activities were shown to be linear with respect to increasing enzyme concentration (42).

The four peptides which appear after digestion have been characterized on the basis of size, time of formation and immunological properties. Quantitative gel scanning has indicated that the two intermediate molecular weight peptides (2 and 3) are derived from peptide 1 and that peptide 3 is further digested to form peptide 4 (Fig. 2). Almost all of the initial synthetase is accounted for by the four peptides. Hence, the two final digestion products (peptides 2 and 4) represent different regions of the unmilled subunits (240,000 daltons). Since both are directly or indirectly derived from peptide 1, it was postulated that peptide 1 consists of two different fragments of the synthetase subunit.

Immunological studies have confirmed the above conclusions (Figs. 8 and 9). Two-dimensional crossed-rocket immunoelectrophoresis, using SDS gel strips in the first dimension (23, 24), demonstrated that peptide 1 forms two nonidentical or partially nonidentical immunoprecipitates, that peptides 3 and 4 are cross-reacting, and that peptides 2 and 3 are nonidentical or partially nonidentical.

To study the organization of domains on the fatty acid synthetase, homogeneous preparations of the digestion products need to be analyzed. One method for determining which partial activities are found on these peptides involves immunotitration of the native enzyme with antibody specific for each of the digestion products. It was previously demonstrated that all of the partial activities in the core of the pigeon liver enzyme can be immunotitrated (19, 20), and that the antibody to the native enzyme is produced if SDS-denatured rat liver synthetase is employed as an immunogen (53). This paper reports that antibody raised to one of the denatured digestion products can also immunotitrate overall enzyme activity (Fig. 6). By extending this work to immunotitration of partial activities, it will be possible to determine which partial activity(ies) is being inhibited by antibody generated to each of the peptides.

Future work from this laboratory will utilize this approach to systematically investigate the individual activities found on each of these peptides.
Elastase Digestion of Pigeon Liver Fatty Acid Synthetase

Limited elastase digestion of pigeon liver fatty acid synthetase with retention of all partial enzyme activities.
S S Rabinowitz, M LaPorte and J W Porter


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