SPECIFIC MODIFICATION OF FATTY ACID SYNTHETASE FROM LACTATING RAT MAMMARY GLAND BY CHYMOTRYPSIN AND TRYPSIN[1]

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SUMMARY

Fatty acid synthetase from lactating rat mammary gland after limited proteolysis with chymotrypsin or trypsin synthesizes longer chain fatty acids than those produced by the native enzyme. Of the seven partial reactions of the multienzyme complex, only the thioesterase activity was decreased. The results suggest that modification of the fatty acid synthetase product specificity by chymotrypsin and trypsin results from a specific action of these proteases on the thioesterase component. Trypsin, but not chymotrypsin, cleaved a catalytically active thioesterase from the complex; it thus appears that limited trypsinization will be a useful tool for the isolation of the thioesterase component of the multienzyme.

In higher organisms, the enzymes involved in the synthesis of fatty acids from acetyl- and malonyl-CoA are arranged in a multienzyme complex, whereas in plants and most microorganisms the enzymes are present as discrete, monofunctional proteins (1). Despite the fact that fatty acid synthetase multienzyme complexes have been purified from numerous sources, little progress has been made in the isolation and study of the individual components of the complex.

Limited proteolysis under nondenaturing conditions has yielded useful information regarding the structure and functional properties of a number of enzymes which consist of large polypeptide chains (2-4). In this communication we report the results of experiments which demonstrate the potential of

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proteolytic enzymes in unraveling the structure of the fatty acid synthetase multienzyme complex.

MATERIALS AND METHODS

Fatty acid synthetase was purified from lactating rat mammary gland (5). Assay mixtures contained 0.1 M potassium phosphate buffer (pH 6.6), 1.5×10^{-4} M NADPH, 5×10^{-5} M acetyl-CoA and 5.4×10^{-5} M [2-^{14}C] malonyl-CoA (50 nCi) in a volume of 0.5 ml and were monitored spectrophotometrically. A unit of activity is defined as the amount of enzyme catalyzing the malonyl-CoA dependent oxidation of 1 nmole NADPH per min at 30°. The products were identified by gas-liquid radiochromatography (6).

The partial reactions of the fatty acid synthetase (acetyl transferase, malonyl transferase, condensation-CO_2 exchange reaction, S-acetoacetyl-N-acetyl cysteamine reductase, S-β-hydroxybutyryl-N-acetyl cysteamine dehydrase and S-crotonyl-N-acetyl cysteamine reductase) were assayed with model substrates by the procedures described by Kumar et al (7) with only minor modifications. All assays were performed under optimal cofactor concentrations; reaction rates were a linear function of protein concentration and time. The assay system for the thioesterase reaction contained 0.2 M potassium phosphate buffer (pH 6.6), 50 µg bovine serum albumin and 5 µM [1-^{14}C] palmityl-CoA (10 nCi) in a final volume of 1.0 ml. 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 and 1.0 ml ethanol. The [1-^{14}C] palmitic acid released was extracted with petroleum ether (2 x 3 ml), the solvent evaporated and the radio-

TABLE I

THE EFFECT OF CHYMOTRYPSIN AND TRYPSIN ON ACTIVITY AND PRODUCT SPECIFICITY OF FATTY ACID SYNTHETASE

Protease	% Inhibition of fatty acid synthetase	Products (moles %)			
		C_{14}	C_{16}	C_{18}	C_{20}
None	0	4.7	90.2	5.1	0
Chymotrypsin, 0.6 µg	15	4.4	84.6	11.0	0
Chymotrypsin, 2.1 µg	47	3.9	61.3	34.2	0.6
Chymotrypsin, 4.2 µg	61	2.9	30.1	61.2	5.8
Trypsin, 2.1 µg	60	4.7	70.5	21.7	3.0
Trypsin, 4.2 µg	94	0	46.8	46.1	7.1

Rat mammary gland fatty acid synthetase (100 units, 0.1 mg protein) was incubated at 30° with protease in a final volume of 0.15 ml containing 0.1 M potassium phosphate buffer (pH 7.0), 1 mM dithiothreitol and 1 mM EDTA. Incubations with chymotrypsin were for 10 mins, those with trypsin were for 20 mins. At the end of the incubation 20 µl portions were removed and assayed immediately for fatty acid synthetase activity and the chain length of the fatty acids produced was determined. Calculations of moles % assume one unlabeled acetyl group per fatty acid molecule.

activity determined by liquid scintillation spectrometry. A unit of activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 nmole palmityl-CoA per min at 30°.

Bovine pancreatic trypsin (210 U/mg) and chymotrypsin A (55 U/mg) were obtained from Worthington. Each enzyme was found to be free of contamination with the other by potentiometric assay (8,9). Soybean trypsin inhibitor was obtained from Calbiochem.

RESULTS

Preliminary experiments with chymotrypsin and trypsin revealed that both proteases altered the product specificity of the fatty acid synthetase (Table I). Whereas palmitic acid was the main product of the native enzyme, the proportion of stearic and arachidic acids synthesized increased in proportion to the extent of proteolysis. The shift in product specificity was accompanied by a

TABLE II

THE EFFECT OF CHYMOTRYPSIN AND TRYPSIN ON THE PARTIAL ACTIVITIES OF FATTY ACID SYNTHETASE

	% of Control Activity	
Enzyme Activity	Chymotrypsinized	Trypsinized
Fatty acid synthetase	13	60
Acetyl transferase	106	105
Malonyl transferase	100	100
Condensing enzyme	90	94
Keto-reductase	90	97
Dehydrase	98	95
Enoyl-reductase	100	111
Thioesterase	29	60

For chymotrypsinization, rat mammary gland fatty acid synthetase (5,670 units, 4.65 mg protein) was incubated for 10 min at 30° with 30 µg chymotrypsin in 9.0 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 1 mM EDTA. Fatty acid synthetase was precipitated with ammonium sulfate (33% saturation) and redissolved in 1.0 ml of 0.25 M potassium phosphate buffer containing 1 mM dithiothreitol and 1 mM EDTA. Samples were dialyzed for 3 hours against the same buffer prior to assay of enzyme activities. For trypsinization, rat mammary gland fatty acid synthetase (10,100 units, 10.0 mg protein) was incubated for 1 hr at 30° in 4.0 ml of potassium phosphate buffer, 0.1 M (pH 7.0) containing 1 mM dithiothreitol 1 mM EDTA and 5 µg trypsin. Digestion was arrested by the addition of 15 µg trypsin inhibitor and the enzyme activities measured directly.

progressive decrease in the capacity of the complex to catalyze the overall reaction of fatty acid synthesis. Similar results were obtained when the concentration of protease was kept constant and the duration of incubation increased.

To determine which components of the multienzyme were affected by trypsin or chymotrypsin, we examined each partial reaction of the complex treated with these proteases. The results are given in Table II as a percentage of controls which were treated identically except that the protease was omitted. The fatty acid synthetase activity of these control preparations was identical to the original native enzyme. Proteolysis resulted in a decrease in the overall capacity for fatty acid synthesis and in the thioesterase activity, but no significant effect on the other partial reactions was observed. It seems likely,

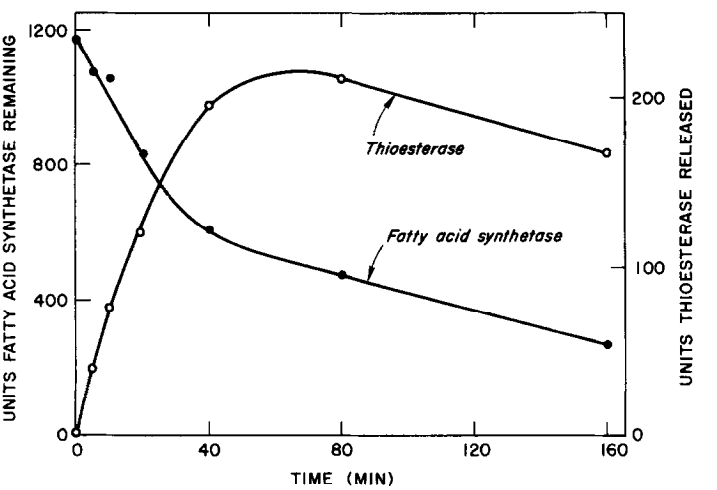

Fig. 1. Time course for inactivation of fatty acid synthetase and release of thioesterase by trypsin. Rat mammary gland fatty acid synthetase (0.95 mg, 1180 units of fatty acid synthetase, 560 units of thioesterase) was incubated at 30° in 0.1 M potassium phosphate buffer pH 7.0 containing 1 mM dithiothreitol, 1 mM EDTA and 0.7 µg trypsin. Final volume was 1.0 ml. Reactions were terminated by the addition of 1.4 µg trypsin inhibitor. A portion of each sample was kept for determination of fatty acid synthetase activity, the remainder was adjusted to 33% saturation with respect to ammonium sulfate, left at 0° for 30 min and then the precipitated protein was removed by centrifugation. Portions of the 33% saturated ammonium sulfate supernatant fraction were taken for determination of thioesterase activity; no fatty acid synthetase activity was present in this fraction.

therefore, that the decrease in thioesterase activity induced by proteolysis was responsible for the change in product specificity of the complex. This result is in agreement with the model scheme of Sumper et al (10) in which a decreased probability for transfer of the acyl group off the enzyme would increase the likelihood of synthesis of longer chain fatty acids.

In subsequent experiments we had occasion to precipitate trypsinized fatty acid synthetase with ammonium sulfate. All of the residual fatty acid synthetase activity was precipitated by 33% saturated ammonium sulfate along with the acetyl transferase, malonyl transferase, condensing enzyme, keto-reductase, dehydrase and enoyl-reductase. However, a considerable amount of thioesterase activity remained in the supernatant. This thioesterase activity could be precipitated by increasing the salt concentration to 80% saturation. Control experiments showed that neither the trypsin nor trypsin inhibitor (used to stop trypsinization) possessed thioesterase activity, confirming that the thioesterase activity recovered in the 33% supernatant originated from the multienzyme complex. Similar experiments with chymotrypsin did not yield significant thioesterase activity in the 33% saturated ammonium sulfate supernatant fraction.

The time course for inactivation of fatty acid synthetase and release of thioesterase in a typical trypsinization reaction is shown in Fig. 1. The progressive decrease in fatty acid synthetase activity observed on trypsinization was accompanied by a release of the thioesterase component from the complex. After trypsinization for 80 min, approximately 38% of the thioesterase activity of the native enzyme was recovered in the 33% saturated ammonium sulfate supernatant fraction. The amount of ammonium sulfate transferred to the thioesterase assay in this type of experiment is small, usually less than 0.02 M final concentration, and has no effect on the thioesterase activity. In the experiment shown in Fig. 1, the amount of protein remaining in the 33% saturated ammonium sulfate supernatant was too small to be measured reliably. In subsequent large scale experiments we have consistently isolated the thioesterase with specific activities of over 1000 units per mg protein. This represents a significant

increase over the specific activity of the thioesterase associated with the multienzyme complex, usually about 500 units per mg protein.

DISCUSSION

Under conditions of limited proteolysis, both chymotrypsin and trypsin selectively attack the thioesterase component of the fatty acid synthetase multienzyme complex. Trypsin, which has a narrow specificity for lysyl and arginyl bonds (8) actually cleaves the thioesterase component from the complex in a catalytically active form. When the fatty acid synthetase is treated with chymotrypsin, a discrete, active thioesterase is not obtained. Presumably the broader specificity of chymotrypsin (9) results in rapid inactivation of the thioesterase, either on the multienzyme, or after cleavage from the complex. We have found that the thioesterase released from the multienzyme complex by trypsinization is also susceptible to further proteolysis and it is likely that this is the reason we have been unable to recover more than about 40% of the thioesterase activity in the dissociated form.

Lornitzo et al (11) have found that both subunits of the pigeon liver fatty acid synthetase contain thioesterase activity. The theory that the multienzyme complex contains two thioesterases, which may or may not be identical, is further supported by studies with phenylmethylsulfonyl fluoride, a thioesterase inhibitor (12). At this point, we cannot say whether one or more thioesterases are released from the complex by trypsinization since less than 50% of the activity is recovered in the dissociated form. The released thioesterase does seem to consist of a single molecular species, since all the thioesterase activity is eluted from a column of Sephadex G75 as one component which appears homogeneous as judged by analytical ultracentrifugation. The molecular weight is approximately 32,000.

This study represents the first successful attempt to isolate one of the partial activities of the fatty acid synthetase multienzyme complex. A detailed study of the properties of the thioesterase component will be reported separately.

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