SITES OF CLEAVAGE OF GLUCAGON BY INSULIN-GLUCAGON PROTEASE

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Summary

To study the mechanism of degradation of glucagon with purified insulin-glucagon protease, glucagon was reacted with the enzyme at various times of incubation. The proteolysis was followed by the production of flourescamine-reacting material as well as reaction with dansyl chloride, cleavage by acid hydrolysis, and identification by thin layer chromatography. For quantitative measurement of the degradation products, [^{14}C] dansyl derivatives were produced, identified by autoradiography, and counted. In the degradation products in addition to histidine, the dansyl derivatives of tyrosine, phenylalanine, two leucines, alanine and lysine were identified. For comparison, glucagon was also reacted with chymotrypsin and the degradation products consisted of threonine, serine, two leucines and valine. Thus, insulin-glucagon protease degrades glucagon in a manner distinct from that of chymotrypsin.

Introduction

An enzyme which can degrade both insulin and glucagon has been previously isolated from skeletal muscle (1). This enzyme has been highly purified by a series of procedures including affinity chromatography and shown to be a single enzyme (2). The proteolytic nature of the enzyme has been shown by its ability to produce an increase in ninhydrin positive materials when incubated with insulin but this required prolonged incubation (18-24 hours) to produce a significant increase (3). Using a more sensitive assay for proteolysis with fluorescamine, the rapid (within 2 minutes) and direct proteolysis of insulin and glucagon by the purified enzyme

has been demonstrated (4). This enzyme appears to be highly specific for these two peptides in that a large number of peptides and proteins including insulin A and B chains, proinsulin, ACTH, growth hormone, albumin, and collagen derivatives were not degraded as measured by changes in fluorescamine reactivity (4).

The degradation products resulting from the reaction of this enzyme with its substrates have not been identified. For this reason, the following study was done. Glucagon was chosen as the initial substrate to be studied because of the higher V_{max} of this reaction and thus a greater amount of material could be obtained for study. ^{14}C dansylation which offers a qualitative and a quantitative method in the 10^{-14} mole range was used to identify the amino end groups of the degradation product.

Materials and Methods

The insulin-glucagon protease was prepared from rat skeletal muscle and purified as described previously (2). Due to instability of the purified enzyme, the final step of affinity chromatography on insulin-agarose was performed on the day of the experiment. After elution from the insulin agarose by 0.2M NaCl-0.02M acetate, pH 6.2 the enzyme was dialyzed against 0.01M phosphate, pH 7.5 for 3 hours. The enzyme preparation was then passed over a small cellulose powder column. Glucagon (lot #258-030-138-4) was the gift of the Eli Lilly and Company, Indianapolis, Indiana. ^{14}C-dansyl chloride purchased from Schwartz/Mann with a specific activity of 100 $\mu C/\mu$ mole was substituted for unlabeled dansyl chloride. The ^{14}C-dansyl chloride was diluted, handled, and stored as described by Briel (5). The amino acid mixture and individual dansylated amino acids were purchased from Pierce Chemical Company, Rockford, Illinois. Polyamide sheets were obtained from Schleicher and Schuell, Inc., Kenne, New Hampshire.

For the study of glucagon degradation, the incubation mixture consisted of

the following: 100 μg of enzyme protein in 0.01M phosphate buffer, pH 7.5, glucagon in concentration of 4.14×10^{-5}M in total incubation, volume 5 ml. The reaction mixture was then incubated at 37° for 60 min. Aliquots were removed at intervals for reaction with fluorescamine to assay for completeness of the reaction by the method described previously (4).

The method used for dansylation was similar to that of Gray (6). 1 ml of an incubation mixture of glucagon and enzyme was lyophylized and then redissolved in 100 μl of distilled H_2O. 25 μl was then removed with a Hamilton syringe and placed in a 3 mm x 30 mm tube. The water was then removed in a vacuum dessicator. After drying, 15 μl of 0.03M Na_2CO_3 was added to the dried residue, followed by 25 μl of ^{14}C-dansyl chloride. The pH of the mixture was adjusted to 9.5 with Na_2CO_3. The tubes were then sealed with parafilm and incubated in a 37° water bath for 1 hour, after which time, the mixture was dried in a vacuum dessicator. 50 μl of 6.7N HCl was added to the residue for acid hydrolysis. The tubes were sealed under vacuum and hydrolyzed in a 105° oven for 18 hours. The reaction mixture was then dried and chromatographed on a 3 x 3 double coated polymide thin layer plate. The dansylated products were dissolved in 1 μl of pyridine water (1:1 v/v) and spotted on the thin layer sheets with a Hamilton syringe. On the back side of the plate, the non-radioactive dansylated amino acids were spotted. The plates were then chromatographed in a solution consisting of water and formic acid (100:1.5 v/v) followed by chromatography in a second system consisting of benzene: acetic acid, which was at a right angle to the first direction. The spots were localized and identified under UV light. For further resolution of glutamic acid, aspartic acid, α-lysine and C-lysine, arginine and α-histidine, a third solvent system consisting of ethyl acetate-methanol-acetic acid (20:1:2, v/v) and a fourth solvent system of 0.05M trisodium phosphate: ethanol (3:1 v/v) were employed to identify these amino acids.

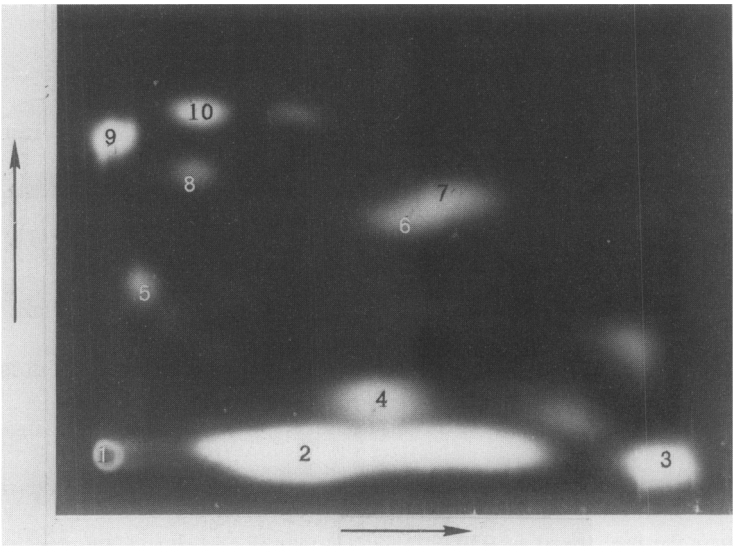

Figure 1: Chromatography of Dns-amino acids on polyamide thin layer plates. The direction of chromatography is indicated by the arrows. 1st direction: water/formic acid 100:3; 2nd direction: benzene/acetic acid 9:1. The numbers on the chromatogram represent the dansylated amino acids for the 60 minute incubation as follows: (1) Starting point (2) Dns-OH (3) α-Dns-Histidine and C-Dns-C-Lysine (4) O-Dns-Tyrosine (5) di-Dns-Lysine (6) Dns-Alanine (7) Dns-NH_2 (8) Dns-Phenylalanine (9) di-Dns-Tyrosine (10) Dns-Leucine.

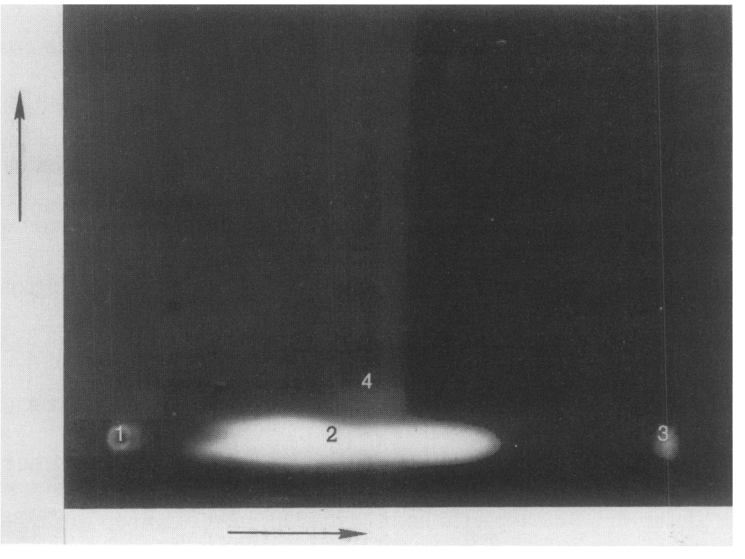

Figure 2: The method of chromatography was the same as in Figure 1. The numbers represent the following dansylated amino acids present in the initial incubation mixture. (1) Starting point (2) Dns-OH (3) α-Dns-Histidine and C-Dns-Lysine (4) O-Dns-Tyrosine.

The individual poly-soft packet of dental X-ray film was then exposed to the thin layer plate for a period of 1 day. The labeled dansyl amino acids were then cut from the plates and placed in individual scintillation vials and each eluted with 3 ml of redistilled chloroform at room temperature for 1 hour with frequent shaking. The segments of the thin layer plates were removed and the chloroform evaporated from the vials. The residue was then dissolved in 10 ml of Liquiflourtoluene scintillation solution (42:1000) and counted in a Searle Isocap/300 spectrometer.

Results and Discussion

The incubation of the purified insulin-glucagon protease with glucagon resulted in the appearance of five new N-terminal amino acids: tyrosine, leucine, lysine,

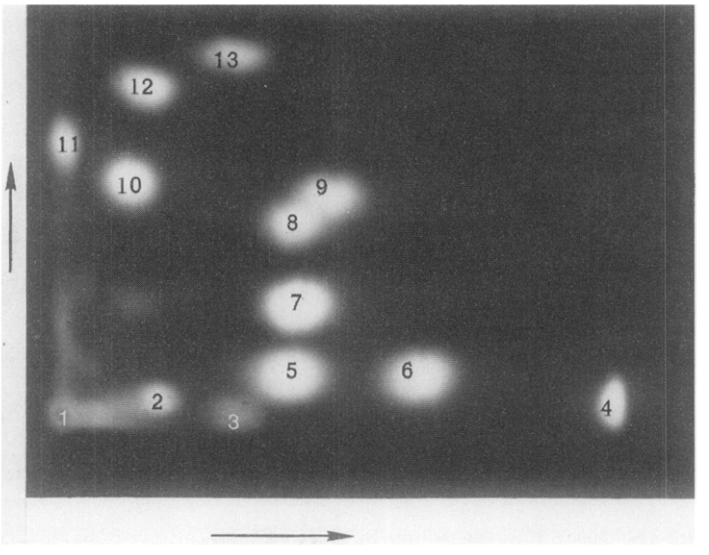

Figure 3: The reference standard was spotted on the back of the double coated polyamide plates. The numbers represent the following Dansylated amino acids: (1) Starting point (2) and (3) Dns-OH (4) Dns-Arginine and α-Dns-Histidine, C-Dns-Lysine, α-Dns-Lysine (5) Dns-Glutamic acid (6) Dns-Serine (7) Dns-Glycine (8) Dns-Alanine (9) Dns-NH_2 (10) Dns-Phe (11) di-Dns-Tyrosine (12) Dns-Isoleucine (13) Dns-Proline.

TABLE I

FORMATION OF [^{14}C]-DANSYL AMINO ACIDS FROM INCUBATION MIXTURE OF INSULIN-GLUCAGON PROTEASE WITH GLUCAGON

	COUNTS PER MINUTE		
	T_0	T_{60}	RATIO $\frac{\text{Amino Acid}}{\alpha\text{-Histidine}}$
α-Histidine	5,300	5,501	1.00
C-Lysine	800	150	
O-Tyrosine	10,505	5,543	1.01
Leucine	0	10,450	1.90
di-Lysine	0	10,289	0.94
di-Tyrosine	0	10,337	0.94
Alanine	0	5,413	0.98
Phenylalanine	0	5,376	0.98

alanine, and phenylalanine (Figure 1) as compared to the control incubation mixture at zero time (Figure 2) and compared to the non-radioactive dansylated amino acids standard (Figure 3). Quantitation of the ^{14}C-labeled dansyl derivative compared with the N-terminal histidine group indicated, as shown in Table I, formation of 2 tyrosines, 2 leucines, 1 lysine, 1 alanine, and 1 phenylalanine.

Thus it appears that 6 peptide bonds in glucagon are cleaved by this enzyme. These results agree with the studies of the production of fluorescamine-reactive material by incubation of the enzyme with glucagon. In previous studies (4) and in the present one, a six-fold increase over basal value in fluorescamine-reactive products is produced by the enzyme's reaction with glucagon, following which

increase, a plateau is reached. These results suggest that the sites of cleavage of glucagon are ser-lys (11-12), tyr-leu (13-14), arg-ala (18-19), trp-leu (25-26) and either thr-phe (5-6) or asp-phe (21-22) and either asp-tyr (9-10) or lys-tyr (12-13).

Using the same procedure, glucagon was reacted with chymotrypsin (data not shown) and as previously shown (7), threonine, serine, 2 leucines, and valine were identified as new N-terminal amino acids. Thus this enzyme clearly attacks sites distinct from those cleaved by chymotrypsin.

These sites of cleavage are also different from other proteolytic enzymes which can degrade glucagon (7). These results, however, do not explain the apparent marked specificity of the enzyme for glucagon and insulin. Attempts are currently being made to identify the sites of cleavage of insulin.

Acknowledgements

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