Purification and Characterization of a Protease with Specificity for Insulin from Rat Muscle

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SUMMARY

A soluble enzyme partially purified from rat muscle is described which degrades insulin proteolytically with a high degree of specificity. The enzyme attacked proinsulin and proinsulin intermediates at only 3 per cent and 10 per cent of the rate with insulin, respectively. Proinsulin competitively inhibited insulin degradation with an inhibition constant of 0.28 μM, whereas the K_m for insulin was 0.18 μM. Insulin derivatives with amino or carboxyl terminal residues removed were attacked with K_m's about five times greater. Removal of an octapeptide from the B chain of insulin resulted in a K_m the same as for insulin. The enzyme is inhibited by the sulfhydryl reagents, N-ethylmaleimide and p-hydroxymercuribenzoate, but not by an inhibitor of the pancreatic proteases, phenylmethylsulfonyl fluoride. The enzymatic activity was not inhibited by a large excess of a number of other proteins and polypeptide hormones. These results suggest that the enzyme is a sulfhydryl-dependent protease which is quite specific for insulin. parameters studied. DIABETES *20*:151-55, March, 1971.

Although insulin degradation in rat liver by proteolysis[1,2] or reductive cleavage[3,4] has been described, the specificity of the first reaction has not been examined, and the second is very broad.[5] Previous work from this laboratory has indicated that a soluble enzyme from rat diaphragm[6] and from rat skeletal muscle[7] rapidly degrades insulin at physiological concentrations. This communication gives additional data on the properties and mode of action of the enzyme, together with evidence suggesting it possesses a high degree of specificity for the insulin molecule.

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MATERIALS AND METHODS

I-125 insulin was obtained from Cambridge Nuclear Corporation (Cambridge, Massachusetts), I-131 insulin was obtained from Abbott Laboratories (North Chicago, Illinois). I-125 insulin* as well as I-131 insulin[6] has been shown to be as active biologically as insulin in the isolated fat cell. The derivatives, d-GP insulin† d-AA insulin and d-OP insulin were kindly furnished by Dr. Frederick Carpenter of the University of California (Berkeley, California). These compounds were iodinated with I-125 according to a modification of the method of Hunter and Greenwood[8] as follows:

To a test tube containing 5 μg. of each of the compounds dissolved in 0.1 ml. of 0.2 M phosphate buffer, pH 7.4, was added 2 millicuries of fresh high specific activity Na I-125, catalogue No. CN5353, Cambridge Nuclear Corp., and the tube capped with a rubber diaphragm. The iodination reaction was started by the addition of 0.025 ml. of 0.4 per cent chloramine T and stopped exactly fifteen seconds later by the addition of 0.1 ml. of 0.24 per cent sodium metabisulfite. To each preparation was added 2.0 ml. of 0.3 per cent BSA and the mixture transferred to a dialysis bag. The preparations were dialyzed three times against one liter of distilled deionized water for three hours at 4° C. The above method is that used for the preparation of I-125 insulin (Cambridge Nuclear Corp.).

Each preparation was further purified by precipitation with 50 per cent saturated $(NH_4)_2SO_4$, then redissolved in 0.3 per cent BSA; after a second precipitation with $(NH_4)_2SO_4$, it was finally redissolved in 0.3 per

* Kitabchi, A. E., Personal communication.

† Abbreviations: d-GP insulin (des-glycinyl^{A1}des-phenylalanyl^{B1} insulin): d-AA insulin (des-asparaginyl^{A21}des-alanyl^{B30} insulin): d-OP insulin (des-octapeptide^{B23-30} insulin): p-HMB (p-hydroxymercuri-benzoate): NEM (N-ethylmaleimide): BSA (bovine serum albumin): GSH (reduced glutathione).

cent BSA. Each iodinated insulin derivative was kept frozen at —20° and prior to use was dialyzed for twenty-four hours against 50-100 volumes of distilled deionized water.

Beef proinsulin and proinsulin intermediates* were the gifts of Dr. Donald Steiner, University of Chicago (Chicago, Illinois). BSA (Fraction V) and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Company (St. Louis, Missouri).

Enzymatic assay

Unless otherwise specified, the enzymatic assay was carried out according to the following method:

The incubation mixture contained enzyme, 0.005 M EDTA, 0.1 M tris buffer, pH 7.5, 0.3 per cent BSA, 0.1 nM I-131 insulin or I-125 insulin. The total volume was 1.0 ml. After completion of incubation at 37°, 1.5 ml. of 10 per cent trichloracetic acid was added and the mixture immediately centrifuged at 1,150 × g in a refrigerated centrifuge at 5° for ten minutes. The resultant supernatant was poured into a separate tube, the last drop being absorbed by a cotton-tipped applicator which was then added to the supernatant. The supernatant and precipitate were then counted in a Packard Auto Gamma Spectrometer and the fraction of the radioactivity in the supernatant corrected for the amount present in control tubes without enzyme. A unit of activity is defined as that amount of enzyme which will destroy 0.001 picomoles insulin/ml./min.

Method of enzyme purification

The rear leg muscles of rats were removed, trimmed of fat and connective tissue, minced, and then homogenized for five fifteen-second intervals in a Waring Blender at high speed in 0.35 M sucrose (5 ml./gm. tissue) and the homogenate centrifuged at 100,000 × g for one hour. The supernatant was dialyzed three times for at least four hours each time against twenty volumes of distilled deionized water and then lyophilized. The dry powder was dissolved in one-eighth the volume of the 100,000 × g supernatant and undissolved material removed by centrifugation at 10,000 × g. The protein content was then determined by the A_{280}/A_{260} absorption method[9] and sufficient 0.015 M acetate buffer, pH 5, added to bring the final protein concentration to 10 mg./ml. (The volume of this solution is hereafter referred to as "v".) Sufficient $Ca_3(PO_4)_2$ gel prepared according to the Keilin and Hartree method[10] was then added to obtain a ratio of gel to protein of 0.28:1. Best results were obtained with freshly prepared gel or gel washed twice before use with distilled water. The gel-protein mixture was stirred at 4° for twenty minutes, followed by centrifugation at 10,000 × g for ten minutes. The supernatant was discarded and the gel resuspended and stirred for twenty minutes in v ml. of 0.05 M phosphate buffer, pH 6.2. Following sedimentation of the gel by centrifugation, as above, it was resuspended and stirred for twenty minutes in v ml. of 0.05 phosphate buffer, pH 7.5. Removal of the gel was accomplished by centrifugation, and the supernatant containing the enzyme was rapidly frozen at —70° in small aliquots until used.

Assay of protease activity

Insulin (1.5 mg. in 0.3 ml. of 0.1 M acetic acid) was added to 1.8 ml. of gel purified enzyme (35 units enzyme per ml.) in 0.05 M phosphate buffer, pH 7.5, together with 0.02 ml. of 0.1 M disodium EDTA. The pH was adjusted to 7.50 with 0.1 M NaOH and the mixture incubated at 37° for sixteen hours. After incubation, 0.05 ml. aliquots were removed, mixed with 0.01 ml. of 0.01 M NEM, and, after appropriate dilution, were assayed for remaining immunoreactive insulin according to the method of Morgan and Lazarow.[11] The remainder of the incubation mixture was lyophilized to dryness, and 0.2 ml. of 0.64 M acetic acid was added. After thoroughly mixing, the tubes were centrifuged in a Sorvall angle head centrifuge at 10,000 × g for ten minutes, after which 0.05 ml. of the supernatant was analyzed for ninhydrin reactive material by the method of Moore and Stein,[12] as modified by Hirs.[13] A standard containing 2 mM leucine was run simultaneously.

RESULTS

The 100,000 × g supernatant solution from homogenates of five tissues was assayed for insulin degradation. The activity found in each of these tissues was the following: liver, 4,300 units/gm., kidney, 1,900

TABLE 1

Purification of insulin-specific protease from rat muscle. For details of purification method see Materials and Methods.

Fraction	Specific Activity*	Purification	Yield
Muscle homogenate	1.8	1.0	100
100,000 × g supernatant	7.5	4.2	85
$Ca_3(PO_4)_2$ gel adsorbed and eluted	91.0	50.5	47

* mμμ moles degraded/min./mg. protein

*Proinsulin intermediates are a mixture of two partially cleaved products of beef proinsulin: one lacking residues 31 and 32, and the second lacking residues 58 and 59. The ratio of the first to the second species is 40:60.

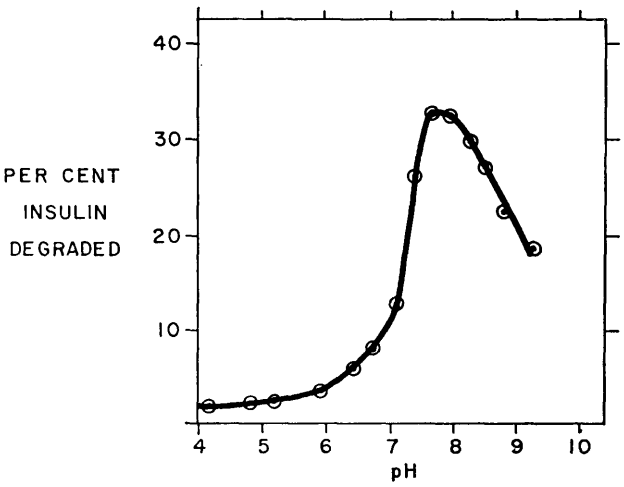

FIG. 1. Variation of enzyme activity with pH. Enzymatic activity at each pH was determined using 0.1 M acetate buffer from pH 4 to 5.3, 0.1 M phosphate buffer from pH 5.9 to 7.1 and 0.1 M tris buffer from pH 7.4 to 9.2.

units/gm., epididymal fat pad (lipid-free infranatant solution), 480 units/gm., diaphragm, 230 units/gm., and rear leg muscle, 240 units/gm. The enzyme was purified from the latter tissue because of the abundance of skeletal muscle and because the activity in a very similar tissue, diaphragm, had already been partially characterized.[6] Table 1 summarizes the method of purification of the enzyme. The purification method routinely results in a forty- to sixtyfold purification starting with muscle homogenate. The enzyme cannot be purified by $(NH_4)_2SO_4$ fractionation, and freezing and thawing has been found to partially inactivate the purified enzyme.

TABLE 2

Activity of purified enzyme in the presence of various agents*

Substance added	Concentration (μM)	Relative Activity (Per Cent)
None	—	100
Insulin A Chain†	0.1	100
Insulin B Chain†	0.1	100
BSA	3.0	100
Globin	10.0	100
Glucagon	2.0	100
Human Growth Hormone	2.0	100
Phenylmethylsulfonyl fluoride‡	1,000.0	100
p-HMB	200.0	2
NEM	1,000.0	0
GSH	1,000.0	210

* I-131 insulin concentration, 10^{-4} μM
† Prepared according to the method of Craig, et al.[27]
‡ Preincubated with the enzyme for 50 min. at 25° in 0.05 M phosphate buffer, pH 7.5, prior to assay.

In figure 1 are presented the results of an experiment to determine the variation of enzymatic activity with pH. The maximum of activity was obtained at pH 7.5 to 7.7 with declining activity on each side of this rather narrow optimum range. When phosphate and tris buffers were compared at the same pH (7.5), it was found that enzymatic activity in phosphate buffer was only 70 per cent of the activity in tris buffer.

Using the purified enzyme, various proteins have been tested for their ability to inhibit the degradation of insulin. The results of these experiments, which are shown in table 2, indicate that neither glucagon, human growth hormone, BSA, nor the A and B chains of insulin inhibit the degradation of intact insulin.

Of the small molecular weight compounds which inhibit insulin degradation, the sulfhydryl reagents, NEM and p-HMB, blocked the activity of the enzyme. The inhibitor of chymotrypsin and trypsin studied by Fahrney and Gold,[14] phenylmethylsulfonyl fluoride, did not inhibit enzymatic activity. GSH stimulated enzymatic activity, which is consistent with its inhibition by sulfhydryl reagents.

In table 3 are presented the results of an experiment in which the ability of the purified enzyme to release ninhydrin-positive material is examined. As can be seen, p-HMB completely abolished protease activity, which is consistent with the inhibition noted in table 2. Thus, the enzyme is apparently a sulfhydryl-dependent protease. Immunoassay of the reaction mixtures indicated that 50 per cent of the insulin was degraded in the noninhibited reaction. The net amount of ninhydrin-reactive material released would indicate approximately five amino terminal residues have been produced by enzymatic degradation. When each of the mixtures was analyzed by paper chromatography in butanol: acetic acid:water (4:1.5), the noninhibited reaction mixture demonstrated increased ninhydrin-reactive components traveling with R_f's of 0.065 and 0.043; in addition, a

TABLE 3

Demonstration of proteolytic attack upon insulin by purified enzyme. For details of methodology see Materials and Methods.

Conditions of Incubation*	μMoles leucine equivalents Per Tube†
Control (0°)	0.47 ± .02
Experimental (37°)	0.98 ± .02
Experimental + 0.2 mM p-HMB 37°)	0.35 ± .03

* Time of incubation 16 hr.
† Average mean of three determinations ± Standard Error of the Mean.

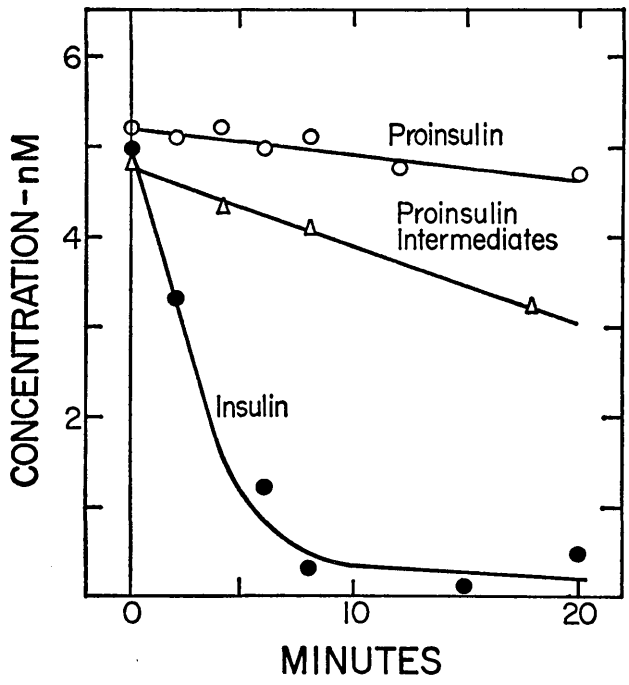

FIG. 2. Rates of enzymatic attack of insulin, proinsulin, and proinsulin intermediates. The reactions containing 3 mg./ml. of bovine serum albumin were run in 0.1 M tris buffer, pH 7.5. Aliquots containing 0.3 ml. were removed from solution at the indicated times and transferred to 1.5 ml. of 6 per cent trichloroacetic acid. After centrifugation at 10,000 x g for 10 min., the supernatant was poured off and the tube drained on filter paper. The precipitate was dissolved in 3.0 ml. of 6 per cent bovine serum albumin containing 0.1 M tris buffer, pH 7.5. A standard quantity of I-131 insulin was added to the solution and the mixture treated with guinea pig anti-insulin serum. Chromatography on filter paper by the method of Yalow and Berson[26] distinguished the undegraded hormone. A standard curve for each substance was run at the same time.

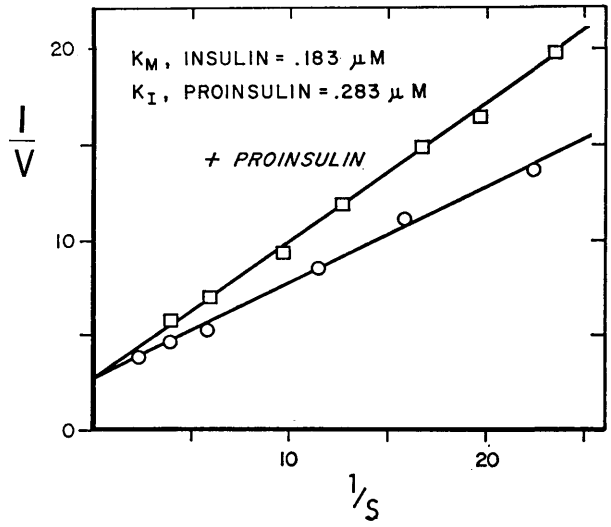

FIG. 3. Competitive inhibition of enzymatic activity by proinsulin. Incubations were carried out with the indicated total concentrations of I-131 insulin and insulin in the presence and absence of 10^{-6} M bovine proinsulin. Velocity is expressed as nanomoles of insulin degraded per ml. per min. Substrate concentration is expressed as nanomoles of insulin per ml. The assay of activity was that described in Materials and Methods.

new component having an R_f of 0.60 was present in the noninhibited mixture. Insulin in this system had an R_f of 0.35.

In figure 2 the results of an experiment are presented comparing the rate of insulin breakdown with that of proinsulin and proinsulin intermediates as determined by immunoassay. The lines drawn for proinsulin and proinsulin intermediates are fitted by the method of least squares, the slopes of which indicate that these two derivatives are degraded at 3 per cent and 10 per cent of the initial rate with insulin, respectively.

In figure 3 is presented a kinetic analysis of the degradation of insulin and its inhibition by proinsulin. As can be seen, proinsulin is a competitive inhibitor of insulin breakdown. The K_m for insulin degradation, as indicated, was 0.18 μM, and the K_I for proinsulin was 0.28 μM. Though the data are not presented, no difference was found between the rate of attack of radioiodinated insulin and noniodinated insulin; this was determined in experiments where the total insulin concentration was kept constant, but the ratio of the two was varied. This result is in accord with the previously reported biological data upon I-131 insulin.[6]

The kinetics of degradation of several derivatives smaller than insulin were examined and compared with the Michaelis constants obtained with the intact insulin molecule. The results obtained with d-GP insulin are pictured in figure 4, and the summary of the Michaelis constants for this and the two other derivatives studied are presented in table 4. The K_m's for d-AA insulin and d-GP insulin were approximately five times the K_m for insulin, whereas the K_m for d-OP insulin was essentially the same as insulin itself.

DISCUSSION

The data in table 3 clearly indicate that the partially purified enzyme preparation of rat muscle contains an enzyme that hydrolytically cleaves insulin. This enzyme has a remarkable degree of specificity, attacking the closely related protein, bovine proinsulin, and bovine proinsulin intermediates at only one-thirty-third and one-tenth the rate of insulin, respectively. However, proinsulin serves as a competitive inhibitor of insulin degradation with a binding (inhibition) constant of the

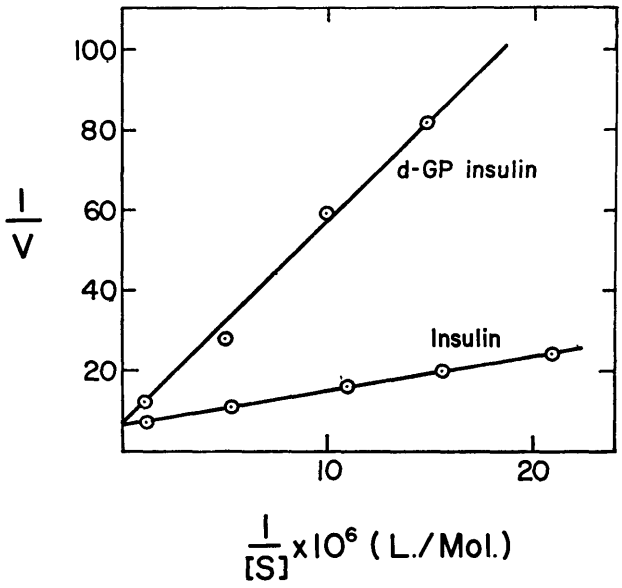

FIG. 4. Kinetic study of the enzymatic attack of d-GP insulin and insulin. Incubations were carried out utilizing the indicated concentrations of I-125 insulin and insulin as well as I-125 d-GP insulin and d-GP insulin. Velocity is expressed as nanomoles degraded per ml. per min. Substrate concentration is expressed as nanomoles per ml. The assay of activity is that described in Materials and Methods.

same order of magnitude as the K_m for insulin. The latter finding suggests a structure for proinsulin similar to that of insulin. This result is in accord with both biological[15,16] and physical data.[17] The fact that proinsulin is not appreciably degraded by the enzyme suggests a multiplicity of recognition sites upon the enzyme for the substrate; that part of the insulin molecule attacked by the enzyme must be sufficiently distorted by residues 31-60 in proinsulin to prevent appreciable proteolysis.

It has been observed that trypsin rapidly attacks certain basic residues in both beef and porcine proinsulins converting them to dealanated insulins.[18,19] The remaining basic arginyl residue at B22 in insulin is much less susceptible to attack.[20] The behavior of the present enzymes is approximately opposite to that of trypsin attacking insulin at thirty-three times the rate with proinsulin. The name, "insulin-specific protease," therefore, seems appropriate for the enzyme.

The studies herein reported on the rates of attack of insulin derivatives with different amino and carboxyl terminal residues removed seem to correlate with the studies of Adams et al.[21] on the three-dimensional structure of crystalline insulin. The latter results have indicated that the amino terminal glycine of the A chain contributes to insulin's tertiary structure through hydrogen bonding to the A 14 tyrosine residue. Its removal would result in partial tertiary structure disruption, and the increase in K_m noted with d-GP insulin seems in accord with this conclusion. Residues B21-30 in the insulin crystal were found largely extended and loosely packed around the A chain.[21] Removal of residues B23-30 in d-OP insulin might not, therefore, effect a large change in the conformation of the rest of the molecule, and the fact that the K_m determined was the same as that for insulin seems in agreement with this conclusion. Recently reported data[22] suggest a change in the conformation of insulin occurs, when the A 21 asparagine residue is removed, as measured by biological activity. The increase in K_m noted with d-AA insulin seems in agreement with this result. It is very probable that the slow enzymatic attack of proinsulin is not such as to convert it to insulin. Such a conversion would permit rapid attack by the enzyme, and, as the data of figure 2 indicate, this does not occur.

TABLE 4

Michaelis-Menten constants for insulin and insulin derivatives

Substrate	K_m
1. Insulin	1.8×10^{-7} M
2. d-AA insulin	8.3×10^{-7} M
3. d-GP insulin	9.4×10^{-7} M
4. d-OP insulin	1.7×10^{-7} M

The enzyme is inhibited by the sulfhydryl reagents, NEM and p-HMB. Sulfhydryl-dependent dipeptidases have been described in the pituitary[23,24] as well as in rat liver.[25] These enzymes are thought to be lysosomal in origin, however, with very broad specificity. Lysosomes have been excluded from the soluble preparations used by differential centrifugation of the homogenate prepared in slightly hypertonic sucrose. As a newly purified and characterized soluble intracellular muscle enzyme, this protease seems unique in that its specificity is for a specific protein structure and seems to attack multiple peptide bonds within that structure.

Use of the enzyme for specifically removing insulin from human plasma permitting the independent measurement of proinsulin has been described.[28]

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