Biochimica et Biophysica Acta, 377 (1975) 421—430

BBA 67422

PROTEOLYTIC DEGRADATION OF INSULIN AND GLUCAGON*

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(Received July 30th, 1974)

Summary

The degradation of insulin and glucagon by a highly purified enzyme isolated from rat skeletal muscle was investigated.

A sensitive assay for proteolytic degradation of insulin and glucagon using fluorescamine to detect an increase in primary amine groups was established.

As measured by an increase in fluorescamine reactive materials, insulin was rapidly degraded by this highly purified enzyme without requiring initial disulfide cleavage. Associated with the increase in fluorescamine reactive materials was a decrease in immunoassayable insulin.

Glucagon was also proteolytically degraded by this enzyme but a number of other peptides and proteins including proinsulin, and A and B chains of insulin were not degraded.

Thus, we have demonstrated that insulin (and glucagon) can be proteolytically degraded by an enzyme isolated from an insulin sensitive tissue, skeletal muscle. Proteolytic degradation by this enzyme requires the intact insulin molecule rather than separate A and B chains.

Introduction

The mechanism of the degradation of insulin has long been of great interest. Mirsky [2] first showed that insulin could be degraded in a number of tissues in the body by a relatively specific process which resulted in proteolytic breakdown of the molecule. Many other workers have also shown that insulin is proteolytically degraded by tissue homogenates or partially purified systems [3,4]. Most of these studies, however, have not excluded the possibility of

* Presented in part at the 34th Annual Meeting of the American Diabetes Association in Atlanta, Georgia, June 16, 1974 [1].

initial cleavage of the disulfide groups by glutathione insulin transhydrogenase followed by proteolytic degradation of the separate chains [5].

Proteolytic degradation of insulin as measured by the production of ninhydrin-positive materials by a partially purified enzyme preparation which had no apparent glutathione insulin transhydrogenase activity has been reported [6]. The relatively low sensitivity of this assay, however, required prolonged incubation (18—24 h) before significant proteolysis could be demonstrated. Similarly prolonged incubation was necessary with a highly purified enzyme preparation [7]. In both of these cases, therefore, it was possible that slow breakdown into A and B chains occurred prior to the proteolysis.

With the introduction of fluorescamine, a compound which made possible the development of a highly sensitive assay for primary amine groups [8], and the use of a highly purified enzyme preparation [7], we have now been able to demonstrate a rapid and direct proteolytic degradation of insulin. The same enzyme preparation also degrades glucagon [9] but does not degrade several other peptides, including A and B chains of insulin.

Materials and Methods

Materials. Male Holtzman rats (200—300 g) were used for all enzyme preparations. Porcine insulin, proinsulin, and glucagon were gifts from Dr R. Chance, Eli Lilly Co., Indianapolis, Ind. Insulin-S-sulfo-A-chain, insulin-S-sulfo-B-chain, and ammonium sulfate (ultrapure grade) were purchased from Schwarz/Mann, Orangeburg, N.Y. Porcine adrenocorticotropic hormone (100 I.U./mg) was purchased from Sigma Chemical Co., St. Louis, M. Human growth hormone was obtained from the National Institutes of Health, Bethesda, Md. Gastrin 2-17 (hexadecapeptide amide hexa-ammonium salt) was purchased from Imperial Chemical Industries, Ltd, Macclesfield, Cheshire, England. Fluorescamine (Fluram^{TM}) was obtained from Roche Diagnostics, Nutley, N.J. Highly specific pancreatic glucagon antiserum (30K) was purchased from The University of Texas, Southwestern Medical School, Dallas, Texas.

Crystalline bovine albumin was obtained from Miles Laboratories, Inc., Elkhart, Ind. All other chemicals were of reagent grade and were purchased from commercial sources. Insulin-agarose was synthesized by reacting CNBr-activated agarose with insulin in 0.2 M sodium citrate/0.7 M NaCl, pH 5.0 [10].

Enzyme preparation and purification. 6—8 male rats were killed by decapitation. The hind leg muscles were removed, cleaned of loose connective tissue, and homogenized in 0.35 M sucrose, 5 ml/g of tissue, in a Waring blendor for 90 s. The homogenate was centrifuged for 15 min at 13 000 × *g*. The supernatant fraction was then centrifuged at 100 000 × *g* for 30 min. The 100 000 × *g* supernatant solution was fractionated with ammonium sulfate: 0.21 g of solid ammonium sulfate was added per ml of supernatant, maintaining pH 6.2 by the addition of 0.1 M NH_4OH or 0.1 M HCl as required. The suspension was centrifuged at 17 000 × *g* for 20 min. The precipitate was discarded, and an additional 0.21 g solid ammonium sulfate was added per ml of supernatant solution. The protein suspension was centrifuged as above. The precipitate was dissolved in 20 mM sodium acetate, pH 6.2, one-fifteenth the

original volume. The solution was immediately dialyzed against 10 volumes of the same buffer for 4 h with six changes. All procedures were carried out at 4°C.

The enzyme was purified further by gel filtration chromatography on a Sephadex G-200 column equilibrated with 0.1 M NaCl in 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2. Fractions comprising the peak enzyme activity were pooled, and the protein solution was dialyzed against 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2, until the dialyzate was free of Cl^- (tested with $AgNO_3$ solution). The dialyzed protein solution (designated G-200 preparation) was stored frozen with no loss of enzyme activity.

The G-200 preparation was chromatographed on a column of QAE-Sephadex equilibrated with 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2. After application of the sample the column was washed first with the same buffer and then with 0.1 M NaCl in 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2. The enzyme was eluted with 0.2 M NaCl in 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2. Fractions containing the peak activity were pooled, and the solution was dialyzed against 20 mM sodium acetate/0.1 mM dithiothreitol, pH 6.2, until the dialyzate was free of Cl^-. This preparation (designated QAE preparation) could then be stored frozen for at least 3 days with no loss of activity.

The final purification steps, all of which were performed on the same day, included dialysis against 20 mM sodium acetate, pH 6.2, to remove the dithiothreitol. The dialyzed protein was chromatographed on an insulin-agarose column equilibrated with the same buffer. The column was washed with the acetate buffer, and the enzyme was eluted with 0.2 M NaCl in 20 mM sodium acetate, pH 6.2. Fractions containing the peak activity were pooled and were dialyzed against 0.05 M potassium phosphate buffer, pH 7.5. Finally, the dialyzed enzyme solution was passed over a column of cellulose powder. Protein concentration of the purified enzyme solution was about 24 μg/ml. The purified enzyme was then used immediately for the studies described below.

Assay of enzyme activity with fluorescamine. The volume of standard or sample, containing 0.1—50 nmol of material, was 0.2 ml. To each tube 2.0 ml of 0.1 M potassium phosphate buffer, pH 6.8 was added. Then 1.0 ml of a freshly prepared solution of fluorescamine in acetone (0.1 mg/ml) was added with immediate mixing. The fluorescence was measured within 10 min on an Aminco-Bowman Spectrophotofluorimeter, excitation 390 nm, emission 475 nm.

Assay of enzyme activity with radioactive substrates. Enzymatic activity was assayed utilizing the solubilization of degradation products of [^{125}I]-insulin to 5% trichloroacetic acid as described previously [7]. Briefly, the assay system consisted of insulin in the indicated concentration with 0.5% bovine serum albumin in 0.1 M Tris · HCl, pH 7.4, in a total volume of 1 ml. After incubation at 37°C, 1 ml of 10% trichloroacetic acid was added and the mixture centrifuged. The supernatant and precipitate were counted in a Packard auto gamma spectrometer. The percent degraded was determined by the increase in trichloroacetic acid-soluble products over the control tubes incubated without enzyme.

Radioimmunoassay of insulin and glucagon. Radioimmunoassay of sam-

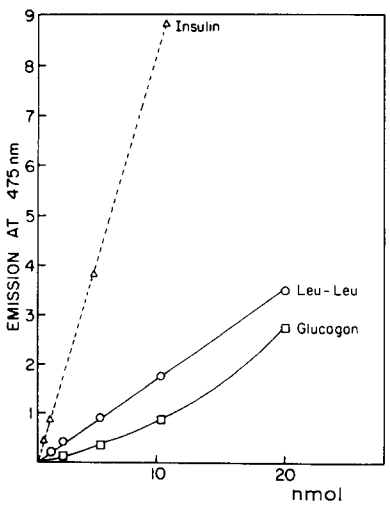
ples for insulin was done by the method of Morgan and Lazarow [11] as described previously [12].

Glucagon radioimmunoassays were performed using the highly specific 30K antiserum of Unger [13].

Results

Standard curve. The fluorescence produced by varying amounts of insulin, glucagon, and a dipeptide, leucylleucine is shown in Fig. 1. The response with insulin and leucylleucine in amounts ranging from 1 to 20 nmol of material is linear. The glucagon response is not linear but has a distinct, reproducible curve. Fig. 2 shows the fluorescence produced by varying amounts of leucylleucine in the presence of constant amounts of insulin and glucagon. In the range from 1 to 20 nmol the glucagon or insulin does not appreciably affect the curve. If the basal fluorescence produced by the insulin and glucagon is subtracted the curves are superimposable over this range. Therefore, the increase in fluorescence produced by the breakdown of insulin and glucagon can be expressed in leucylleucine equivalents.

Enzyme purification. A summary of the enzyme purification results is shown in Table I. Each enzyme preparation was assayed as described under Materials and Methods in the absence or presence of glutathione. Under these conditions, glutathione had very little effect on the enzyme specific activity. The final enzyme preparation contained one major band on polyacrylamide gel [7,9]. All enzymatic activity was contained in this major band as demonstrated by slicing and eluting unstained gels. Insulin and glucagon-degrading activity was found only in the section of the gel corresponding to the major



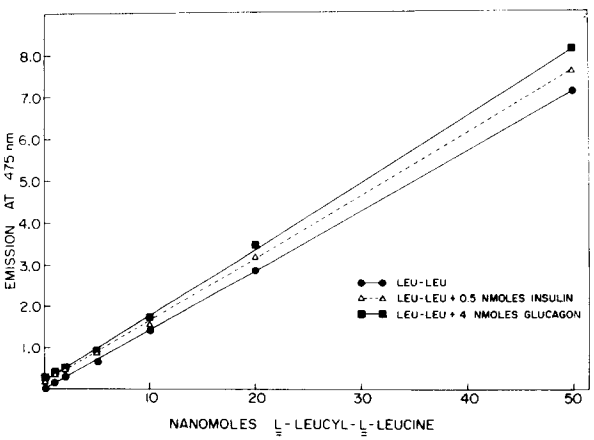


Fig. 1. The fluorescence produced by the reaction of fluorescamine with varying amounts of insulin, glucagon, or leucylleucine. See Materials and Methods for details of reaction procedure. The fluorescence was measured on an Aminco-Bowman spectrophotometer, excitation 390 nm, emission 475 nm.

Fig. 2. The fluorescence produced by varying amounts of leucylleucine in the presence of a constant amount of insulin or glucagon. Assay conditions were as described in Fig. 1.

TABLE I

PURIFICATION OF DEGRADATION ACTIVITY FROM RAT SKELETAL MUSCLE

	Specific activity*		Recovery activity (%)	Recovery protein (%)
	Without glutathione	With glutathione		
Homogenate	1.55	1.51	(100)	(100)
100000 × *g* supernatant	10.9	9.2	90	18
Ammonium sulfate	44.6	48.9	82	2.8
Sephadex G-200	78.5	82.3	39	0.77
QAE-Sephadex	341.2	379	18	0.08
Affinity chromatography	2019	2429	10	0.007

* Specific activity is expressed in fmol insulin degraded per mg protein per min in the absence or presence of 1 mM glutathione. Assay was performed with radioactive substrate as described under Materials and Methods. Protein was determined by the method of Lowry et al. [14].

band, and addition of eluted material from other areas of the gel had no stimulatory nor inhibitory effect on degrading activity.

Proteolytic assay. In order to establish the proper conditions for optimal sensitivity of the assay, purified enzyme was incubated with glucagon and the products reacted with fluorescamine at different pH values. The greatest increase in fluorescence was seen at pH 6.8 (Table II) and, therefore, this was chosen as the standard assay pH.

The incubation of insulin alone, glucagon alone, or purified enzyme alone at 37° C for 60 min results in no change in fluorescamine-reactive material (data not shown).

Increasing amounts of enzyme protein results in a linear increase in glucagon breakdown products (Fig. 3).

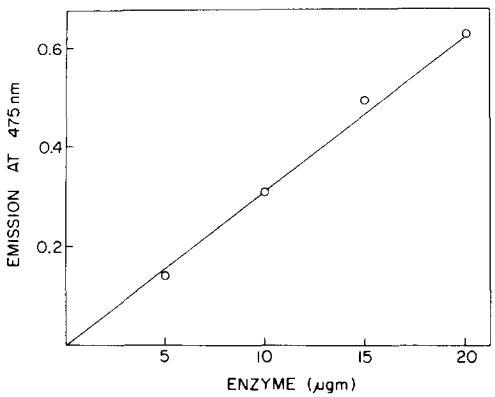
Degradation of glucagon. Incubation of glucagon with purified enzyme results in a decrease in immunoassayable glucagon and an increase in fluorescamine-reactive material (Fig. 4). The amount of starting material was identical

TABLE II

EFFECT OF pH ON THE FLUORESCENCE OF DEGRADATIVE PRODUCTS

Purified enzyme (approx. 50 μg protein) was incubated with $5 \cdot 10^{-5}$ M glucagon in 0.05 M phosphate, pH 7.5, at 37°C in a total volume of 6 ml. At the indicated times, 6 aliquots of 0.2 ml each were removed and added to tubes containing *N*-ethylmaleimide in a final concentration of 0.001 M. Duplicate aliquots from each incubation time were then reacted with fluorescamine at various pH values as described under Materials and Methods except that 0.1 M borate buffer was used for pH 8 and pH 9.

Time (min)	Percent of starting fluorescence		
	pH 6.8	pH 8.0	pH 9.0
0	100	100	100
10	135	110	103
20	171	115	118
40	213	132	123



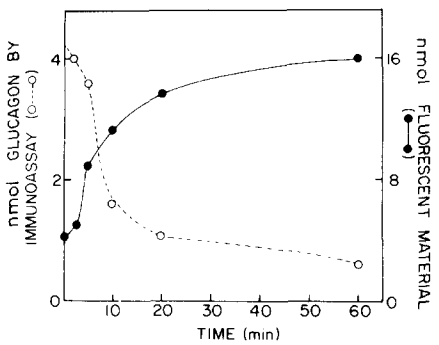


Fig. 3. Effect of increasing amounts of enzyme protein on glucagon degradation. The incubation system consisted of $2 \cdot 10^{-5}$ M glucagon and the indicated amount of enzyme protein in 0.05 M phosphate buffer, pH 7.5, in a total volume of 0.25 ml. This was incubated at 37° C in a shaking metabolic incubator for 5 min and the reaction terminated with *N*-ethylmaleimide in a final concentration of 0.001 M. The fluorescence produced by reacting the samples with fluorescamine (see Materials and Methods) was determined on an Aminco-Bowman spectrophotometer. The results are expressed as increase in fluorescence over that at 0 time.

Fig. 4. The effect of the purified enzyme on glucagon as a function of time. The incubation system consisted of $2 \cdot 10^{-5}$ M glucagon and highly purified enzyme (approx. 100 μg enzyme protein) in 0.05 M potassium phosphate buffer, pH 7.5, in a total volume of 5 ml at 37° C. At the indicate times duplicate 0.2-ml aliquots were removed and transferred to tubes containing *N*-ethylmaleimide in a final concentration of 0.001 M. These were assayed for fluorescamine-reactive materials as described under Materials and Methods. The fluorescence produced is expressed as nmol glucagon equivalent determined from a glucagon standard curve (Fig. 1). At the same indicated times, duplicate 0.1-ml aliquots were removed and added to 0.001 M *N*-ethylmaleimide. These samples were then diluted with 0.2 M glycine buffer, pH 8.8, with 0.25% albumin, and assayed for glucagon immunoreactivity using specific glucagon antiserum (30 K of Unger).

(4 nmol) whether determined by glucagon immunoassay or fluorescent assay using glucagon as a standard.

Fig. 5 shows further studies on the degradation of glucagon. The calculated amount of glucagon added to the incubation mixture was 4.5 nmol. The fluorescence is equivalent to 5 nmol glucagon (from glucagon standard curve) or to 3 nmol leucylleucine equivalent (from leucylleucine standard curve). As early as 2 min an increase in the amount of fluorescamine-reactive material is seen. By 20 min a plateau is reached. When expressed as leucylleucine equivalents, the increase is 15 nmol which represent a 6-fold increase in fluorescence over the basal fluorescence. In numerous experiments where the degradation of glucagon was carried to a plateau, the maximal increase in fluorescence was 6-fold. This would suggest that the degradation of glucagon by this enzyme is relatively limited and that five bonds are cleaved.

Fig. 5 also shows that this enzyme preparation does not produce an increase in fluorescence when incubated with either adrenocorticotropic hormone or bovine serum albumin.

Degradation of insulin and related polypeptides. Fig. 6 shows the proteolytic degradation of insulin. An increase in fluorescamine-reactive materials is seen by 2 min and continues through 60 min of incubation. (In terms of

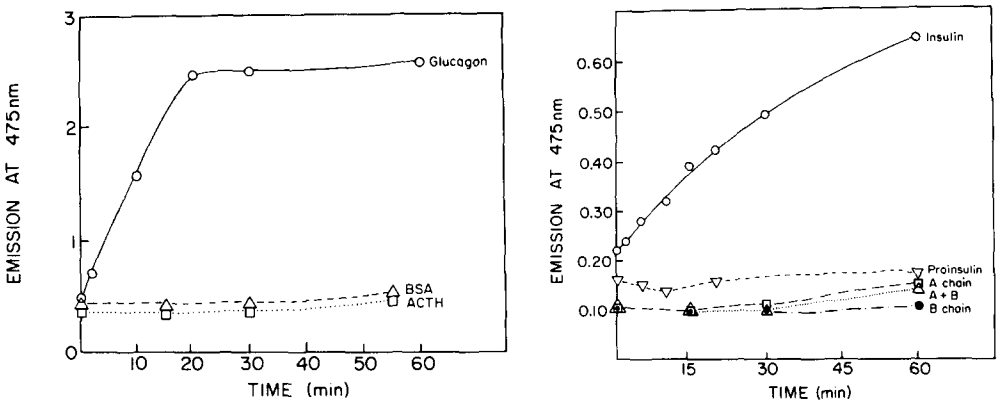


Fig. 5. The effect of the purified enzyme on glucagon, bovine serum albumin and adrenocorticotropic hormone. The incubation mixture contained $3 \cdot 10^{-5}$ M glucagon, $3 \cdot 10^{-5}$ M adrenocorticotropic hormone or $3 \cdot 10^{-6}$ M bovine serum albumin and 50 μg enzyme protein in 0.05 M phosphate buffer, pH 7.5, in a total volume of 2 ml at 37°C. Aliquots were removed at the indicated times, the reaction stopped with *N*-ethylmaleimide, and fluorescence determined as described under Fig. 4.

Fig. 6. The effect of the purified enzyme on insulin and related polypeptides. The method of procedure was as described in Fig. 5. Initial substrate concentrations were $3.3 \cdot 10^{-6}$ M insulin, $6.6 \cdot 10^{-6}$ M A chain, $6.6 \cdot 10^{-6}$ M B chain, $3.3 \cdot 10^{-6}$ M A and B chain each, $3 \cdot 10^{-6}$ M proinsulin.

leucylleucine equivalents this is a net increase in fluorescence equivalent to the production of 3 nmol by 60 min from a starting 0.45 nmol of insulin.)

This figure also shows the failure of the enzyme to degrade either proinsulin or isolated B chain. A chain is degraded very poorly or not at all, since by 60 min there is only a slight increase in fluorescence. A and B chains together are degraded no better than each separately.

Fig. 7 demonstrates that the increase in fluorescamine-reactive material is

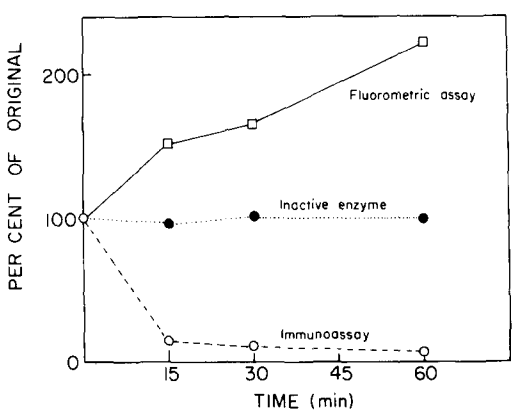


Fig. 7. The effect of the enzyme on insulin as measured by the disappearance of immunoassayable insulin and the production of fluorescamine-reactive material. $1 \cdot 10^{-5}$ M insulin was incubated at 37°C with enzyme (50 μg protein) in 0.05 M phosphate buffer, pH 7.5. Aliquots were removed for fluorescence assay and for immunoassay as described in Fig. 4 except the aliquots for immunoassay were diluted in 0.13 M borate buffer, pH 8.5, with 0.25% bovine serum albumin and assayed for insulin by a double antibody technique. A parallel incubation was performed and assayed as described above except 0.001 M *N*-ethylmaleimide was added at 0 time. No change in either immunoassayable insulin or fluorescamine-reactive material was seen with time (Inactive Enzyme Curve).

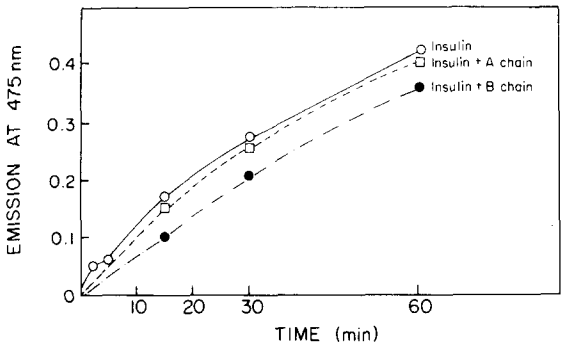


Fig. 8. The effect of insulin A chain and insulin B chain on the proteolytic degradation of insulin. $3.3 \cdot 10^{-6}$ M insulin was incubated as described in Fig. 6 alone or with $3.3 \cdot 10^{-6}$ M A chain of B chain. Results are expressed as increase in fluorescence over fluorescence at 0 time.

associated with a decrease in immunoreactive insulin. This figure also shows that incubation of inactive enzyme with insulin produces no change in either fluorescamine-reactive material or immunoreactive insulin.

Fig. 8 shows the effects of incubating insulin alone and insulin with A and B chains. B chain inhibits the degradation of insulin slightly as measured by the proteolytic method as it does when measured by the trichloroacetic acid method.

Degradation of other peptides. Several other peptides were incubated with the purified enzyme as described under Fig. 5. These peptides included gastrin, growth hormone, α_1-chain of collagen, and a CNBr peptide (α_1-CB2) derived from the α_1-chain of collagen (mol. wt 3000). No increase in fluorescamine-reactive material was seen after 60 min of incubation.

Assay for glutathione insulin transhydrogenase. Using the method of Chandler and Varandani [15] for assay of glutathione insulin transhydrogenase in which insulin 50 μg/ml ("high" insulin) is incubated with and without glutathione (1 μmol/ml), no insulin-degrading activity was detected in the QAE preparation or in the final affinity chromatography-purified enzyme. When the same preparations were assayed for insulin degradation using insulin concentration at $1 \cdot 10^{-10}$ M ("low" insulin), the preparation produced an increase in

TABLE III

EFFECT OF GLUTATHIONE ON INSULIN DEGRADATION

Insulin was incubated with enzyme in the absence and presence of 1 μmol glutathione and the percent degraded was determined as described under Materials and Methods.

Incubation mixture	QAE preparation	Insulin agarose preparation
Low insulin	0.72*	2.95
+ glutathione	0.80	3.55
High insulin	< 0.01	< 0.01
+ glutathione	< 0.01	< 0.01

* Results expressed as percent insulin degraded per min per mg protein during 10 min incubation. Protein determined by fluorescamine reaction [8].

trichloroacetic acid-soluble products as described previously. The addition of glutathione (1 μmol/ml) to the low insulin concentration assay produced a slight (15%) increase in the enzyme activity (Table III). This is in keeping with the previously described sulfhydryl dependency of the enzyme [7].

Discussion

The degradation of insulin by tissue homogenates has been studied extensively for many years. Mirsky [2] systematically conducted a large number of studies and concluded that insulin could be degraded by many tissues with a high degree of specificity. He further concluded that this degradation could occur without reductive cleavage of the disulfide bonds. Discovery and characterization of an enzyme, glutathione insulin transhydrogenase, which could degrade insulin by a non-proteolytic mechanism, i.e. reductive cleavage of the disulfide bonds [16,17], led many workers to conclude that separation into A and B chains was a necessary first step in the degradation of insulin [18]. The difficulty in isolating and characterizing a specific proteinase supported their belief.

Initial work on the enzyme studied here indicated that insulin could be proteolytically degraded by a specific enzyme, which was named insulin specific protease, without requiring disulfide cleavage [6,19]. This confirmed the work of Mirsky and was also in agreement with other investigators [20] who demonstrated two types of insulin degradation in liver, one requiring glutathione and one not. The demonstration of proteolysis by this partially purified insulin specific protease, however, required prolonged incubation (18—24 h) in order to show an increase in ninhydrin-positive materials [6,19]. Because of this prolonged incubation period, it was suggested that the insulin degrading activity and the proteolytic activity were produced by two different enzyme species with cleavage into A and B chains being required for proteolysis [21, 22]. Insulin degradation was routinely measured in these studies by the production of trichloroacetic acid-soluble materials which could not distinguish between proteolysis and disulfide cleavage.

In order to help circumvent these objections, a highly purified enzyme was prepared by affinity chromatography on insulin sepharose utilizing the known specificity of the enzyme for its substrate [7]. Even with the highly purified enzyme, prolonged incubation was required to produce an increase in ninhydrin-positive materials both because of the low sensitivity of the assay and the low velocity of the reaction. Again, slow reductive cleavage followed by proteolysis could not completely be excluded.

With the introduction of a more sensitive method for measuring amino acids and peptides using the reaction between fluorescamine and primary amines [8], it has now been possible to demonstrate the rapid and direct proteolysis of insulin by a purified enzyme preparation. That initial disulfide cleavage is not necessary has been shown by (1) the rapid increase in primary amine groups, (2) the absence of glutathione insulin transhydrogenase activity in preparation, and (3) the failure of the enzyme to degrade separate A and B chains of insulin. Thus, it is apparent that insulin can be degraded by an enzyme which is clearly different from glutathione insulin transhydrogenase.

This work further establishes the usefulness of the fluorescamine reaction as an assay of proteolytic activity. The production of fluorescamine-reactive materials has been used previously as an assay for cathepsin D [23] and trypsin [24].

The enzyme studied in this paper is not completely specific for insulin. It also degrades glucagon, but fails to degrade a number of other peptides and proteins, including insulin B chain. Thus, a classification of this enzyme cannot be based on activity against this standard substrate. If this enzyme is representative of the entire group of neutral proteinases, then a classification system for these enzymes may of necessity have to be based on their natural substrates. The peptide bonds susceptible to this enzyme are at present unknown although preliminary data on the bonds cleaved in glucagon has been obtained (manuscript in preparation). This work has been handicapped by the instability of the purified enzyme and the low velocity of the reaction. When the information on peptide bond susceptibility is available, it may help explain the mechanism of action and the specificity of this enzyme. Studies of this enzyme may help clarify the nature of the neutral proteinases and their physiological importance.

Acknowledgements

The valuable technical assistance of Mrs Mary A. Bobal is gratefully acknowledged.

This work was supported by Veterans Administration projects 1942—03 and 1942-04, and by a Clinical Investigatorship grant (to W.C.D.) and in part by U.S.P.H.S. Grant AM-15509 from the National Institute of Arthritis, Metabolism and Digestive Diseases; General Clinical Research Center Grant RR 00211, National Institutes of Health, Bethesda, Md.

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