Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 635–639, February 1979
Biochemistry

Initial site of insulin cleavage by insulin protease

(hormone/degradation/proteolysis/dansylation)

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Communicated by Henry Lardy, November 8, 1978

ABSTRACT **Exposure of insulin to insulin protease (insulinase, EC 3.4.22.11), a degradative enzyme with considerable specificity toward insulin, results in alterations in the properties of the insulin molecule. Limited degradation by the enzyme results in a decrease in the ability of insulin to bind to membrane receptors with less change in the immunoprecipitability or trichloroacetic acid precipitability of the hormone. Limited degradation by insulin protease also alters insulin so that the molecule becomes susceptible to attack by nonspecific endopeptidases which have no effect on unaltered insulin. These data demonstrate the production of an intermediate in the proteolytic degradation of insulin. By labeling with [^{14}C]dansyl chloride, an insulin intermediate with three amino-terminal residues, glycine, phenylalanine, and leucine, was identified. Analysis of this intermediate demonstrated that it was composed of an intact A chain and a B chain cleaved between residues B16 and B17, with the three peptide chains held together by disulfide bonds. Based on these findings, we hypothesize that a stepwise degradation of insulin occurs *in vivo* and that an early step in the process is the cleavage between B16 and B17 that renders the molecule susceptible to further degradation by nonspecific proteases.**

The initial step in the action of insulin is the binding of the hormone to a specific receptor on the cell membrane. These specific binding sites have been identified and characterized extensively (1, 2). Although the insulin receptors do not apparently degrade the hormone (3), one of the characteristic properties of insulin-sensitive tissue is the ability to degrade insulin (4). Several workers have demonstrated interrelationships between binding and degradation (5, 6), and some have suggested a relationship between degradation and insulin action, either initiation (7, 8) or termination (9).

Because of these considerations, an understanding of the mechanism of insulin degradation is important. We now report that the proteolytic degradation of insulin by the relatively specific enzyme insulin protease (insulinase, EC 3.4.22.11) proceeds through a series of intermediates (10, 11) and that the initial cleavage of insulin is between residues 16 and 17 in the B chain, resulting in a molecule consisting of three peptide chains held together by disulfide bonds.

METHODS

Enzyme Preparation. Purified insulin protease was prepared from rat skeletal muscle as described (12). Kidney neutral endopeptidase (13) was prepared from the 100,000 × *g* pellet of rat kidney homogenates (10) after solubilization of the pellet in 0.1 M Tris·HCl/0.2% Triton X-100 at pH 7.5. After centrifugation to remove the insoluble material, the supernatant was dialyzed extensively against 20 mM sodium acetate (pH 6.2). A heavy precipitate was removed by centrifugation and the supernatant was chromatographed on a 2.6 × 51 cm Sephadex G-200 column equilibrated in 20 mM acetate (pH 6.2). This partially purified enzyme degrades small peptides such as glucagon or the separate A and B chains of insulin but has no activity against the intact insulin molecule (11, 13). Each preparation used in the present study was tested for, and found to be free of, any insulin-degrading activity.

Assays for Insulin Degradation. Insulin degradation was assayed by the loss of precipitability in 5% trichloroacetic acid (14), by loss of immunoprecipitability in the presence of excess antibody against insulin (3), and by loss of ability to bind to liver cell membranes (3).

Identification of Products. [^{14}C]Dansyl amino acids were labeled, separated, and identified as described (15). Reduction and carboxymethylation of insulin have been described (16). Experimental procedures are given in detail in the figure legends.

Materials. Porcine insulin and glucagon were gifts of R. Chance of Eli Lilly. ^{125}I-Labeled insulin was purchased from Immunonuclear Corp. (Stillwater, MN) and purified by passage over a Sephadex G-50 column. [^{14}C]Dansyl chloride was purchased from Schwartz/Mann (specific activity 100 Ci/mol).

RESULTS

Trichloroacetic acid solubility was used as a measure of insulin degradation; the susceptibility of ^{125}I-labeled insulin to a nonspecific endopeptidase before and after exposure to the specific enzyme insulin protease is shown in Fig. 1. Unaltered ^{125}I-labeled insulin is not susceptible to the nonspecific endopeptidase. At all times other than 0, the amount of trichloroacetic acid-soluble material is far greater after the combination of insulin protease and nonspecific endopeptidase than after insulin protease alone. This finding suggests that the ^{125}I-labeled insulin is altered by insulin protease in such a way that it is still acid-precipitable but becomes susceptible to nonspecific hydrolases.

In order to examine the effect of insulin protease on ^{125}I-labeled insulin, we studied the elution pattern of ^{125}I-labeled material from a Sephadex G-50 column after exposure to insulin protease for various periods of time (Fig. 2). There is a progressive loss of the insulin peak (61–92 ml, peak 2) with the subsequent formation of a second peak (92–104 ml, peak 3). With time, there is also an increase in the smaller molecular weight components (122–140 ml, peak 4). Little if any change is seen in the material eluting in the void volume (35–45 ml, peak 1).

Table 1 shows the extent of degradation at each of the incubation times shown in Fig. 2. Degradation was measured by four different methods. Degradation as measured by the loss of receptor-binding ability is far greater than that measured by trichloroacetic acid solubility. Loss of immunoprecipitability is intermediate in sensitivity. The extent of degradation as

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Abbreviation: MalNEt, *N*-ethylmaleimide.

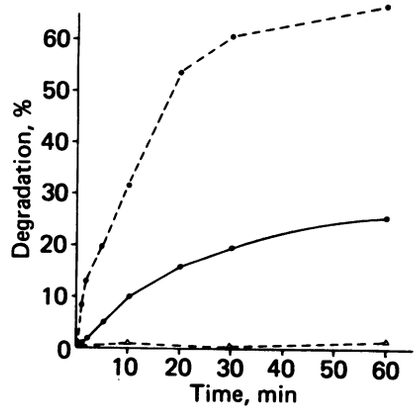

FIG. 1. Degradation as measured by production of trichloroacetic acid-soluble fragments of ^{125}I-labeled insulin after exposure to insulin protease alone (●—●) or insulin protease plus nonspecific endopeptides (● - - - ●). ^{125}I-Labeled insulin (0.1 nM) was incubated with insulin protease in 50 mM Tris·HCl/0.5% bovine serum albumin at pH 7.5 for the indicated times at 37°C. *N*-Ethylmaleimide (MalNEt) (1 mM final concentration) was added to inactivate insulin protease. The nonspecific endopeptidase, which is not affected by MalNEt, was added to one set of tubes (triplicates) and an equal volume of buffer alone was added to another set (triplicates). The tubes were incubated for another 30 min. An equal volume of 10% trichloroacetic acid was then added and the acid solubility was determined. The experiment shown is an example typical of at least five similar experiments. Included in the experiment were appropriate controls to show that MalNEt completely inactivated insulin protease with no effect on the nonspecific endopeptidase, and that the nonspecific enzyme had no effect on insulin alone (△) or on insulin incubated with MalNEt-inactivated insulin protease.

measured by susceptibility to the nonspecific endopeptidase is similar to that measured by the receptor-binding assay.

In an attempt to identify the degradation products of insulin protease susceptible to the nonspecific endopeptidase, the experiment in Table 2 was done. This table shows the change in elution pattern of ^{125}I-labeled insulin degradation products after exposure to both insulin protease and the nonspecific endopeptidase. The insulin protease activity was stopped by addition of MalNEt at the designated time followed by addition of the nonspecific endopeptidase. At 0, 5, and 15 min, aliquots were removed for trichloroacetic acid precipitation, chromatography on Sephadex G-50, and incubation with the nonspecific endopeptidase followed by molecular sieve chromatography. Table 2 shows the net change in each of the peaks after insulin protease alone and insulin protease plus nonspecific endopeptidase. After incubation with insulin protease alone, there is a decrease in peak 2 (insulin) and an increase in peak 3 (smaller molecular weight material) and peak 4 (small product). The combination of insulin protease and nonspecific endopeptidase produces a further decrease in peak 2, suggesting an alteration in the properties of the material in peak 2, since the endopeptidase does not affect intact insulin. There is also a decrease in peak 3, relatively little change in peak 4, and the appearance of a new peak with the elution volume of iodotyrosine (peak 5). The increase in trichloroacetic acid solubility of the ^{125}I-labeled material after 5 min of incubation with insulin protease alone was 0.76%; after incubation with insulin protease for 5 min followed by endopeptidase for 30 min, it was 3.44%. After 15 min with insulin protease, the trichloroacetic acid solubility was 2.22%; after insulin protease followed by endopeptidase, it was 8.68%. The amount of trichloroacetic acid-soluble material agrees reasonably well with the amount of labeled material in peaks 4 and 5, suggesting that these peaks are composed of trichloroacetic acid-soluble material.

The above data suggest that the degradation of insulin by

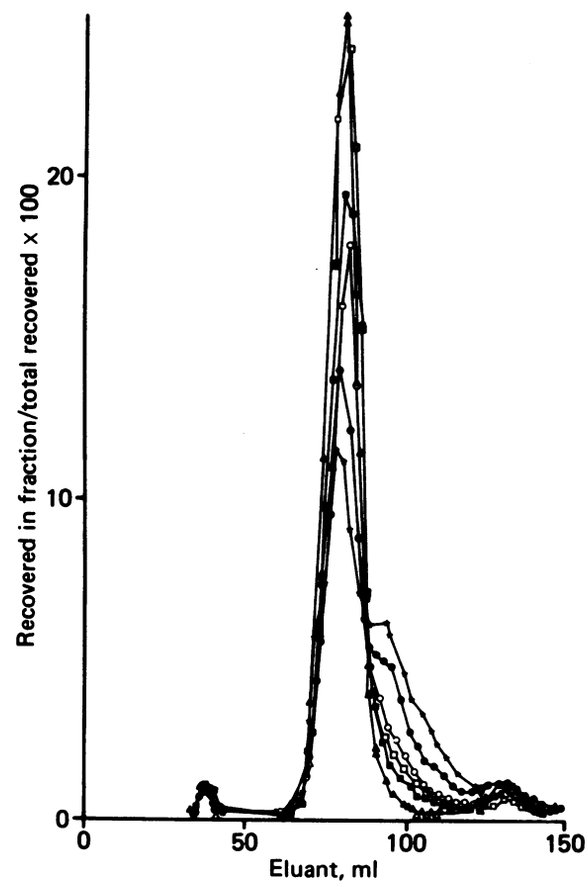

FIG. 2. Chromatography of ^{125}I-labeled insulin after exposure to insulin protease for various times. Aliquots were removed, 10 mM MalNEt was added, and the aliquots were stored in liquid N_2. Each aliquot was assayed for degradation (see Table 1) and chromatographed on a 1.5 × 82 cm Sephadex G-50 column in 1 M acetic acid. All samples were chromatographed on the same column on successive days. The order of chromatography was random (actual order: 5 min, 0 time, 1 min, 60 min, 30 min, 3 min, and 10 min). Peak 1 is defined as 35–45 ml (void volume), peak 2 as 61–92 ml, peak 3 as 92–104 ml, and peak 4 as 122–140 ml. Elution volume for markers was 80 ml for ^{125}I-labeled insulin (6000 M_r), 88 ml for ^{125}I-labeled glucagon (3500 M_r), and 100 ml for ^{125}I-labeled insulin A chain (2500 M_r). ▲, 0 min; △, 1 min; ■, 3 min; □, 5 min; ○, 10 min; ●, 30 min; ★, 60 min.

insulin protease proceeds through one or more intermediates prior to the appearance of small fragments. Furthermore, the initial cleavage may be of primary importance since it may alter the receptor-binding ability of the insulin molecule and increase susceptibility of insulin to nonspecific hydrolases. In order to

Table 1. Degradation of ^{125}I-labeled insulin by insulin protease

Time of incubation, min	Assay			
	Trichloroacetic acid	Immunoprecipitability	Receptor binding	Nonspecific peptidase
0	0*	0*	0*	0*
1	0.5	0	1.5	2.0
3	0.9	1.6	2.9	5.2
5	1.6	3.5	2.4	8.1
10	2.1	6.7	11.2	11.9
30	6.1	19.5	27.6	26.6
60	9.6	27.1	42.1	38.9

* The change in each of these reflects the percent degradation of aliquots from the experiment in Fig. 2.

Table 2. Elution pattern of ^{125}I-labeled insulin on Sephadex G-50 after exposure to insulin protease or insulin protease plus a nonspecific endopeptidase*

Incubation conditions	Peak 1 (45–55 ml)†	Peak 2 (61–92 ml)†	Peak 3 (92–104 ml)†	Peak 4 (122–138 ml)†	Peak 5 (140–155 ml)†
Insulin protease (5 min)	0.49	97.15	1.8	0.85	0
Insulin protease (5 min) plus endopeptidase (30 min)	0.27	94	0.2	2.32	2.62
Insulin protease (15 min)	0	95.5	2.84	2.6	0
Insulin protease (15 min) plus endopeptidase (30 min)	0	87.54	1.1	2.02	5.84

* Values are percent of labeled material recovered in each peak as compared with untreated ^{125}I-labeled insulin.
† Elution volume corresponds to Fig. 2.

identify this intermediate, which is immunoprecipitable and trichloroacetic acid precipitable, and to identify the initial cleavage site, we incubated insulin with insulin protease and then chromatographed the product on Sephadex G-50. The insulin peak (corresponding to 61–92 ml, Fig. 2) was reacted with [^{14}C]dansyl chloride and the amino-terminal residues were determined. In addition to the glycine and phenylalanine of insulin, a new leucine was identified (Fig. 3). Insulin incubated with inactivated insulin protease and treated as described showed only glycine and phenylalanine (data not shown).

Because the insulin molecule contains six leucines (two in the A chain and four in the B chain), this experiment did not identify the exact cleavage site. In order to identify the specific initial cleavage, we did the following experiment. Unlabeled insulin, with a trace amount of ^{125}I-labeled insulin, was incubated with insulin protease, chromatographed, and dansylated as described above. The excess [^{14}C]dansyl chloride was removed by chromatography and the labeled material was reduced and carboxymethylated (16). After chromatography on a Sephadex G-50 column (1.5 × 84 cm), equilibrated in 1 M acetic acid, four peaks of ^{14}C-labeled material were identified. An aliquot from each peak was hydrolyzed and the amino-terminal residues were identified as described above. The first peak was incompletely resolved and contained a mixture of ^{14}C-labeled amino acids (glycine, phenylalanine, and leucine). Peaks 2, 3, and 4 had only one ^{14}C-labeled residue each, glycine, phenylalanine, and leucine, respectively. Each peak was pooled and then chromatographed on a Sephadex G-25 column in 1 M acetic acid. The peptides obtained were then hydrolyzed, and the amino acid mixtures were reacted with [^{14}C]dansyl chloride and separated by thin-layer chromatography. The amino-acid compositions of the three peptides are shown in Table 3. The relative proportion of amino acids was determined by scraping the thin-layer plates and measuring the ^{14}C radioactivity in each spot. The ratio of counts in each spot relative to the counts in the amino-terminal residue was taken as the residues per peptide. The amino-acid compositions identify these peptides as intact A chain and two peptides derived from B chain cleaved at B16–B17 (Fig. 4).

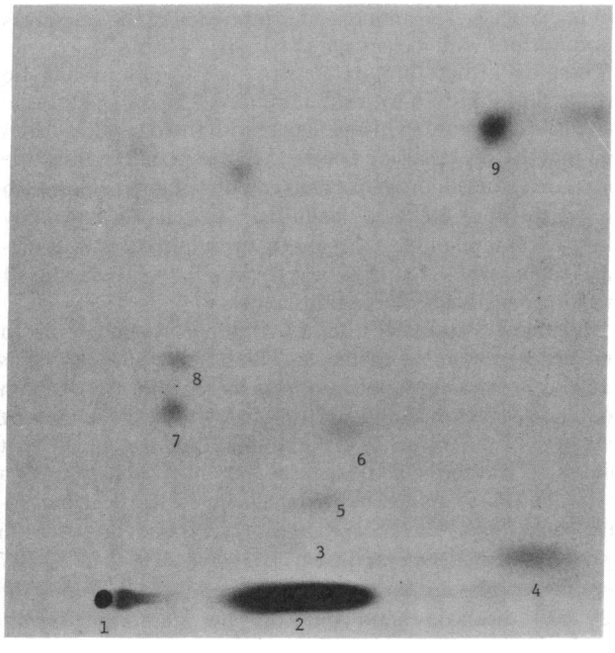

FIG. 3. Autoradiography of [^{14}C]dansylated amino acids. Porcine insulin (34 nM) was incubated with insulin protease in 50 mM potassium phosphate (pH 7.5) for 10 min at 37°C. After addition of 10 mM MalNEt, the mixture was chromatographed on Sephadex G-50; the insulin peak was reacted with [^{14}C]dansyl chloride and hydrolyzed, and the product was chromatographed on polyamide thin-layer plates as described (15). The ^{14}C-labeled amino acids were located by autoradiography and identified by comparison with reference standards chromatographed on the back of the double-coated polyamide plates. (Dns, dansyl.) 1, Starting point; 2, Dns-OH; 3, Dns-*O*-tyrosine; 4, Dns-ε-lysine; 5, Dns-glycine; 6, Dns-Dns-NH_2; 7, Dns-phenylalanine; 8, Dns-leucine; 9, Dns-derivative (unidentified).

DISCUSSION

There are two processes by which insulin can be degraded. The disulfide bonds can be reduced by the enzyme glutathione-insulin transhydrogenase (EC 1.8.4.2), resulting in the production of A and B chains (17). These peptides, which are biologically inactive, are then susceptible to further degradation by nonspecific cellular hydrolases. This process (i.e., initial disulfide reduction and subsequent proteolysis) has been termed the sequential degradation of insulin (18), and at one time was felt to be the only mechanism of insulin degradation.

The other insulin-degrading process is the direct proteolytic degradation of the insulin molecule by the enzyme insulin

Table 3. Amino-acid composition of peptides obtained after degradation of insulin by insulin protease

Amino acid	NH_2-terminal Gly	Amino acid	NH_2-terminal Phe	Amino acid	NH_2-terminal Leu
Gly	1.00 (1)	Phe	1.00 (1)	Leu	1.00 (1)
Ile	1.87 (2)	Val	1.96 (2)	Val	0.94 (1)
Val	0.93 (1)	Asn	0.75 (1)	½ Cys	0.83 (1)
Glu	1.81 (2)	Gln	0.79 (1)	Gly	2.07 (2)
Gln	1.79 (2)	His	1.73 (2)	Glu	0.84 (1)
½ Cys	3.56 (4)	Leu	2.86 (3)	Arg	0.79 (1)
Thr	0.96 (1)	½ Cys	1.08 (1)	Phe	1.88 (2)
Ser	1.83 (2)	Gly	1.02 (1)	Tyr	0.72 (1)
Leu	1.95 (2)	Ser	0.79 (1)	Thr	0.75 (1)
Tyr	1.66 (2)	Glu	0.94 (1)	Pro	0.87 (1)
Asn	1.69 (2)	Ala	0.83 (1)	Ala	0.81 (1)
		Tyr	0.93 (1)	Lys	0.73 (1)

Numbers in parentheses represent integral values.

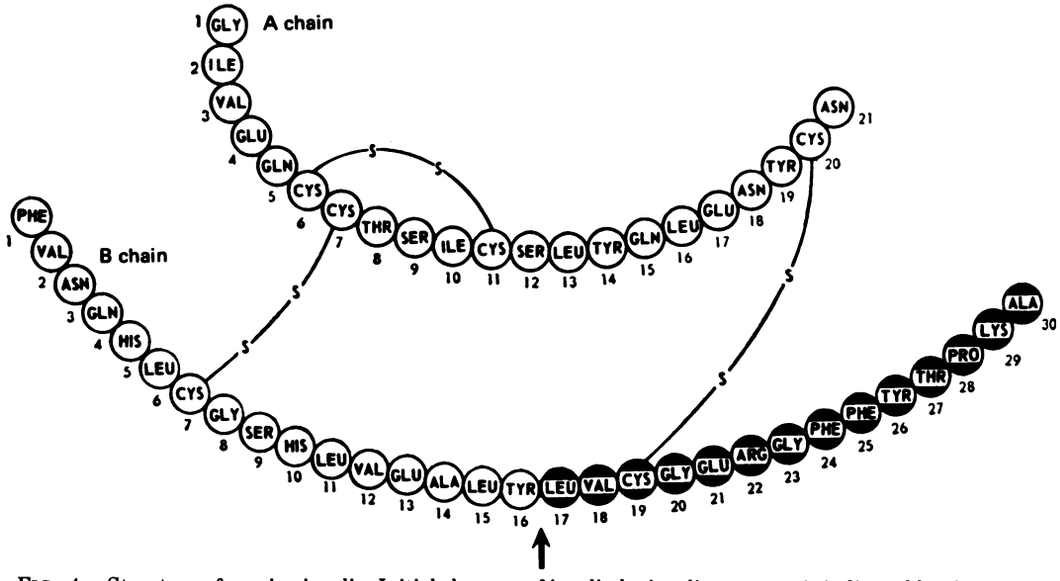

FIG. 4. Structure of porcine insulin. Initial cleavage of insulin by insulin protease is indicated by the arrow.

protease (19, 20). We now show that the degradation of insulin by insulin protease can also occur in a sequential fashion. Brief exposure of insulin to insulin protease (specific) alters the molecule so that it is susceptible to nonspecific endopeptidases which do not attack the intact insulin molecule. Ultimately, the degradation of insulin results in the production of amino acids and small peptides, regardless of the mechanisms involved. Because these small peptides are soluble in 10% trichloroacetic acid, the conversion of labeled insulin to trichloroacetic acid-soluble material has been widely used as a measurement of insulin degradation. This method can also be used to assay purified glutathione-insulin transhydrogenase because most of the radioactive label of iodinated insulin is on the A chain, which is trichloroacetic acid soluble (21).

It has been shown repeatedly, however, that exposure of insulin to tissues, isolated cells, or subcellular fractions alters various properties of the molecule without necessarily producing small fragments (3, 11, 22, 23). Thus, immunoprecipitability of insulin is decreased more than trichloroacetic acid precipitability and the ability of the hormone to bind to receptors is decreased to an even greater degree after exposure to degrading systems. These findings have suggested that relatively subtle changes in the insulin molecule can result in decreased biological activity (measured by receptor binding) without altering other properties of the hormone.

We have now shown that similar results occur when insulin is degraded by purified insulin protease. This suggests the production of an intermediate in the degradation of insulin that is trichloroacetic acid precipitable and partially immunoprecipitable, but that has a decreased ability to bind to receptors. This intermediate is also susceptible to nonspecific hydrolases which do not degrade intact insulin.

Molecular sieve chromatography of labeled insulin exposed to insulin protease for various times shows clearly that lower molecular weight fragments are susceptible to the nonspecific endopeptidase. Chromatography after exposure to the nonspecific enzyme shows a decrease in peak 3 and an increase in smaller molecular weight materials (peak 4).

Careful inspection of the data in Table 2, however, shows that not all of the material that is susceptible to the nonspecific endopeptidase is found in peak 3. A significant amount elutes in the insulin peak (peak 2). From Table 2, approximately 8% of the insulin peak is susceptible to the nonspecific enzyme after exposure to the specific insulin protease for 15 min, demonstrating that an intermediate also elutes in the insulin peak.

Reaction with [^{14}C]dansyl chloride of peak 2 obtained after insulin protease treatment reveals, in addition to the glycine and phenylalanine of insulin, a new amino-terminal leucine. The intermediate containing the amino-terminal leucine could not be separated from insulin by chromatography on either Sephadex G-50 or G-25. Chromatography after reduction of the disulfide bonds resulted in the identification of intact A chain (amino-terminal glycine) and two fragments of B chain, one with amino-terminal phenylalanine and one with amino-terminal leucine. The amino-acid composition of the fragments was consistent with a cleavage at B16–B17 (Table 3).

These data, therefore, demonstrate a stepwise proteolytic degradation of insulin by insulin protease. The initial cleavage is at B16–B17, resulting in a molecule with three peptide chains held together by disulfide bonds. If at this point the disulfide bonds are reduced, intact A chain can be obtained along with the fragments of B chain. The initial cleavage is followed by other, as yet unidentified, breaks that result in other intermediates, such as those found in peak 3, prior to the production of trichloroacetic acid-soluble fragments.

The initial cleavage of insulin by insulin protease occurs in a critical region of the molecule. The B16 tyrosine residue is involved in the binding of insulin to its receptor (24); thus, a cleavage between B16 and B17 could alter greatly the binding properties of the molecule. It is also tempting to speculate that this initial cleavage could be in some way involved in the action of insulin. It has been postulated that some of the effects of insulin could result from generation of active fragments of insulin by cellular degradative processes (7). Hydrolysis of the B16–B17 peptide bond by insulin protease followed by reduction of the A20–B19 disulfide bond (possibly by glutathione-insulin transhydrogenase) would result in a peptide (shaded area, Fig. 4) containing the residues B22–B26. These residues have been suggested as the active portion of the insulin molecule since synthetic peptides with this sequence have insulin-like action (25, 26). Thus, the combined action of the "degradative" enzymes, insulin protease and glutathione-insulin transhydrogenase, could actually be involved in the production of some of the biological actions of insulin. The recent report that inhibition of glutathione-insulin transhydrogenase (8) prevents at least some of the effects of insulin would be consistent with this hypothesis.

We acknowledge the excellent technical assistance of Ms. M. Goessling and Ms. K. Nash. A portion of this work was done as partial requirement for a doctoral degree in biochemistry (F.B.S.). This work was supported in part by research funds from the Veterans Administration and by a grant from the National Institutes of Health (AM 18022) and General Clinical Research Center Grant (RR00211) to the University of Tennessee. W.C.D. is a recipient of a Research Career Development Award from the National Institutes of Health (5K04-AM 00187).

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