Degradation of Insulin and Insulin-Like Growth Factors by Enzyme Purified From Human Erythrocytes

Comparison of Degradation Products Observed With A14- and B26-[^{125}I]monoiodoinsulin

ROBERT I. MISBIN AND ERNESTO C. ALMIRA

An insulin-degrading enzyme has been purified from human erythrocytes. This enzyme degraded ^{125}I-labeled insulin-like growth factor I (IGF-I) more slowly than ^{125}I–IGF-II and degraded IGF-II more slowly than ^{125}I-insulin. The time course of ^{125}I-insulin degradation suggested the presence of intermediates, each of which was itself shown to be a substrate for the enzyme. One of these intermediates appeared to be made up entirely of B-chain residues and had His^{B10} as its NH_2-terminal. The final major radiolabeled degradation product of A14-[^{125}I]monoiodoinsulin was a peptide with Tyr^{A14} at the A-chain NH_2 terminal. This peptide could be reduced with dithiothreitol, suggesting that it contained amino acid residues from both A- and B-chains. It was partially precipitated by trichloroacetic acid and anti-insulin antibody but bound poorly to IM-9 lymphocytes. The final major degradation product of B26-[^{125}I]monoiodoinsulin was a peptide whose NH_2-terminal was Tyr^{B26} and could not be reduced by dithiothreitol. It was partially precipitated by anti-insulin antibody but was precipitated poorly, if at all, by trichloroacetic acid and bound poorly to IM-9 lymphocytes. The results show that this enzyme degraded insulin by sequential cleavage of peptide bonds on both A- and B-chains. We identified Leu^{A13}-Tyr^{A14}, Ser^{B9}-His^{B10}, and Phe^{B25}-Tyr^{B26} as three of the bonds that are cleaved. *Diabetes* 38:152–58, 1989

Insulin binding to a specific receptor on the cell surface is the step that initiates its effects on metabolic processes (1). After binding, the insulin molecule is taken up by the cell and degraded. The exact site and mechanism of this degradation are not well understood. Morphological studies and the use of degradation inhibitors such as chloroquine have suggested that much of the internalized insulin is degraded in lysosomes or the Golgi apparatus (2–7). Several studies, however, have provided evidence for the existence of two pathways for insulin degradation in cells, one that is inhibited by chloroquine and one that is not (3,8–10). The pathway that is not sensitive to chloroquine is presumed to be due to insulin protease (10), an enzyme that is distributed widely in mammalian tissues and has been studied extensively (11–19).

Studies on the degradation of labeled insulin by whole cells have shown that most of the cell-associated radioactivity is intact insulin (20). Although peptide intermediates of insulin degradation have been identified in cells and their incubation medium (10,21–24), the major degradation products of ^{125}I-labeled insulin in insulin-sensitive cells appear to be iodide and iodotyrosine (8). Peptide intermediates appear to derive from modification of insulin B-chain, the consequence of which is loss of immunoreactivity and even greater loss of receptor-binding activity (8,21). These peptide intermediates apparently are degraded rapidly within cells, and a sufficient amount has not yet been isolated to permit structural analysis. Numerous peptide products have also been observed with insulin protease isolated from skeletal muscle (25,26). Shii et al. (27) have isolated an insulin protease from human erythrocytes and purified it to homogeneity. In this study we use this preparation to compare the degradation of A14- and B26-[^{125}I]monoiodoinsulin (gift of B. Frank, Lilly, Indianapolis, IN) to further characterize the nature of insulin-degradation products.

MATERIALS AND METHODS

Insulin protease was isolated and purified from human erythrocytes by a modification of the methods of Shii et al. (27). First, 1.2 L of heparinized blood was centrifuged for 15 min at 1500 × *g* at 5°C, and the plasma and white blood cells were removed by aspiration. The remaining erythrocytes were resuspended, washed 3 times with saline, and lysed with 2.4 L of water at 4°C. Membranes were removed by

From the Division of Endocrinology and Metabolism, University of Florida College of Medicine, Gainesville, Florida.

Address correspondence and reprint requests to Dr. Robert I. Misbin, Division of Endocrinology and Metabolism, University of Florida College of Medicine, JHMHC, Box J-226, Gainesville, FL 32610.

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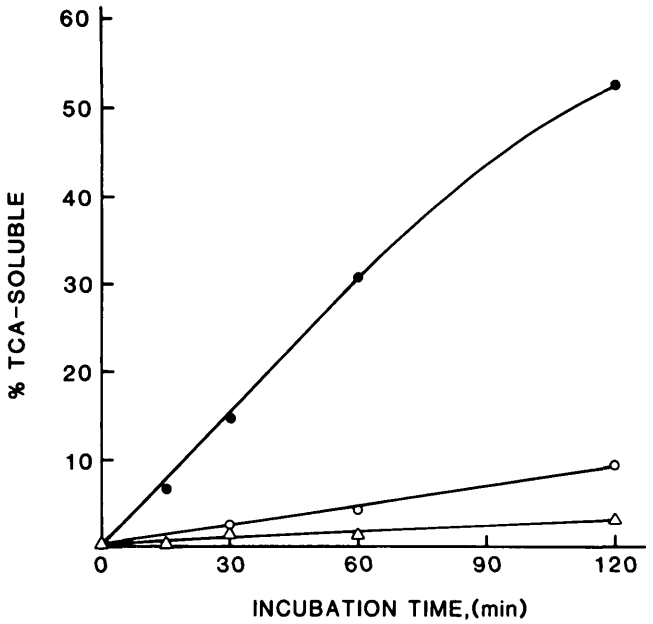

FIG. 1. Degradation of insulin (●), insulin-like growth factor I (IGF-I; ○), and insulin-like growth factor II (IGF-II; △). Enzyme preparation (11 μg/ml) was incubated at 37°C with A14-[^{125}I]monoiodoinsulin, ^{125}I–IGF-I, and ^{125}I–IGF-II at 22 pM. Hormone degradation was measured as appearance of trichloroacetic acid (TCA)–soluble radioactivity.

centrifugation at 12,000 × *g* for 1 h. The supernatant (lysate) was fractionated by ammonium sulfate precipitation. The 30–60% fraction was dissolved in 20 mM Tris-HCl, pH 7.4, and dialyzed against the same buffer plus 1.0 mM dithiothreitol (DTT).

The dialyzed sample was applied to a DEAE-Sephadex A-50 column (3 × 12 cm) previously equilibrated with 20 mM Tris-HCl, ph 7.4, and the column was washed with 1.5 L of the same buffer plus 1.0 mM DTT and 100 mM NaCl. The column was then eluted with a salt gradient (400 ml of 100–400 mM NaCl in the same buffer). The peak of insulin-degrading activity (which eluted at 200–300 mM NaCl) was pooled, brought to 40% ammonium sulfate, and applied to a pentylagarose column (1.5 × 10 cm) equilibrated with 40% ammonium sulfate in 10 mM imidazole-HCl, pH 7.1, plus 1.0 mM DTT. The column was eluted with a decreasing gradient of ammonium sulfate (400 ml of 40 to 0% ammonium sulfate in the imidazole buffer). The peak of insulin-degrading activity (which eluted from 20 to 0% ammonium sulfate) was pooled and concentrated to 10 ml on an Amicon stirred-flow concentrator with a YM-30 membrane. The concentrated sample was desalted on a Sephadex G-25 column (1.5 × 6 cm) equilibrated with 10 mM imidazole-HCl plus 5 mM phosphate buffer, pH 7.1, and 1.0 mM DTT. This material was then applied to a chromatofocusing column (0.9 × 60 cm column of polybuffer exchanger PBE 94) and eluted with 200 ml polybuffer 74, pH 4.5, containing 1.0 mM DTT. After the pH values of the fractions were measured, the fractions were immediately neutralized with 1 M tris(hydroxymethyl) aminomethane. The peak of insulin-degrading activity (which eluted from pH 5.3 to 5.0) was concentrated to 1 ml on an Amicon centricon 30 and applied to an Ultrogel AcA-34 column (1.5 × 90 cm) equilibrated with 20 mM phosphate-buffered saline containing 1.0 mM DTT. The final enzyme preparation had an insulin-degrading activity of 6.3 pmol · $mg^{-1} \cdot h^{-1}$ (measured at 17 pM insulin), which represented a 3200-fold purification over the activity in the erythrocyte lysate. The yield from 0.55 L of blood was 0.2 mg of purified enzyme.

Incubations were performed at 37°C in 20 mM phosphate-buffered saline, pH 7.4, containing 0.1% radioimmunoassay-grade bovine serum albumin. The incubation contained ~6 ng/ml A14- or B26-[^{125}I]monoiodoinsulin, to which unlabeled porcine insulin was added to reach a final concentration of 5 nM. Incubations of ^{125}I-labeled insulin-like growth factor I (IGF-I; Amersham, Arlington Heights, IL) and IGF-II (T. Merimee, University of Florida, Gainesville, FL) were performed at 22 pM. In IGF experiments, the concentration of ^{125}I-insulin was also 22 pM. Approximately 11 μg of purified insulin protease was used per milliliter of incubation. Incubations were terminated at various times by addition of concentrated acetic acid to a final concentration of 3 M. Samples were frozen until further analysis.

^{125}I-insulin degradation was assayed by the methods of precipitation in 5% trichloroacetic acid, immunoprecipitation with anti-insulin antibody, binding to IM-9 lymphocytes, and

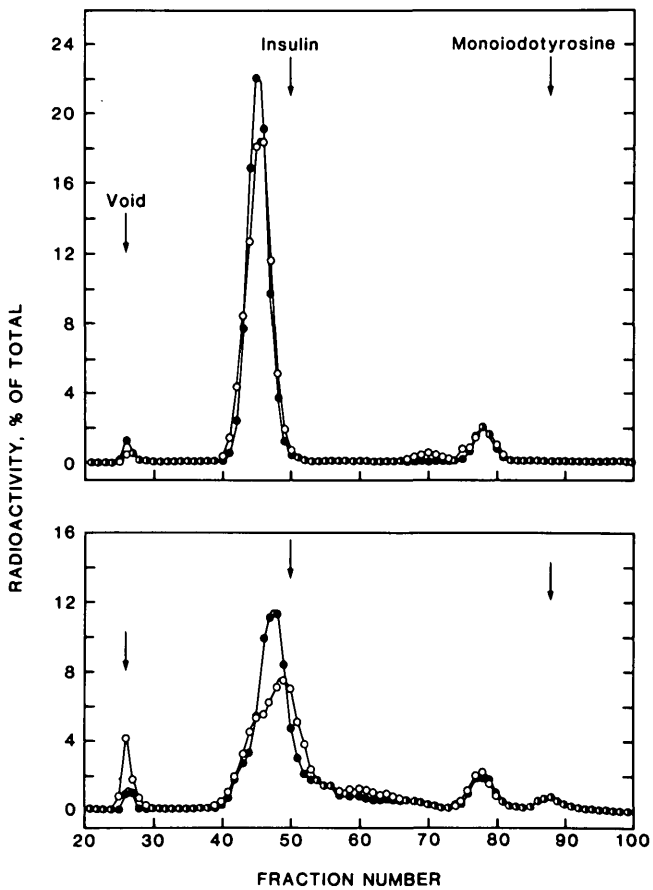

FIG. 2. Gel-filtration chromatography on Sephadex G-50 of insulin-like growth factor I (IGF-I; *top*) and insulin-like growth factor II (IGF-II; *bottom*) incubated for 2 h with enzyme preparation indicated in Fig. 1. Reaction was terminated with addition of 3 M acetic acid at 0 min (●) and 120 min (○). Mixture was then eluted through Sephadex G-50 column (1.5 × 72.5 cm) in 1 M acetic acid; 1.5-ml fractions were collected and counted in γ-counter. Columns were calibrated with blue dextran (void volume), ^{125}I-insulin, and monoiodotyrosine as shown by *arrows*.

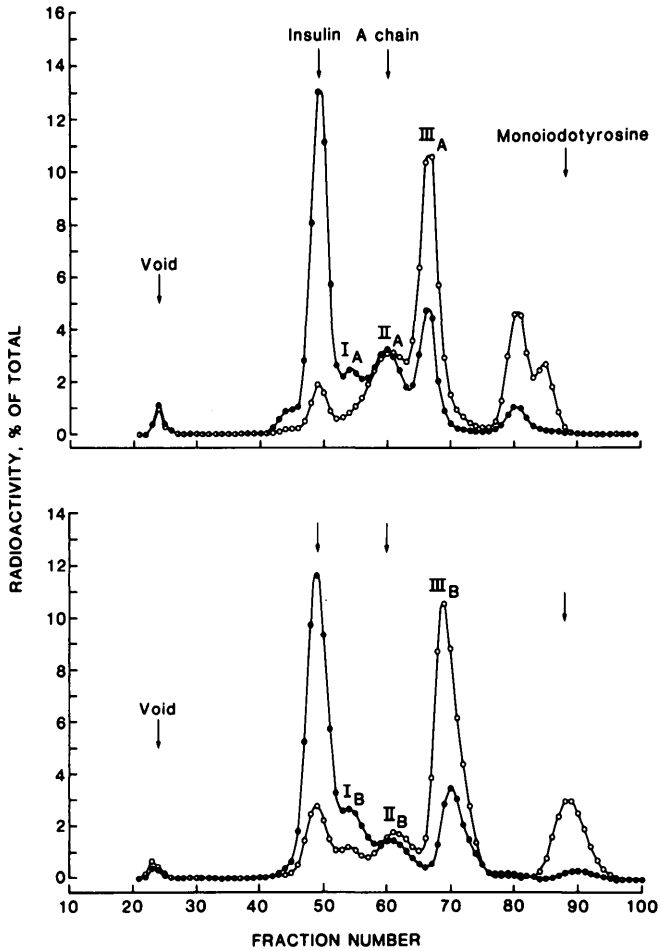

FIG. 3. Generation of radiolabeled fragments of A14- (*top*) and B26-[^{125}I]monoiodoinsulin (*bottom*). ^{125}I-insulin was incubated with enzyme. Reaction was terminated with addition of 3 M acetic acid at 15 min (●) and 60 min (○). Reaction mixture was then eluted through Sephadex G-50 column in 1 M acetic acid. Columns were calibrated with blue dextran (void volume), ^{125}I-insulin, insulin A-chain, Na^{125}I, and monoiodotyrosine as shown by *arrows*.

gel filtration through Sephadex G-50 (8). Insulin fragments were further purified by retention on C-18 reverse-phase columns (Millipore, Milford, MA). The position of the ^{125}I-Tyr relative to the NH_2-terminal of the purified peptides was determined by sequential Edman degradation on an Applied Systems, (ABI, Foster City, CA) 470A protein sequencer. Sample aliquots of 50–200 μl were loaded onto cartridge filters preconditioned with 30 ml ABI Biobrene-plus with three cycles of the manufacturer's program 02RPRE. The number of cycles sequenced varied from 9 to 28. All sequencing was performed with the ABI 02RPTH program. Control experiments were done with reduced A14-[^{125}I]-A-chain, B26-[^{125}I]-B-chain, and trypsinized B26-[^{125}I]monoiodoinsulin, and radiosequence analysis gave peaks of >90% of the total radioactivity in the 14th, 26th, and 4th cycles, respectively, as expected.

RESULTS

A time course of degradation of insulin, IGF-I, and IGF-II by measurement of trichloroacetic acid–soluble radioactivity is shown in Fig. 1. The degradation rate of IGF-II was less than that of insulin, and IGF-I was degraded more slowly. These results are similar to those that reported with insulin protease isolated from rat skeletal muscle (28). Compared with immunoprecipitation, the trichloroacetic acid method underestimated by ~25% the rate of degradation, but the difference was approximately the same among all three tracers. Gel filtration patterns of partially degraded IGF-I and IGF-II are shown in Fig. 2.

A time course was performed comparing degradation of A14- and B26-[^{125}I]monoiodoinsulin. The gel filtration pattern of degraded insulin is shown in Fig. 3. Peaks of radioactivity derived from A14-[^{125}I]monoiodoinsulin are designated A, and peaks derived from B26-[^{125}I]monoiodoinsulin are designated B. With both labels, we observed small peaks of radioactivity immediately after the insulin peak after 15 min of incubation that were decreased (IA and IB) or unchanged (IIA and IIB) after 60 min of incubation. By 60 min of incubation most of the radioactivity appeared in low-molecular-weight peptides. The A14 fragments (IIIA) were partially precipitated by trichloroacetic acid (50% relative to ^{125}I-insulin) and anti-insulin antibody (22% relative to ^{125}I-insulin) but bound poorly to IM-9 lymphocytes (25% specific binding relative to ^{125}I-insulin). The B26 fragment (IIIB) was soluble in 5% trichloroacetic acid. It was partially precipitated by anti-insulin antibody (29% relative to ^{125}I-insulin) but showed no specific binding to IM-9 lymphocytes. As shown in Fig. 4, reduction and carboxymethylation caused a shift to the right in the elution pattern of the IIIA. This result suggests

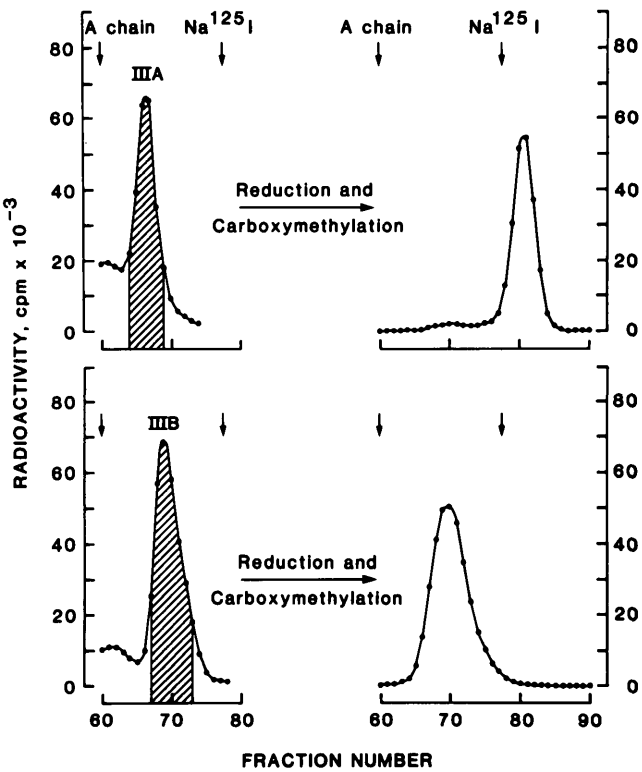

FIG. 4. Reduction of insulin fragments (*top*, A14-[^{125}I]monoiodoinsulin; *bottom*, B26-[^{125}I]monoiodoinsulin). Graphs at *left* show part of elution pattern of degraded ^{125}I-insulin obtained after 60 min of incubation in Fig. 3. *Hatched* fractions were pooled and treated with 10 mM dithiothreitol followed by 30 mM iodoacetamide to reduce and carboxymethylate disulfide bonds (51). Material was then rechromatographed on same column as shown in graphs at *right*.

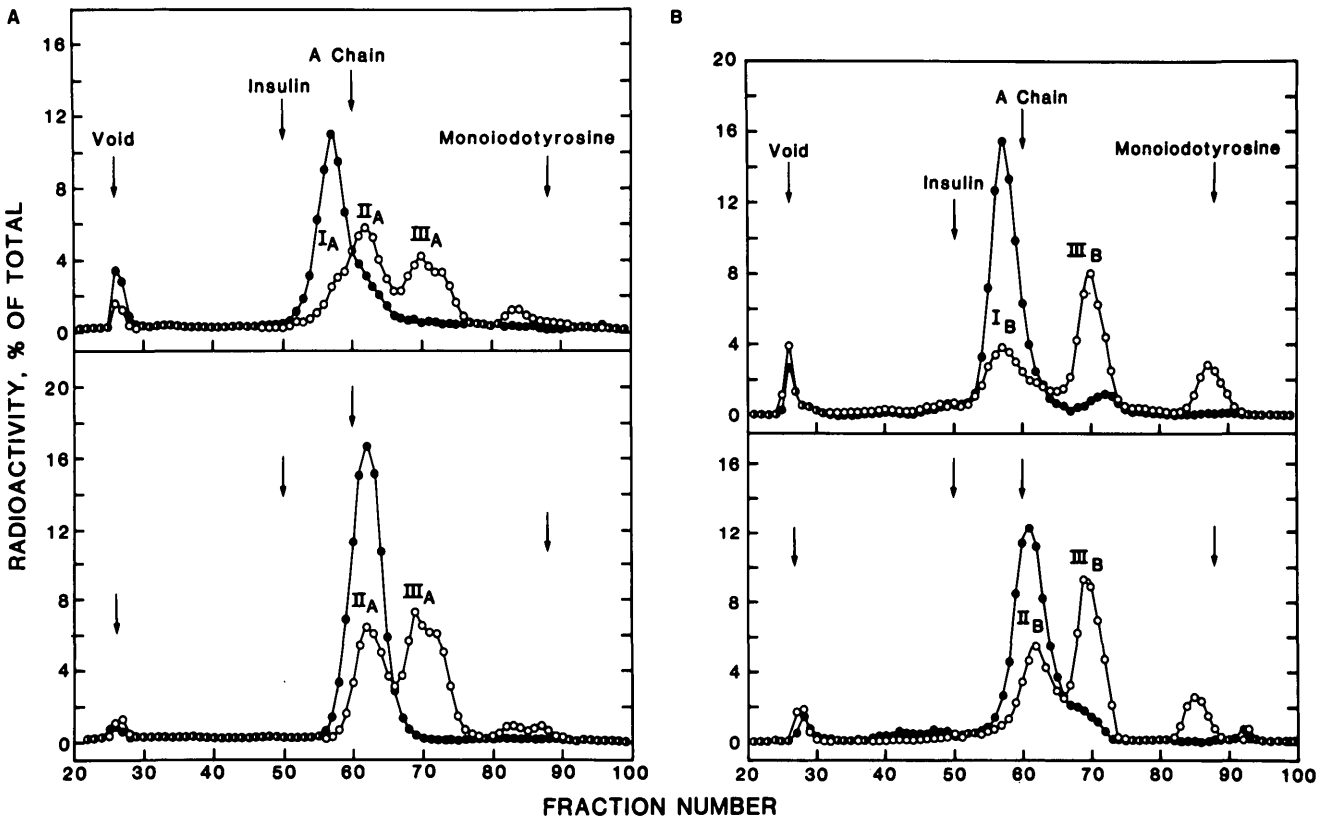

FIG. 5. Incubation of radiolabeled fragments of A14- and B26-[^{125}I]monoiodoinsulin with enzyme preparation. *A*: fragments IA (*top*) and IIA (*bottom*) purified by repeated gel filtration on Sephadex G-50 column were incubated in absence (●) or presence (○) of enzyme preparation and rechromatographed on same column. *B*: fragments IB (*top*) and IIB (*bottom*) were purified, incubated, and rechromatographed as in *A*.

that the fragment is derived from both A- and B-chains linked to each other by a disulfide bond. By contrast, the elution pattern of the IIIB was not affected by reduction; IIIB therefore does not appear to contain A-chain amino acids. Radiosequence analysis (Edman degradation) revealed that both IIIA and IIIB had ^{125}I-Tyr at their NH_2-terminals.

The observation that fragments IA, IB, IIA, and IIB appeared early in the incubation and did not accumulate suggested that they might be intermediate products of insulin degradation. To examine this possibility directly, we determined if these fragments themselves could be substrates for insulin protease (Fig. 5). With A14-[^{125}I]monoiodoinsulin we found that fragment IA was indeed the precursor of fragment IIA and that IIA was the precursor of fragment IIIA (Fig. 5*A*). With B26-[^{125}I]monoiodoinsulin, however, we found that fragments IB and IIB were precursors of fragments IIIB, but that fragment IB was not a precursor of IIB (Fig. 5*B*). Reduction and carboxymethylation of the intermediates showed a decrease in molecular size with fragments IA, IIA, and IIB but not with fragment IB (Fig. 6). Radiosequence analysis showed that the NH_2-terminal of fragment IB was His^{B10}. Retention time of the insulin fragments on a C-18 reverse-phase column (Fig. 7) and susceptibility of the fragments to reduction and further degradation by insulin protease are summarized in Table 1. The results show that fragments IA and IB are different despite their eluting in a similar position from Sephadex G-50.

TABLE 1
Characteristics of insulin-degradation products

Label	Fragment	Susceptibility to reduction	Susceptibility to insulin protease	NH_2-terminal
A14	IA	Yes	Yields IIA and IIIA	
	IIA	Yes	Yields IIIA	
	IIIA	Yes		Tyr^{A14}
B26	IB	No	Yields IIIB	His^{B10}
	IIB	Yes	Yields IIIB	
	IIIB	No		Tyr^{B26}

Determination of NH_2-terminal of each insulin fragment performed by determining number of cycles of sequential Edman degradation performed on peptide sequencer required to liberate the radioactive residue. From number of cycles required for liberation of ^{125}I-Tyr, the NH_2-terminal amino acid residue was inferred from primary structure of porcine insulin.

DISCUSSION

Insulin degradation has been studied extensively with extracts of various tissues from mammalian species. Although different laboratories have referred to these activities as insulin protease or insulin-degrading enzyme, the relationship between these activities has not always been clear. The results of substrate specificity reported here help to resolve this problem. With insulin-degrading enzyme isolated by the method of Shii et al. (27), we found that the degradation of IGF-II was less than that of insulin, while the degradation of IGF-I was barely detectable. These results are similar to those reported for insulin protease isolated from rat skeletal muscle (28) and with an enzyme from IM-9 cultured human

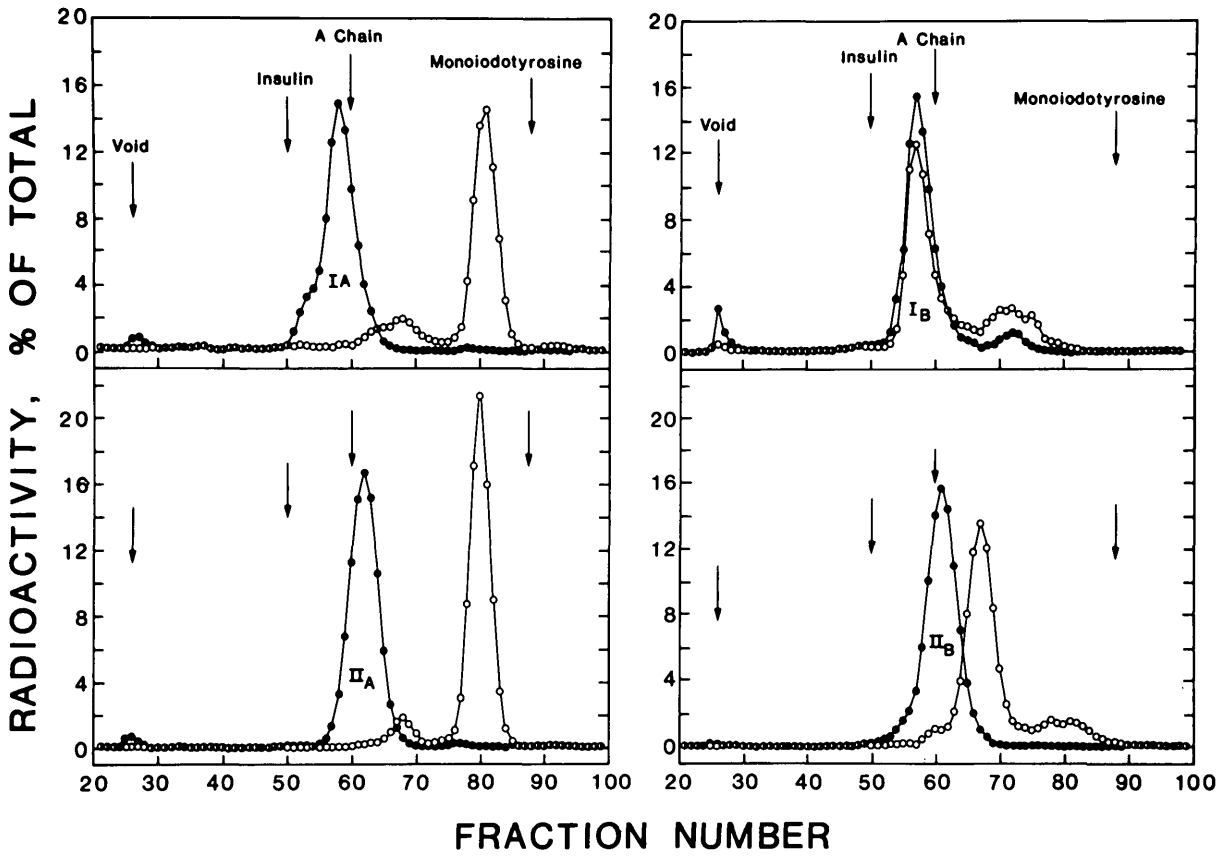

FIG. 6. Reduction of insulin fragments. Gel filtration of A14-[^{125}I]monoiodoinsulin fragments IA and IIA (*left*) and B26-[^{125}I]monoiodoinsulin fragments IB and IIB (*right*) on Sephadex G-50 column before (●) and after (○) reduction and carboxymethylation of disulfide bonds as described in Fig. 4.

lymphocytes (22). With results of Yokono et al. (30) these data provide evidence for similarities among the various insulin-degrading activities reported in the literature. It should also be noted that inhibition of insulin degradation by a crude preparation of IGF (referred to as nonsuppressible insulin-like activity) was first observed in a preparation of liver plasma membranes (31) and insulin protease (32), and that IGF-II inhibited insulin degradation in the serum of a patient with diabetes and severe insulin resistance (33). Why IGF-II is a better substrate for insulin protease than IGF-I is not known. IGF-I and IGF-II are more similar to each other in structure than they are to insulin. Furthermore, the receptors for insulin and IGF-I are more similar to each other with respect to structure and ligand specificity than they are to the receptor for IGF-II (34). Despite our observation that insulin and IGF-II compete for the same protease (28), incubation of cells with insulin actually increases the rate of IGF-II degradation because of translocation of IGF-II receptors from microsomes to the cell surface (35). To what extent, if any, insulin protease accounts for IGF-II degradation in intact cells is not known.

The results of this study provide evidence for sequential degradation of insulin by erythrocyte insulin protease. We have identified intermediates early in the incubation that are slightly smaller than insulin in molecular size and probably result from cleavage of single peptide bonds without disruption of the interchain disulfide bonds. This type of product was first suggested by Duckworth et al. (14) to result from cleavage of Tyr^{B16}-Leu^{B17} and has subsequently been called clipped insulin. The concentrations of the intermediates fall as the incubation proceeds, and final products of smaller molecular size accumulate, two of which could be characterized with the radioactive label used in these experiments. The proposed sites of insulin degradation are illustrated in Fig. 8. Fragment IIIA is a peptide whose A-chain NH_2-terminal is Tyr^{A14} and whose A-chain is linked to a portion of the B-chain. Fragment IIIB is a peptide whose NH_2-terminal is Tyr^{B26} and that appears to be derived entirely from the B-chain (no shift in molecular weight on reduction). It appears that the bonds Ser^{A9}-His^{B10}, Phe^{B25}-Tyr^{B26}, and Leu^{A13}-Tyr^{A14} are broken by erythrocyte insulin protease in addition to other bonds in both A- and B-chains that we were unable to specify. These bonds have also been reported to be cleaved by rat skeletal muscle insulin protease (25,36–38).

The insulin fragments that we found here to be produced by insulin protease are smaller and with less immunoactivity than what we and others have reported to be produced in preparations of intact cells (8). One possible explanation for this discrepancy is the existence of nonspecific protease in intact cells that would further degrade insulin fragments to iodotyrosine as soon as they are formed. An alternative explanation is that the larger fragments may be bound or sequestered within the cell so that they are no longer available for further action by insulin protease in the cytosol. Indirect evidence for this explanation is provided by the preliminary report of Kitabchi et al. (39) of insulin fragments produced

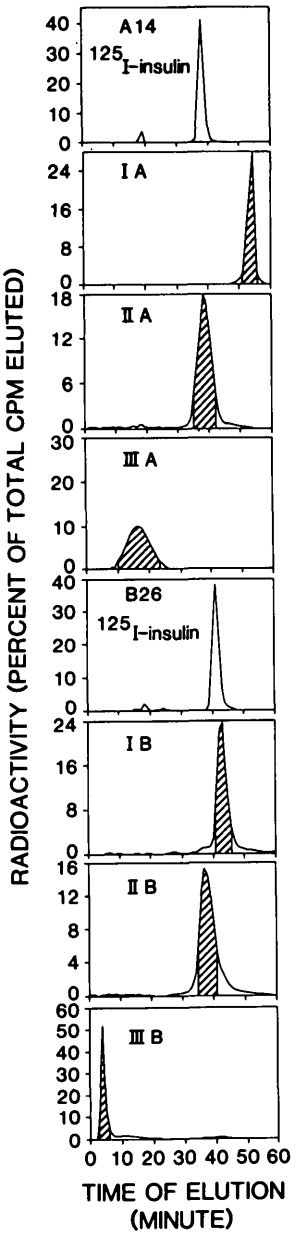

FIG. 7. Retention times of insulin fragments on SepPak C-18 reverse-phase column. Data obtained from elution profiles with flow rate of 1.0 ml/min. A solvent consisted of 0.1% trifluoroacetate in water, and B solvent was 0.1% trifluoroacetate in 90% acetonitrile 10% water. Column was first equilibrated in 20% B/80% A. After sample (usually in 1 M acetic acid) has been applied, 5-min isocratic elution with 20% B/80% A was used. Thereafter, B solvent was increased linearly to 100% B over 90 min. Fractions in *hatched* areas were pooled and treated with 10 mM dithiothreitol followed by 30 mM iodoacetemide. Treated material was then eluted again through C-18 reverse-phase column and applied to protein sequencer.

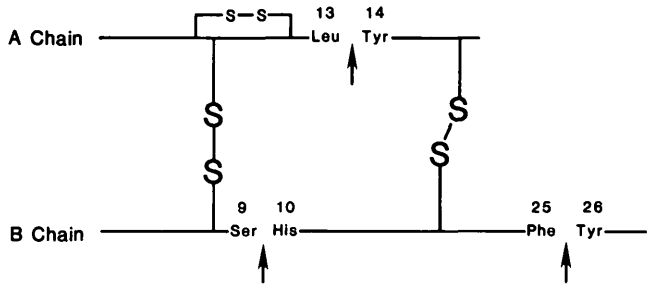

FIG. 8. Proposed sites of insulin degradation by erythrocyte enzyme.

by human fibroblasts that stimulate pyruvate dehydrogenase activity in isolated mitochondria.

Although we have used human erythrocytes as a source of insulin-degrading enzyme, there is no evidence that insulin degradation by erythrocytes is of physiological importance. It has long been recognized that the liver is primarily responsible for removal of insulin from the circulation (40–43). Elegant studies by Ferrannini et al. (44) have shown that the kidney accounts for >80% of extrasplanchnic insulin removal in humans. These studies of insulin degradation were performed with circulating human blood cells because they provided a convenient source of tissue rather than because they represented important sites of insulin degradation (45). The work of Shii and co-workers provides compelling evidence that the erythrocyte insulin-degrading enzyme is similar or identical to that found in other tissues (27) and that this enzyme is responsible for degradation of internalized insulin (46). Thus, studies of the erythrocyte insulin protease may provide information that is applicable to insulin degradation at physiologically important sites.

The role of insulin degradation with respect to insulin action and carbohydrate metabolism is not clear. Studies with insulin mimickers such as anti-receptor antibody have shown that the insulin molecule itself is not necessary for activation of certain receptor-mediated processes (47). Although some authors have suggested that intracellular insulin may regulate certain metabolic processes, only Kitabchi et al. (39) provide evidence for biological activity of insulin fragments at an intracellular site. The B-chain fragments had previously been shown to have insulin-like activity in vivo and in isolated cells, although high concentrations are required for the effects (48,49). Thus, the literature provides little evidence that insulin degradation has any physiological function other than to terminate insulin's signal once it has interacted with its receptor. On the other hand, if insulin degradation has no function other than to remove insulin from the circulation, it is hard to understand why insulin must escape uptake by the liver before it enters the peripheral circulation. Insulin protease also has the unusual property of substrate specificity both for the peptides it degrades and for the particular bonds on those peptides that it is capable of cleaving. That insulin fragments are released by liver cells (8) and are found in peripheral blood (50) leads to the speculation that they may exert some effect on other tissues, perhaps even on neural and vascular tissues, which are not thought to be major sites for the action of insulin but are adversely affected by insulin deficiency.

ACKNOWLEDGMENTS

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