Comparison of the Enzymatic and Biochemical Properties of Human Insulin-degrading Enzyme and *Escherichia coli* Protease III*

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The enzymatic and biochemical properties of human insulin-degrading enzyme and *Escherichia coli* protease III have been compared. Both enzymes were found to degrade insulin in such a way that its receptor binding activity was rapidly lost but its precipitability in trichloroacetic acid was only slightly decreased. Both enzymes were also found to be inhibited by chelating agents. The bacterial enzyme, which could be purified in large amounts, was found to contain 0.6 mol of zinc per mol of enzyme but no detectable manganese. The mammalian enzyme but not the bacterial one was inhibited by a sulfhydryl alkylating agent. The two enzymes also differed in substrate specificity. The mammalian enzyme degraded insulin much better than insulin-like growth factor II, whereas the bacterial enzyme degraded them equally. The mammalian enzyme could be labeled by cross-linking to insulin = bombesin II > insulin-like growth factor I and II > relaxin, whereas the bacterial enzyme was labeled by insulin-like growth factor II > insulin = insulin-like growth factor I > relaxin > bombesin. Finally, sucrose gradient centrifugation and cross-linking studies both in vitro and in vivo indicated that active human enzyme partially existed as a homo- or heterodimer, whereas the bacterial enzyme was active as a monomer.

Insulin binds to its receptor on the surface of the cell to initiate its diverse biological actions (Roth, 1990; Hollenberg, 1990; Rothenberg et al., 1990). The hormone-receptor complex is then internalized in an endocytic vesicle (Levy and Olefsky, 1990). After acidification of the vesicle, the insulin dissociates and most of the receptors are recycled back to the cell surface (Levy and Olefsky, 1990). In contrast, the majority of the insulin molecules are degraded (Levy and Olefsky, 1990; Duckworth, 1990). Evidence has accumulated indicating that the process of insulin degradation begins in these endocytic vesicles (Pease et al., 1985; Hamel et al., 1988; Doherty et al., 1990). A specific enzyme, called insulin-degrading enzyme (IDE), has been proposed to play a role in this process (Duckworth, 1988, 1990). Evidence in support of this hypothesis comes from studies showing that isolated IDE cleaves insulin at a limited number of sites, consistent with insulin peptide intermediates found in cells and endocytic vesicles (Duckworth, 1988, 1990; Hamel et al., 1988; Duckworth et al., 1988; Assoian and Tager, 1982; Williams et al., 1990). In addition, when radioactive insulin is cross-linked to proteins in intact cells, IDE becomes labeled (Hari et al., 1987). Also, the injection of monoclonal antibodies directed against IDE lowers the level of insulin degradation in cells (Shii and Roth, 1986). Most recently, IDE has been shown to be at least partly present in endosomes (Hamel et al., 1991). The above data are therefore all consistent with a role for the enzyme in insulin metabolism. However, it is possible that other enzymes may also contribute to insulin degradation. In addition, IDE may have other roles in the cell, such as participating in the general turnover of proteins.

Although the existence of a specific insulin-degrading enzyme was reported over 30 years ago (Mirsy, 1957), progress in the study of IDE has been limited by the low levels of this molecule and its lack of stability in vitro. Small amounts of purified IDE have been obtained from human red blood cells using conventional column chromatography, and these studies have revealed the presence of a single polypeptide of 110 kDa on denaturing polyacrylamide gels (Kirschner and Goldberg, 1983; Shii et al., 1986). In recent years, monoclonal antibodies against this enzyme have been generated (Shii and Roth, 1986), and affinity columns composed of these antibodies have yielded enough homogeneous human IDE for partial sequence analysis (Affholter et al., 1988). This protein sequence was used to isolate a full-length cDNA clone that encodes IDE (Affholter et al., 1988). The amino acid sequence of the human enzyme deduced from this clone showed no significant identity to any known mammalian proteases and did not contain the consensus active site sequence (HEXXH) present in most zinc metalloendopeptidases (Vallee and Auld, 1990). However, an *Escherichia coli* protease, protease III or Pi (Swamy and Goldberg, 1981), showed 46% sequence similarity to human IDE (Affholter et al., 1988; Finch et al., 1986). In addition, three regions in the sequences of these two enzymes are between 54 and 80% identical, implying that these regions may be important for the enzymatic functions of IDE and protease III. One of these conserved regions contains a potential metal-binding site (HXXEH).

In the present studies we have utilized the gene coding for protease III (Dykstra and Kushner, 1985) to genetically engineer bacteria to overexpress this enzyme. These cells have allowed us to readily purify large quantities of the enzyme. To see if the bacterial protease III is a good model to study human IDE, the biochemical and enzymatic activities of the two enzymes were compared. In addition, we show that protease III contains almost stoichiometric amounts of zinc.

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1 The abbreviations used are: IDE, insulin-degrading enzyme; IGF, insulin-like growth factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.
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**EXPERIMENTAL PROCEDURES**

**Materials**—The following were purchased: standard SDS molecular weight markers from Bio-Rad; prestained SDS molecular weight markers from Bethesda Research Laboratories; bacitracin, N-ethylmaleimide, ethidium bromide, and EDTA from Sigma; DEAE-Sephadex, Sepharose CL-6B, and phenyl-Sepharose CL-4B from Pharmacia LKB Biotechnology Inc.; MemSep™ membrane chromatography cartridges from Millipore; Ultrogel AcA-34 from Pharmacia. Other reagents were as previously described (Shi and Roth, 1986; Affnolt et al., 1990a, 1990b; Steele-Perkins et al., 1988).

**Partial Purification of Human IDE**—Packed human red blood cells (500 ml) were washed three times in an equal volume of phosphate-buffered saline, pH 7.4, by centrifugation at 4000 x g for 10 min. The washed cells were lysed in 1 liter of 10 mM sodium phosphate buffer, pH 7.4, containing 25 μM PMSF by freezing the cells in a dry ice ethanol bath and then thawing them at 4 °C. Membranes were removed by centrifugation at 12,000 x g for 1 h. Aliquots of the supernatant were frozen at -70 °C and used as the source for IDE in the purification and assays described below. For further purification, 1340 ml of the supernatant was absorbed to 1.2 liters of DEAE-Sephadex preequilibrated in 10 mM sodium phosphate buffer, pH 7.4, 25 μM PMSF. The resin was washed with 2 liters of 10 mM sodium phosphate buffer, pH 7.4, 25 μM PMSF, and the bound proteins were eluted with 1 liter of 0.5 M KCl in 100 mM sodium phosphate buffer, pH 7.4. The eluant was fractionated by ammonium sulfate precipitation, and the proteins which precipitated between 40 and 60% ammonium sulfate were dissolved in 20 mM HEPS, pH 7.6.

The sample was then further purified on a Sepharose CL-6B column (2.5 x 30 cm) equilibrated in 20 mM HEPS, pH 7.6. For each run, 10 ml of the above sample was loaded. The peak of insulin-degrading activity eluted between 290 and 350 ml from the start of the run. This material was further purified on a Water's 600E protein purification system with a DEAE MemSep™ 1000 cartridge preequilibrated in 90% Buffer A (20 mM HEPES, pH 7.6) and 10% Buffer B (0.5 M KCl in Buffer A). The column was washed with 15 ml of this buffer and then eluted for 60 min by a nonlinear gradient (Gradient 4) of 90% Buffer A and 10% Buffer B to 60% Buffer A and 40% Buffer B. The peak of insulin-degrading activity from this column was further purified on a phenyl-Sepharose column (1.5 x 4 cm) by binding at 2 M NaCl, 20 mM HEPS, pH 7.6, and eluting with a linear gradient of ammonium sulfate (2 to 0 M) and increasing ethylene glycol (0-50%). Fractions of IDE off this column were used in the cross-linking studies.

**Overexpression of Protease III**—The protease III gene (Dykstra and Kusher, 1988) was subcloned into the EcoRV and HindIII sites of a modified Tacterm vector, referred to as ptts6, which contains an isopropyl-1-thio-D-galactopyranoside-inducible tac promoter (Fa- luh and Yanofsky, 1986). The protease III/ptts6 plasmid was used to transform competent JM101 bacteria, and expression of protease III was induced by the addition of 2 mM isopropyl-1-thio-D-galactopyranoside-inducible tac promoter (Faluh and Yanofsky, 1988). The bacteria were pelleted for 15 min at 4 °C and then washed with 50 ml of a chilled solution containing 20% sucrose, 0.1 M EDTA, and 50 mM Tris-HCl, pH 7.6. The bacteria were again pelleted for 15 min and resuspended in 20-30 ml of 0.5 M MgCl₂ in water. The cells were incubated at 4 °C for 30 min and then micro-centrifuged for 15 min at 10,000 x g. The supernatant was collected and represented the periplasmic shokcate.

A variety of purification procedures has been utilized to isolate protease III. The basic procedure is as follows. The periplasmic shokcate was first fractionated by ammonium sulfate precipitation. The precipitated material (35% of 55% saturated ammonium sulfate) was resuspended in 1.5 l of 10 mM HEPS, 20 mM NaCl, 0.1% Tween 20 (pH 7.6) and applied to an Ultrogel AcA34 column (1.5 x 90 cm) equilibrated in the same buffer. The peak of protease III, determined by SDS gel electrophoresis, was diluted 1:2 with 200 mM Tris-HCl, 0.1% SDS, and applied to a phenyl-Sepharose (Pharmacia) preequilibrated in a solution consisting of 5 mM HEPS, 10 mM NaCl, 0.05% Tween 20, and 100 mM Tris-HCl, pH 8.0. The purity of protease III in the flow-through from this column is shown in Fig. 1. In a typical experiment, 0.5 mg of purified enzyme was obtained from 1 liter of cells. These highly purified preparations were used in the in vitro cross-linking and sucrose gradient experiments.

**Meal Determinations**—The amount of zinc and manganese present in the purified protease III was determined by atomic absorption spectroscopy (Perkin-Elmer, model 2380). All assays were performed in duplicate with 1-10 μg of purified protease III every 2 weeks over a period of 2 months. The first two injections were intraperitoneal using Ribi as an adjuvant. The third injection was performed intraperitoneally using Freund's complete adjuvant, and the fourth injection was performed without adjuvant and was split between the intraperitoneal cavity and the tail vein. Five days after the fourth injection, the mouse was killed and the spleen was used to NS1 as described (Shi and Roth, 1986). The hybridoma cells were grown in 96-well plates and then split into 24-well plates. The supernatants of these cells were screened for the ability to precipitate protease III insulin-degrading activity. The cells secreting the monoclonal antibody with the highest apparent affinity in this assay (2B4D9) were cloned and grown up.

**Homogenous Degradation Assays**—As indicated in the text, the degradation of [125I]-peptide was measured by either the loss of trichloroacetic acid precipitable radioactivity or the loss of ability to bind to receptor. For all the trichloroacetic acid assays, the enzyme was immunomobilized on polyvinyl chloride microtiter plates (Falcon). First, the wells were coated with 20 μl of 50 μg/ml affinity-purified rabbit anti-mouse IgG (Pel-Freeze) in 20 mM NaHCO₃, pH 9.6. After 1-2 h at room temperature, the wells were washed twice with ice-cold washing buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.2% bovine serum albumin, 0.1% Tween 20, 0.1% Triton X-100) and then coated with 45 μl of either 5 μg/ml anti-IDE monoclonal antibody 9B12 or culture medium supernatant containing anti-protease III monoclonal antibody 2B4D9. After 1-2 h at room temperature, the wells were washed twice with washing buffer, and then appropriate amounts of either red blood cell lysate (for IDE) or periplasmic shokcate (for protease III) were added. The wells were washed 4 times in a final volume of 40 μl washing buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.3% BSA) and divided into triplicate wells. After 4 h at 4 °C, the wells were again washed twice.

For the degradation assays, approximately 50,000 cpm of [125I]-histamine in 35 μl (approximately 2 μm) were added to each well. After an appropriate amount of time at 37 °C (typically 30 min), the reaction was stopped by placing the plate on ice, and the reaction mixture was transferred into 160 μl of 9.1% trichloroacetic acid. After 15 min at 4 °C, the samples were centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant and pellet were separated and counted in a Beckman model 5500 γ counter. The percentage of degraded hormone was calculated as the percentage of the total counts in the supernatant.

For the receptor binding assay of insulin degradation, purified enzyme or immunomobilized enzyme was incubated with labeled hormone as above. After the appropriate incubation, the labeled insulin or IGF-II in the supernatant was removed and tested for its ability to bind to immunoimmobilized insulin receptor or the type I IGF receptor (Steele-Perkins et al., 1988), respectively. After 4-8 h at 4 °C, the wells were washed twice with the washing buffer and cut out for counting in a γ counter. The degradation of the hormone is calculated as the percentage decrease in radioactivity bound to receptor in comparison with controls which had been incubated in microtiter wells without protease.

**Immunoblots**—Electrophoresed samples were transferred to nitrocellulose filters (Schleicher & Schuell) for 2 h at 280 mA in a buffer containing 24 mM Tris base, 192 mM glycine, and 20% methanol. The filter was blocked by a 10-min incubation with a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% BSA, and 0.1% Triton X-100. The filters were then incubated with the primary antibody solutions for 12 h at 4 °C. For detecting IDE, the primary antibody solution was 5 μg/ml monoclonal antibody 9B12 in Tris-HCl, pH 7.5, 150 mM NaCl, 3% BSA, and 0.1% Tween 20. For detecting protease III, mouse ascites containing monoclonal antibody 2B4D9 was diluted 1:200 in the above buffer. After the incubation, the filters were washed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl four times over a period of 1 h at room temperature. The bound mouse immunoglobulin was detected using 1:1000 dilution of 1:100 dilution of rabbit anti-mouse immunoglobulin (Promega Biotech) and a colorometric stain as described in the Promega handbook.

**Cross-linking Studies**—Cross-linking of the purified enzymes was
performed by incubating at 0°C for 1 h the enzyme and labeled hormones (200,000 cpm) in 50 μl of buffer containing 20 mM HEPES, pH 7.6, and the indicated concentrations of disuccinimidyl suberate. The cross-linking reaction was terminated by the addition of 2 μl of 1 M Tris-HCl, pH 7.4, and then brought to 0.005% bromphenol blue, 0.5% glycerol, and 0.4% SDS. The samples were heated for 3 min at 100°C and electrophoresed on a 10% SDS-polyacrylamide gel. Gels were stained, dried, and autoradiographed. For the detection of higher molecular weight species of IDE and protease III, nonradioactive hormone or no hormone was added. After cross-linking and electrophoresis, samples were transferred and probed with the appropriate antibodies as described above.

IDE was also cross-linked to its associated proteins in intact cells. Confluent monolayers of either human hepatoma or Chinese hamster ovary cells overexpressing insulin receptors were washed once with ice-cold phosphate-buffered saline, and then 5 ml of 0.3 mM disuccinimidyl suberate in the same buffer was added to the 100-mm dish of cells. After 30 min at 4°C, the cells were washed twice and solubilized with 0.5 ml of lysis buffer (20 mM Tris-HCl, 20 mM HEPES, pH 7.6, 2% Triton X-100, and 25 μM PMSF). The lysate was microcentrifuged, and the supernatant was absorbed with either 9B12 or control IgG and analyzed by Western blots as described above.

Sucrose Gradient Centrifugation—A linear gradient of 5–20% sucrose in 4.5 ml of 50 mM HEPES, pH 7.6, 150 mM NaCl was generated in a polycarbonate centrifuge tube (13 × 51 mm, Beckman). Each sample in 150 μl of 50 mM HEPES, pH 7.6, 150 mM NaCl was loaded on the top of the gradient. The sample was then centrifuged for 15–17 h at 200,000 × g at 4°C (Beckman model L5–50 ultracentrifuge, SW 50.1 rotor, 35,000 rpm). Fractions of 5 drops (about 150 μl) were collected by a gradient tube fractionator (Hoeffer, FS 101) into polypropylene microcentrifuge tubes. Fractions were then assayed for insulin-degrading activity or by immunoblotting as described above. For each independent centrifugation, marker proteins (thyroglobulin, 19.4 S, bovine liver catalase, 11.3 S, bovine serum albumin, 4.6 S)) were analyzed. These protein standards were detected by Bradford assay. These protein standards were detected by Western blots as described above.

RESULTS

Purification of Protease III and Generation of a Monoclonal Antibody to the Enzyme—Periplasmic shockates of genetically engineered bacteria designed to overexpress protease III exhibited a major protein band on Coomassie-stained SDS gels of 100 kDa (Fig. 1A, lane b) that was not present in shockates of the parental cells (not shown). Purification of this protein to homogeneity could be accomplished by conventional chromatography (Fig. 1A, lane a). These purified preparations were highly active in degrading insulin. Two ng of a typical preparation degraded 50% of the insulin in 30 min at 37°C in the receptor binding degradation assay described below. A monoclonal antibody was generated against this preparation, and this antibody was found to specifically recognize in immunoblots a 100-kDa protein in the periplasmic shockates (Fig. 1B) and to precipitate insulin-degrading activity (see below). These results indicate that the 100-kDa protein is protease III.

Degradation of Insulin by IDE and Protease III—Because of the lability of purified IDE, its activity is most conveniently assayed after absorption to microtiter wells coated with monoclonal antibodies to the enzyme. As previously documented (Shii et al., 1986), IDE exhibits much higher insulin-degrading activity when its activity is assessed by the loss in ability of the cleaved insulin to bind its receptor than when degradation is assessed by loss in trichloroacetic acid precipitability (Fig. 2A). Immunomobilized protease III was found to degrade insulin in the same way as IDE, that is insulin incubated with protease III showed a much greater loss in receptor binding ability than trichloroacetic acid precipitability (Fig. 2B). A lower maximal degradation of insulin was observed in both assays with protease III, possibly reflecting either a lower amount of immobilized protease III or a lower specific activity of the enzyme.

Inhibition of IDE and Protease III-degrading Activities by Various Protease Inhibitors—The ability of various reagents to inhibit the degrading activities of the immobilized IDE and protease III was tested by utilizing the receptor binding assay to assess insulin degradation (Table I). 1 mM of a sulfhydryl-alkylating agent, N-ethylmaleimide, was found to inhibit IDE by 81%, while it did not have much effect on the activity of protease III (Table I). Two chelating agents (1,10-phenan-
thornine and EDTA) were found to inhibit both enzymes. However, IDE appeared to be less sensitive to EDTA than protease III with 1 mM EDTA inhibiting IDE only 18% but inhibiting protease III 96% (Table I). The general protease inhibitor bacitracin was partially inhibitory to both enzymes. Finally, a serine protease inhibitor, PMSF, did not inhibit either enzyme.

Metal Determinations—The purified protease III was tested for zinc and manganese by atomic absorption spectroscopy. Manganese was present at less than 0.01 molecules/molecule of protease III whereas 0.6 ± 0.04 (n = 3) molecules of zinc/molecule of enzyme were present.

Interactions of Insulin-related Hormones with IDE and Protease III—As previously noted (Misbin et al., 1983), the insulin-related peptides IGF-I and -II can inhibit the degradation of insulin by IDE, with IGF-II being approximately 100 times more potent than IGF-I (Fig. 3A). The degradation of insulin by protease III was also inhibited by IGF-I and -II (Fig. 3B). However, the relative potencies were quite different for the two enzymes. For example, with protease III, IGF-II was approximately 100 times more potent at inhibiting insulin degradation than insulin itself, whereas they were almost equipotent at inhibiting insulin degradation by IDE (Fig. 3).

The ability of IDE and protease III to degrade IGF-II was directly assessed by utilizing the immunoimmobilized enzymes and loss in ability of the treated IGF-II to bind to the type I receptor. As previously reported (Misbin et al., 1983: Roth et al., 1984), IDE more readily degrades insulin than IGF-II (Fig. 4A). In contrast, protease III degrades insulin and IGF-II almost equally (Fig. 4B).

Prior studies have shown that insulin can be cross-linked directly to IDE with the bifunctional cross-linker disuccinimidyl suberate (Shii et al., 1985). To further compare the interactions of IDE and protease III with insulin and insulin-related peptides, the two enzymes were tested for the ability to be cross-linked to labeled insulin and the insulin-related molecules IGF-I, IGF-II, relaxin, and bombesin II. insulin and bombesin II were found to preferentially label IDE (Fig. 5A), whereas protease III was almost equally labeled by IGF-I, IGF-II, and insulin but very poorly by bombesin II (Fig. 5B).

Quaternary Structure of IDE and Protease III—When the IDE preparation was cross-linked to insulin, an additional band of approximately 160 kDa was observed (Fig. 5A). To test whether this higher molecular weight complex contained IDE, Western blotting was performed on a cross-linked preparation of IDE. In addition to the major IDE band of 110 kDa, a weak band of approximately 160 kDa was observed in Western blots with the monoclonal antibody to IDE when IDE was incubated with 0.5 mM disuccinimidyl suberate (Fig. 6A, lane c). The presence of this band was dependent on the addition of cross-linker (Fig. 6A, lanes a and c). The amount of the 160-kDa protein was not increased by the addition of ligand, higher concentrations of disuccinimidyl suberate, or the use of another cross-linker, disuccinimidyl tartarate (Fig. 6B and data not shown). No higher molecular weight complex was observed with protease III, either by Western blotting or by labeling (Figs. 5B and 6B).

To further study the presence of this higher molecular weight complex, IDE and protease III were analyzed by sucrose gradient centrifugation. When the sucrose gradient fractions were analyzed by Western blotting, protease III was found to elute as a sharp peak in fractions 18–20 with an apparent sedimentation coefficient of approximately 5.3 S (Fig. 7B). In contrast, IDE was spread out over fractions 14–19 with a peak at 6.0 S (Fig. 7A). The peak of insulin-degrading activity of protease III coincided with the peak of protease III immunoactivity (Fig. 7B). In contrast, the peak of insulin-degrading activity of the IDE preparation was in fraction 14, approximately 5 fractions ahead of the peak of IDE immunoactivity (with an apparent sediment coefficient of 9.2 S) (Fig. 7A). This peak of insulin-degrading activity was in the same position when the fractions were either assayed directly or when IDE in the fractions was first captured on microtiter wells coated with antibodies to IDE, indicating that IDE was responsible for this activity.

To test whether IDE was also in a complex in the intact

| Table I
| Effect of various agents on the insulin-degrading activity of IDE and protease III |
| Agent       | Concentration | Inhibition % |
|-------------|---------------|-------------|
|             | IDE           | PTR         |
| N-Ethylmaleimide | 1 mM         | 81          | 6          |
| 1,10-Phenanthroline | 0.1 mM       | 76          | 82         |
| EDTA        | 1 mM          | 47          | 100        |
|             | 10 mM         | 47          | 100        |
| Bacitracin  | 0.1 mg/ml     | 48          | 78         |
| PMSF        | 1 mM          | 0           | 0          |

**Fig. 3.** Inhibition of $^{125}$I-insulin degradation by unlabeled insulin, IGF-I, and IGF-II. Immunoimmobilized IDE (A) from red blood cell lysate or protease III (B) from E. coli shockate was incubated with 50,000 cpm of $^{125}$I-insulin in the presence of the indicated concentrations of unlabeled insulin (O), IGF-I (C), or IGF-II (A). After 30 min at 37°C, the degradation of $^{125}$I-insulin was assessed by trichloroacetic acid precipitation. Results shown are means ± S.D. of triplicate determinations.
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Fig. 4. Degradation of 125I-insulin and 125I-IGF-II by IDE and protease III. Immunoimmobilized IDE (A) or protease III (B) was incubated with either 50,000 cpm of 125I-insulin (●) or 125I-IGF-II (△) for 30 min at 37°C. The extent of degradation of the two hormones was determined by the homologous receptor binding assay. Results shown are means ± S.D. of triplicate determinations.

Fig. 5. Cross-linking of 125I-labeled hormones to IDE and protease III. Partially purified IDE (A) or protease III (B) was incubated with either 125I-relaxin (c), 125I-IGF-I (b), 125I-IGF-II (c), 125I-insulin (d), or 125I-bombesin II (e) in the presence of 0.5 mM disuccinimidyl suberate. The cross-linked samples were electrophoresed on a nonreduced 10% polyacrylamide-SDS gel, and an autoradiograph of the gel is shown. The positions (in kDa) of prestained molecular mass markers are indicated. The labeled 66-kDa protein is bovine serum albumin.

Fig. 6. Immunoblots of IDE (A) or protease III (B) after cross-linking. Partially purified IDE was incubated with either buffer (a), 2 mM (b), 0.5 mM (c), or 0.05 mM (d) disuccinimidyl suberate or buffer (e), 2 mM (f), 0.5 mM (g), or 0.05 mM (h) disuccinimidyl tartrate. Samples were then electrophoresed on a SDS-polyacrylamide gel, transferred to nitrocellulose filters, and blotted with a monoclonal antibody (9H12) to IDE. Purified protease III was incubated with either buffer (a), 2 mM (b), or 0.5 mM (c) disuccinimidyl suberate and then electrophoresed and immunoblotted with the monoclonal antibody to protease III. The positions (in kDa) of the prestained markers are indicated.

Fig. 7. Sucrose gradient fractionation of IDE (A) and protease III (B). Partially purified IDE and protease III were fractionated on a 5–20% sucrose gradient. Each fraction was tested for insulin-degrading activity by the receptor binding assay and immunoblotted from a 10% polyacrylamide-SDS gel (shown in the inset). Positions of marker proteins are indicated. The results shown are representative of three separate experiments.

cell, Chinese hamster ovary cells were incubated with the bifunctional cross-linker disuccinimidyl suberate, washed, lysed, and the IDE was immunoprecipitated. The immunoprecipitates were analyzed on Western blots with the monoclonal antibody to IDE. As observed in vitro, IDE from cells treated with cross-linker but not control cells exhibited an immunoreactive band at 160 kDa as well as the more abundant 110-kDa IDE band (Fig. 8). This 160-kDa band was not present in control precipitates and was not detected in Western blots developed with control antibody. The same higher molecular weight species could also be generated in human...
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Discussion

Although an insulin-specific protease was described more than 30 years ago (Mirskey, 1957), the detailed biochemical characterization of this enzyme has been limited. Difficulties in the isolation of this enzyme are in part due to its low properties of these two proteins.

First, bacteria were genetically engineered to overproduce protease III so that the enzyme could be readily purified in large amounts (Fig. 1). In contrast, we have not been able to overproduce active IDE in E. coli. The bacterial enzyme also appears to be much more stable than mammalian IDE, possibly due to its lack of quaternary structure (see discussion below). One characteristic of IDE that has been extensively studied is its ability to make a limited number of cleavages in the insulin molecule (Duckworth, 1988, 1990). These cleavages greatly affect the ability of insulin to bind to its receptor without causing much decrease in the trichloroacetic acid precipitability of the molecule (Shii et al., 1986). The prior studies of protease III have shown that this enzyme readily cleaves insulin B chain (Dykstra and Kushner, 1985; Cheng and Zipser, 1979) although it was not clear whether this enzyme cleaves intact insulin. In the present studies, we demonstrate that protease III can also degrade intact insulin, and this degradation, like that by IDE, much more readily affects receptor binding than the trichloroacetic acid precipitability of the insulin molecule (Fig. 2B). These results indicate that protease III is like IDE in that it makes a limited number of cleavages in insulin. It is likely that the cleavage sites of the two enzymes are similar since prior studies have shown that protease III cleaves insulin B chain at Tyr-Leu (16-17) and Phe-Tyr (25-26) (Cheng and Zipser, 1979), two of the sites in intact insulin cleaved by IDE (Duckworth, 1988).

Protease III was previously shown to be a metalloprotease since it could be inhibited by chelating agents and since the chelated enzyme could be reactivated by zinc, cobalt, or manganese (Cheng and Zipser, 1979). However, the metal normally present in protease III had not been previously determined. In the current studies we have been able to show that our purified preparations of protease III contain approximately 0.6 mol of zinc per mol of enzyme. Thus, protease III appears to be a zinc metalloendopeptidase even though it does not contain the traditional consensus sequence (HEXXH) for this class of enzymes. Studies of mammalian IDE and a related insulin-degrading enzyme from Drosophila melanogaster (Garcia et al., 1988; Kuo et al., 1990) have given conflicting data on whether this enzyme is a metalloprotease. Although several studies have shown that chelating agents inhibit the activity of the mammalian enzyme (Kirschner and Goldberg, 1983; Shii et al., 1986; Ansorge et al., 1984; Kayalar and Wong, 1989; Duckworth et al., 1990), other studies of the mammalian (Burghen et al., 1972) and Drosophila enzyme (Garcia et al., 1988) did not observe any inhibition by the same agents. In the present studies, we did observe inhibition of human IDE with two chelating agents (Table I). However, the mammalian enzyme was less sensitive to EDTA than the bacterial enzyme (Table I), possibly due to the mammalian enzyme having a higher affinity for metal than the bacterial enzyme. This high affinity could explain some of the prior discrepancies in the literature. It is possible that the presence of a cysteine in a putative metal-binding site of IDE but not protease III (see discussion below) gives IDE the higher affinity for its metal since cysteine residues bind metals with high affinities (Vallee and Auld, 1990).

In agreement with prior studies (Kirschner and Goldberg, 1983; Shii et al., 1986; Cheng and Zipser, 1979), the mammalian enzyme was also very susceptible to inhibition by a sulphydryl-modifying agent whereas protease III was not affected by this inhibitor (Table I). The deduced sequence of protease III indicates that this enzyme only has a single cysteine (Finch et al., 1986) whereas the IDE sequence predicts that this enzyme has 12 cysteines (Affholter et al., 1988). One of the cysteines in IDE is present in a potential metal-binding site (Vallee and Auld, 1990) (HXXH) in the first highly conserved domain of IDE. This metal-binding site is present in protease III (HXXXH), but it lacks the cysteine. The alkylation of this cysteine in IDE could therefore disrupt the activity of IDE. The role of this residue in the enzymatic activity of IDE and in mediating the sensitivity of the enzyme to alkylating agents can be tested by site-directed mutagenesis.

IDE and protease III also differed in their substrate specificity. Protease III appeared to almost equally degrade insulin and IGF-II, whereas IDE showed a clear preference for insulin (Fig. 4). Cross-linking studies also indicated that protease III could be labeled almost equally with insulin and IGFs, whereas IDE again was preferentially labeled with insulin (Fig. 5). IDE was also readily cross-linked to labeled bombyxin II, an insulin-related peptide from silkworm (Maruyama et al., 1990). In contrast, protease III was very poorly labeled by this molecule (Fig. 5). These differences in substrate specificity between protease III and IDE can be utilized to identify the region(s) of these homologous enzymes responsible for ligand specificity by constructing chimeric molecules with parts of each enzyme.

In the cross-linking studies with labeled ligand, a molecule larger than IDE was also observed (Fig. 5). In prior studies of insulin cross-linked to IDE in intact cells, a similar molecular weight species was observed (Hari et al., 1987). In the present work we found that a molecule of very similar M, could be detected in Western blots with a monoclonal antibody to IDE.

Fig. 8. In vivo cross-linking of IDE. Intact Chinese hamster ovary cells were incubated with either 0.3 mM disuccinimidyl suberate (lanes a and b) or buffer (lanes c and d), washed two times, and lysed. Immunoprecipitates of either IDE (lanes a and c) or control IgG (lanes b and d) were analyzed by immunoblotting as in Fig. 6.

**A. B. Becker and R. A. Roth, unpublished studies.**
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(Fig. 6). The presence of this band required the prior crosslinking of the IDE preparation with disuccinimidyl suberate (Fig. 6). In contrast, no similar band was observed with protease III in either Western blots or after cross-linking labeled ligand (Figs. 5B and 6B). These results suggested that IDE might normally exist as either a homo- or heterodimer. Further support for this hypothesis came from the finding that the treatment of intact cells with cross-linker also generated an immunoreactive IDE molecule with the same electrophoretic mobility (Fig. 8). This higher molecular weight species may be the active IDE since the major peak of insulin-degrading activity eluted on a sucrose gradient ahead of the peak of immunoreactive material (Fig. 7). Such a hypothesis may explain the liability of purified IDE since its dissociation into monomers would result in a loss of activity. Several prior studies have also indicated that the molecular weight of IDE under nondenaturing conditions is greater than one would expect if the enzyme was a monomer (Kirschner and Goldberg, 1986). This does not appear to be true of protease III since the major peak of activity of this enzyme on sucrose gradients coincided with its peak of immunoreactive material and both had a sedimentation coefficient (6.2 S) which was consistent with being a monomer. The Drosohila insulin-degrading enzyme may be similar to protease III in that it was previously reported that the enzymatic activity of this molecule elutes as a monomer on sucrose gradients (Garcia et al., 1988).

In summary, the present results indicate that the bacterial protease III is similar to human IDE in that it cleaves insulin in a limited number of sites and in the requirement for a metal ion for activity. In contrast to IDE, it can be readily overproduced and purified and appears to be more stable. Protease III may, therefore, be a useful model for structural and biochemical studies of IDE and other members of this class of proteases.

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