Degradation of Amylin by Insulin-degrading Enzyme*

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A pathological feature of Type 2 diabetes is deposits in the pancreatic islets primarily composed of amylin (islet amyloid polypeptide). Although much attention has been paid to the expression and secretion of amylin, little is known about the enzymes involved in amylin turnover. Recent reports suggest that insulin-degrading enzyme (IDE) may have specificity for amyloidogenic proteins, and therefore we sought to determine whether amylin is an IDE substrate. Amylin-degrading activity co-purified with IDE from rat muscle through several chromatographic steps. Metalloproteinase inhibitors inactivated amylin-degrading activity with a pattern consistent with the enzymatic properties of IDE, whereas inhibitors of acid and serine proteases, calpains, and the proteasome were ineffective. Amylin degradation was inhibited by insulin in a dose-dependent manner, whereas insulin degradation was inhibited by amylin. Other substrates of IDE such as atrial natriuretic peptide and glucagon also competitively inhibited amylin degradation. Radiolabeled amylin and insulin were both covalently cross-linked to a protein of 110 kDa, and the binding was competitively inhibited by either unlabeled insulin or amylin. Finally, a monoclonal anti-IDE antibody immunoprecipitated both insulin- and amylin-degrading activities. The data strongly suggest that IDE is an amylin-degrading enzyme and plays an important role in the clearance of amylin and the prevention of islet amyloid formation.

A pathologic feature of as many as 90% of persons with Type 2 diabetes is the presence of islet amyloid deposits comprised predominantly of the peptide amylin, also known as islet amyloid polypeptide (reviewed in Ref. 1). These deposits are thought to contribute to pancreatic beta cell dysfunction, either by direct cytotoxicity or by reducing beta cell mass (2). Understanding the mechanisms of amyloid formation, and how fibril formation is prevented under normal conditions, is therefore of particular interest in the study of Type 2 diabetes. Because amylin is continually produced in non-diabetic humans with no particular interest in the study of Type 2 diabetes. Because amylin is continually produced in non-diabetic humans with no amyloid formation, the mere presence of amylin is not sufficient to cause fibril formation, a concept that has been supported in studies using transgenic mice expressing human amylin (3). Further studies have suggested that amyloid formation is associated with metabolic perturbations, such as hyperglycemia or high fat intake (4). Therefore, a shift in the amylin balance either by altering synthesis, secretion, or degradation could contribute to amyloid formation. Although there have been numerous studies regarding amylin synthesis and secretion, at present there have been few studies exploring the degradation of amylin.

The levels of proteins are regulated by both synthesis and degradation. The enzyme responsible for the intracellular degradation of insulin is insulin-degrading enzyme (IDE),1 also known as insulysin (E.C. 3.4.24.56). A metallothiolproteinase found primarily in the cytosol, IDE has been detected in lesser amounts in endosomes, peroxisomes, on the plasma membrane, and in an extracellular form (reviewed in Ref. 5). In addition to insulin, a number of other proteins have been identified as IDE substrates, including proteins structurally related to insulin such as proinsulin, insulin-like growth factor II, and relaxin (6), and seemingly unrelated peptides, such as atrial natriuretic peptide (ANP) and glucagon (7, 8). However, insulin, ANP, and glucagon all contain regions that can form beta-pleated sheets, and thus are amyloid-forming peptides (9). Thus, it has been proposed that, rather than displaying specificity for a primary sequence motif, IDE is specific for amyloidogenic peptides (8, 10). Indeed, recently the Alzheimer’s β-amyloid peptide was shown to be degraded by IDE, and IDE was implicated in regulating extracellular levels of β-amyloid peptide (11–13). Therefore, because amylin is an amyloid-forming peptide, it may also be a substrate for IDE. The studies presented here characterize an amylin-degrading activity purified from rat muscle and identify the degrading enzyme as IDE.

EXPERIMENTAL PROCEDURES

Human and rat amylin were from Bachem. 125I-Human amylin was from Peninsula Laboratories. In all cases, human amylin was diluted with deionized water from stock solutions immediately before use to minimize the spontaneous formation of amyloid fibrils. Because of variable amounts of contaminating 125I-labeled bovine serum albumin, in some cases 125I-amylin was purified by reverse-phase HPLC as described below. Biosynthetic human insulin and 125I-insulin (labeled on the A14 position) were generously provided by Ronald Chance and Bruce Frank, respectively, of Lilly Research Laboratories. The covalent

1 The abbreviations used are: IDE, insulin-degrading enzyme; ANP, atrial natriuretic peptide; NHS-ASA, N-hydroxysuccinimidyl-4-azido-

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valeric acid; E-64, trans-epoxysuccininy1-leucylamido14-guanidine/buf-

tane; PMSF, phenylmethylsulfonyl fluoride; ALLN, N-acetyl-Leu-Leu-

norleucinal; ACTH, adrenocorticotropic hormone; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
cross-linking reagent NHS-ASA was from Pierce. All chemicals were reagent grade or better.

**Rat Skeletal Muscle IDE Purification**—Homogenates of hind leg muscle from 100- to 120-g male Harlan Sprague-Dawley rats were prepared and purified by 30–60% ammonium sulfate precipitation and batchwise DEAE-Sephalac purification as described previously (14). The preparation was further purified by chromatography on DEAE-Sephalac, pentyl agarose, and chromatofocusing columns as described (15), except that dithiothreitol was omitted from the buffers and a Mono-P fast-protein liquid chromatography column was used as the chromatofocusing matrix. After concentration of the chromatofocusing peak using a microconcentrator with a 10-kDa cutoff (Centricon-10, Amicon), the sample was applied to a Bio-Gel A-0.5m (Bio-Rad) gel filtration column (1.5 x 84 cm) equilibrated with 50 mM HEPES, 0.15 M NaCl, pH 7.4. The peak of insulin-degrading activity was pooled and stored in aliquots at -70 °C until used. Protein was determined by the bicinchoninic acid assay (Pierce).

**Measurement of Amylin- and Insulin-degrading Activities**—The degradation of insulin and amylin were measured by the trichloroacetic acid solubility assay. All studies were performed in test tubes coated with bovine serum albumin to prevent adsorption of the substrates. An appropriate amount of enzyme was incubated with tracer 125I-insulin or 125I-amylin in 100 mM Tris, pH 7.5, for 15 min at 37 °C (reaction volume 0.5 ml). The reaction was terminated by adding 25 μl of 10% bovine serum albumin and 125 μl of 50% trichloroacetic acid. After chilling in an ice bath for 15 min, the tubes were centrifuged at 3000 g for 15 min, and the radioactivity in the supernatants and pellets was counted.

The degree of degradation is expressed as the percentage of trichloroacetic acid solubility counts.

**Reverse-phase HPLC Analysis**—Samples of intact and degraded 125I-amylin were applied to a Supelcosil ODS column (Supelco) equilibrated in 0.1% trifluoroacetic acid in water (Buffer A). The samples were eluted using a linear gradient of 0–60% 0.1% trifluoroacetic acid in acetonitrile (Buffer B) over 45 min, followed by 60–100% B over 15 min. Fractions of 1 ml were collected, and the radioactivity was measured.

**Covalent Cross-linking**—Partially purified IDE was covalently cross-linked to either 125I-amylin or 125I-insulin using the heterobifunctional amine/photoinactive cross-linker N-hydroxysuccinimidyl-4-azidosalicylic acid (NH4)-SO4 precipitation and insulin. (Table III). In the presence of increasing doses of unlabeled amylin or insulin (Fig. 1). Both amylin and insulin reduced 125I-amylin degradation in a dose-dependent manner (IC50 290 and 80 nM, respectively). Similarly, when 125I-insulin degradation was measured (Fig. 2), both competitor amylin and insulin decreased 125I-amylin degradation (IC50 160 and 27 nM, respectively) with a similar profile to that in Fig. 1, strongly suggesting that the same enzyme is responsible for degrading both amylin and insulin. To verify that the acid-soluble radioactivity was due to amylin degradation, 125I-amylin was incubated with purified IDE for 0 or 30 min, then analyzed by reverse-phase C18 chromatography (Fig. 3). Intact amylin eluted at ~41 min. After degradation by IDE, the disappearance of the amylin peak at 41 min was accompanied by the accumulation of three degradation products eluting at approximately 26, 31, and 35 min. When the peak areas were quantified, the degree of degradation measured by HPLC was similar to that estimated by trichloroacetic acid in the same sample (~30% degraded). In general, there was linear agreement between the two methods within the range of degradation tested (0–40%).

To determine whether the amylin-degrading activity is due to IDE, a panel of inhibitors was used to characterize the enzymatic properties of the amylin-degrading activity (Table II). The activity was greatly inhibited by N-ethylmaleimide, 1,10-phenanthroline, and bacitracin and inhibited to a lesser degree by EDTA and EGTA, consistent with the metallothiol-proteinase characteristics of IDE. Conversely, other cysteine proteinase inhibitors (E-64, leupeptin), inhibitors of acid proteases (pepsatin), serine proteases (aprotinin, leupeptin, PMSF) and calpains (ALLN), were ineffective at inhibiting amylin degradation. In addition, because ALLN at 1 mM is a potent proteasome inhibitor, degradation of amylin by the proteasome in this preparation can be ruled out. Taken together, the amylin-degrading enzyme has the properties of a neutral metallothiolproteinase and the inhibition profile is consistent with the enzymatic properties of IDE.

Because IDE degrades other proteins in addition to insulin, the ability of these substrates to competitively inhibit amylin degradation was examined (Table III). The IDE substrates insulin, glucagon, ANP, and ACTH all effectively inhibited the amylin-degrading activity. In addition to human amylin, rat amylin also inhibited human amylin degradation. In contrast, EGF, which binds IDE but with low affinity, had little effect, and insulin C-peptide, which is not a substrate, had no effect. Taken together, these data demonstrate that the amylin-degrading activity is consistent with the enzymatic characteristics of IDE.

To examine amylin-binding proteins in the IDE preparation, covalent cross-linking experiments were performed. Early ex-
experiments using traditional cross-linking reagents such as the homobifunctional amine-reactive disuccinimidyl suberate were unsuccessful when using amylin as the substrate. This may have been due to the lack of reactive groups on the amylin molecule, which contains a single amino acid with primary amines (the N-terminal lysine). Therefore, a heterobifunctional amine/photoreactive reagent (NHS-ASA) was used. Partially purified IDE (through the ammonium sulfate precipitation step) was incubated with either 125I-amylin or 125I-insulin, and associated proteins were cross-linked with NHS-ASA (Fig. 4). In the absence of NHS-ASA (Me2SO only), no cross-linking of labeled amylin or insulin was detected. In the presence of NHS-ASA, a radiolabeled band at approximately 110 kDa was readily detected using either 125I-amylin or 125I-insulin, and associated proteins were cross-linked with NHS-ASA (Fig. 4). This is consistent with amylin binding by IDE, which migrates on SDS-PAGE at 110 kDa. In the presence of either excess insulin or amylin, the cross-linking to the 110-kDa protein was abolished, indicating the specificity of the binding. No other specifically cross-linked proteins were detected. These data show that a protein at 110 kDa on SDS-PAGE binds both insulin and amylin and is consistent with amylin degradation by IDE.

To definitively establish IDE as the protease responsible for amylin degradation in this preparation, immunodepletion studies were performed. Purified IDE was incubated with the monoclonal IDE-specific antibody 9B12 at various concentrations, then the antibody was precipitated with anti-mouse IgG-coated Protein A-Sepharose. The precipitates were removed, and the supernatants were measured for residual amylin- and insulin-degrading activities (Fig. 5). The removal of IDE with increasing antibody concentration correlated with a loss of both amylin- and insulin-degrading activity. In contrast, an isotype-matched control antibody (anti-tubulin), even at concentrations as high as 25 μg/ml, did not affect either degrading activity.

**FIG. 1.** Amylin degradation is inhibited by excess insulin or amylin. The effect of increasing doses of unlabeled human insulin (closed symbols) or amylin (open symbols) on the degradation of 125I-amylin was determined. The data are expressed as the percentage of degradation in the absence of unlabeled hormone (mean ± S.E., for three independent experiments).

**FIG. 2.** Insulin degradation is inhibited by excess insulin or amylin. The effect of increasing doses of unlabeled human insulin (closed symbols) or amylin (open symbols) on the degradation of 125I-insulin was determined. The data are expressed as the percentage of degradation in the absence of unlabeled hormone (mean ± S.E., for three independent experiments).

**TABLE II**

| Inhibitor       | Concentration | Inhibition (%) |
|-----------------|---------------|----------------|
| Bacitracin      | 1 mg/ml       | 97.5           |
| N-Ethylmaleimide| 1 mM          | 95.6           |
| 1,10-Phenanthroline | 1 mM      | 97.4           |
| EDTA            | 1 mM          | 95.6           |
| EGTA            | 1 mM          | 35.7           |
| PMSF            | 1 mM          | 21.0           |
| Aprotinin       | 1 TIU/ml      | 22.4           |
| Pepstatin-A     | 0.1 mM        | 5.0            |
| Leupeptin       | 1 mM          | 0              |
| E-64            | 1 mM          | 7.9            |
| ALLN            | 1 mM          | 0              |

The degradation of 125I-amylin by purified IDE was measured in the absence and presence of protease inhibitors at the indicated concentrations. The data are expressed as percentage of inhibition of amylin-degrading activity in the absence of inhibitor.
Amylin degradation is competitively inhibited by IDE substrates

The degradation of 125I-amylin by purified IDE was measured in the absence and presence of the indicated peptides (all at 100 nM). The data are expressed as percentage of inhibition of amylin-degrading activity in the absence of additional peptide.

| Inhibitor       | Inhibition % |
|-----------------|-------------|
| Amylin (human)  | 24.5        |
| Amylin (rat)    | 42.7        |
| Insulin         | 72.2        |
| ACTH            | 89.3        |
| ANP             | 52.4        |
| Glucagon        | 44.2        |
| EGF             | 6.4         |
| C-peptide       | 0           |

**FIG. 4. Covalent cross-linking of 125I-amylin.** Partially purified IDE was incubated with 125I-amylin for 30 min, then the covalent cross-linking reagent NHS-ASA was used to analyze amylin binding proteins. The samples were resolved on 7.5% SDS-PAGE, and radioactivity was detected by phosphorimaging analysis.

**FIG. 5.** An anti-IDE antibody immunodepletes both insulin- and amylin-degrading activities. Purified IDE was incubated with the indicated concentration of either the monoclonal IDE-specific antibody 9B12 (closed symbols) or a control isotype-matched antibody specific for α-tubulin (open symbols). The antibodies were precipitated with anti-mouse IgG-coated Protein A-Sepharose, and the residual 125I-amylin (squares) or 125I-insulin (circles) degrading activities were determined. The data are expressed as the percentage of degradation in the absence of antibody (mean ± S.E., for three independent experiments).

**DISCUSSION**

The formation of amyloid deposits is a critical event in the pathogenesis of many diseases, including Alzheimer’s disease and Type 2 diabetes. Much of the research performed to date has focused on the expression of the amyloid-forming proteins. Little is known about the processes responsible for the turnover and clearance of amyloid-forming proteins. Recently, IDE was implicated in the degradation of the Alzheimer’s disease beta-amyloid peptide (11, 12). Further studies have suggested that IDE plays a role in the control of extracellular levels of Alzheimer’s beta-amyloid peptide (13, 16). Therefore, there is emerging evidence that a major role of IDE may be in the clearance of amyloid-forming peptides.

Amylin is co-produced and co-packaged with insulin by pancreatic beta cells. In as many as 90% of persons with Type 2 diabetes, amyloid plaques are found in the area of the beta cells, and contribute to beta cell dysfunction and death. The studies presented here identify IDE as an amylin-degrading enzyme. In a typical series of purification steps used to isolate IDE, amylin-degrading activity co-purified with insulin-degrading activity through all steps. The insulin-degrading activity was inhibited by amylin in a dose-dependent manner, whereas amylin-degrading activity was similarly inhibited by excess insulin, suggesting that both insulin and amylin are degraded by the same protease in the preparation. The inhibitor profile identifies the enzyme as a metallothiolproteinase with properties consistent with those reported previously for IDE. On the other hand, the amylin-degrading activity was not inhibited by serine or acid protease inhibitors nor by an inhibitor of calpains and proteasomes. The amylin-degrading proteinase had affinity for insulin, glucagon, ACTH, and ANP, but not for EGF or insulin C-peptide, all consistent with the specificity of IDE. Using a cross-linking reagent, the association of a 110-kDa protein with radiolabeled amylin was detected. This cross-linking was abolished in the presence of either excess insulin or amylin, indicating that the same protein binds both amylin and insulin. This finding was supported by the observation that radiolabeled insulin cross-linked to the 110-kDa protein was also eliminated with excess insulin or amylin, strongly suggesting that the 110-kDa insulin and amylin binding protein is IDE. Finally, a well-characterized monoclonal antibody directed against IDE co-immunodepleted both insulin- and amylin-degrading activity, providing definitive evidence that IDE is the amylin-degrading proteinase.

Because IDE is an amylin-degrading enzyme, it may play an important role in amylin homeostasis. It is important to remember that in species expressing amyloidogenic amylin (including humans), amylin is continually present, yet does not normally aggregate into amyloid deposits. Therefore, the mere presence of amylin does not predicate islet amyloid formation. This has been explored further in studies using transgenic animals. Although some lines of transgenic mice overexpressing human amylin displayed amyloid deposits (17, 18), others did not (3, 19–21), suggesting that elevated amylin alone may not be sufficient for amyloid formation. Therefore, a perturbation of some other element of amylin processing, such as amylin degradation, may be involved.

Amylin from a number of species, including human, spontaneously aggregates into amyloid fibrils as a result of β-pleated sheet formation around residues 20–29 of the primary sequence. Rodent amylin, on the other hand, does not form amyloid, presumably because proline residues in this area of the molecule alter secondary structure (22). In the studies presented here, IDE degraded both human and rat amylin. It would follow that the recognition motif for IDE is not the β-pleated sheet region itself, but the structure of the peptides in the non-amyloid-forming state. Indeed, most if not all studies of IDE and insulin have been performed under conditions in which insulin does not spontaneously form amyloid fibrils. The role of IDE may, therefore, be in the cleavage of peptides with the potential to form amyloid aggregates. In this case, IDE can be thought of as a scavenger of amyloidogenic peptides. Normally, a balance exists between deposition and degradation of the amyloidogenic peptide. When the levels of the peptide exceed the capacity of IDE to degrade them, either by increased expression of the peptide, or decreased expression or enzymatic activity of IDE, the balance is shifted from degradation to deposition. In the case of Type 2 diabetes, both insulin and amylin secretion are increased due to peripheral insulin resist-
ance. Because IDE has approximately 4-fold greater affinity for insulin than for amylin, amylin degradation will be proportionately impaired. The increased production and relative decrease in degradation may allow sufficient accumulation of amylin to cause islet amyloid formation.

The site of both synthesis of amylin and deposition of islet amyloid, the pancreatic beta cells, is a logical site of amylin degradation. An insulin-degrading enzyme consistent with the properties of IDE has been reported in islets and in a beta cell line (23). Furthermore, recent studies in our laboratory have suggested that IDE is responsible for amylin degradation in a beta cell line and that inhibition of IDE increases the formation of amyloid by exogenous human amylin (24). If this proves to be the case, then IDE would be a critical player in the prevention of amyloid formation by amylin and perhaps other amyloidogenic peptides. A new area of study focusing on turnover of amyloidogenic peptides could lead to novel therapeutic approaches in the treatment of amyloid diseases.

REFERENCES
1. Kahn, S. E., Andrikopoulos, S., and Verchere, C. B. (1999) Diabetes 48, 241–253
2. Johnson, K. H., O’Brien, T. D., Betsholtz, C., and Westermark, P. (1989) N. Engl. J. Med. 321, 513–518
3. Verchere, C. B., D’Alessio, D. A., Wang, S., Andrikopoulos, S., and Kahn, S. E. (1997) Horm. Metab. Res. 29, 311–316
4. Verchere, C. B., D’Alessio, D. A., Palmiter, R. D., Weir, G. C., Bonner-Weir, S., Baskin, D. G., and Kahn, S. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3492–3496
5. Duckworth, W. C., Bennett, R. G., and Hamel, F. G. (1998) Endocr. Res. 19, 608–624
6. Roth, R. A., Mesirow, M. L., Yokono, K., and Baba, S. (1984) Endocr. Res. 10, 101
7. Duckworth, W. C., and Kitabchi, A. E. (1974) Diabetes 23, 536–543
8. Muller, D., Baumeister, H., Back, F., and Richter, D. (1991) Eur. J. Biochem. 202, 285–292
9. Sipe, J. D. (1992) Annu. Rev. Biochem. 61, 947–975
10. Kurochkin, I. V. (1998) FEBS Lett. 427, 153–156
11. Goto, S., and Kurochkin, I. V. (1994) FEBS Lett. 345, 33–37
12. McDermott, J. R., and Gibson, A. M. (1997) Neurochem. Res. 22, 49–56
13. Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) J. Biol. Chem. 273, 32730–32738
14. Duckworth, W. C., Heinemann, M., and Kitabchi, A. E. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3698–3702
15. Shii, K., Yokono, K., Baba, S., and Roth, R. A. (1986) Diabetes 35, 675–683
16. Vekrellis, K., Ye, Z., Qiu, W. Q., Walsh, D., Hartley, D., Chesneau, V., Rosner, M. R., and Selkoe, D. J. (2000) J. Neurosci. 20, 1657–1665
17. Yagui, K., Yamaguchi, T., Kanatsuka, A., Shimada, F., Huang, C. I., Tokuyama, Y., Ohhasawa, H., Yamamura, K., Miyazaki, J., Mikata, A., Yoshida, S., and Makino, H. (1995) Eur. J. Endocrinol. 132, 487–496
18. Janson, J., Soeller, W. C., Roche, P. C., Nelson, R. T., Torcchia, A. J., Kreutter, D. K., and Butler, P. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7283–7288
19. Hoppenr, J. W., Verbeek, J. S., de Koning, E. J., Oosterwijk, C., van Hulst, K. L., Vasser-Vernay, H. J., Hoffnau, F. M., van Gaalen, S., Berends, M. J., Hackeng, W. H., Janze, H. S., Morris, J. F., Clark, A., Capel, P. J., and Lips, C. J. (1993) Diabetologia 36, 1258–1265
20. Fox, N., Schremeniti, J., Nishi, M., Ohagi, S., Chan, S. J., Heisserman, J. A., Westermark, G. T., Leckstrom, A., Westermark, P., and Steiner, D. F. (1993) FEBS Lett. 323, 40–44
21. D’Alessio, D. A., Verchere, C. B., Kahn, S. E., Hoagland, V., Baskin, D. G., Palmiter, R. D., and Ensinck, J. W. (1994) Diabetes 43, 1457–1461
22. Westermark, P., Enstrom, U., Johnson, K. H., Westermark, G. T., and Betsholtz, C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5036–5040
23. Bhathena, S. J., Timmers, K. I., Oie, H. K., Voyles, N. R., and Recant, L. (1985) Diabetes 34, 121–128
24. Bennett, R. G., Hamel, F. G., and Duckworth, W. C. (2000) in The Endocrine Society’s 82nd Annual Meeting, pp. 89, The Endocrine Society, Toronto, Canada