Published in final edited form as:

J Mol Biol. 2010 January 15; 395(2): 430. doi:10.1016/j.jmb.2009.10.072.

Molecular basis for the recognition and cleavages of IGF-II, TGF- α , and amylin by human insulin degrading enzyme

Qing Guo¹, Marika Manolopoulou¹, Yao Bian¹, Alexander B. Schilling², and Wei-Jen Tang^{1,3}

¹Ben-May Department for Cancer Research, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA

²Proteomics and Informatics Services Facility, University of Illinois at Chicago, 835 S. Wolcott Ave., Chicago, IL 60612

Abstract

Insulin-degrading enzyme (IDE) is involved in the clearance of many bioactive peptide substrates, including insulin and amyloid- β (A β), peptides vital to the development of diabetes and Alzheimer's disease, respectively. IDE can also rapidly degrade hormones that are held together by intra-molecular disulfide bond(s) without their reduction. Furthermore, IDE exhibits a remarkable ability to preferentially degrade structurally similar peptides such as the selective degradation of insulin-growth factor-II (IGF-II) and transforming growth factor- α (TGF- α) over IGF-I and epidermal growth factor (EGF), respectively. Here, we used high accuracy mass spectrometry to identify the cleavage sites of human IGF-II, TGF- α , amylin, reduced amylin, and A β by human IDE. We also determined the structures of human IDE-IGF-II and IDE-TGF- α at 2.3 Å and IDE-amylin at 2.9 Å. We found that IDE cleaves its substrates at multiple sites in a biased stochastic manner. Furthermore, the presence of a disulfide bond in amylin allows IDE to cut at an additional site in the middle of the peptide (aa 18-19). Our amylin-bound IDE structure offers insight into how the structural constraint from a disulfide bond in amylin can alter IDE cleavage sites. Together with NMR structures of amylin and the IGF and EGF families, our work also reveals the structural basis of how the high dipole moment of substrates complements the charge distribution of the IDE catalytic chamber for the substrate selectivity. In addition, we show how the ability of substrates to properly anchor their N-terminus to the exosite of IDE and undergo a conformational switch upon binding to the catalytic chamber of IDE can also contribute to the selective degradation of structurally related growth factors.

Introduction

Insulin degrading enzyme (IDE) is an 110 kDa zinc metalloprotease that belongs to the M16A family (EC 3.4.24.56, insulysin, or insulinase)^{1, 2}. Similar to other M16 family proteases, IDE has a conserved inverted zinc-binding sequence HEXXH in which the two histidines are involved in zinc ion coordination and one glutamate residue is involved in catalytic water activation^{1, 2, 3, 4}. Recent structural analysis revealed that human IDE consists of two roughly equally sized N- and C-terminal domains (IDE-N and IDE-C)³. IDE-N and IDE-C are homologous to each other and have an $\alpha\beta\alpha\beta$ -roll structure, shared among proteases within

© 2009 Elsevier Ltd. All rights reserved.

³Corresponding author, 773-702-4331 (Tel), 773-702-6260 (Fax), wtang@uchicago.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the M16 family, which include mitochondrial processing peptidase, pitrilysin, and mitochondrial presequence peptidase^{5; 6; 7}. IDE-N and IDE-C are joined by an extended 28 amino acid loop, and together they form an enclosed chamber to selectively enclose and cleave certain peptides. IDE was recently described as a prototypical cryptidase because of its ability to use a sizable catalytic chamber (crypt) to selectively recognize peptides targeted for proteolysis¹.

Since the discovery of IDE based on its ability to rapidly degrade insulin, IDE was shown to play a major role in the degradation and clearance of insulin *in vivo*^{1; 2; 8; 9}. This is consistent with the observation that IDE gene knockout mice have an elevated level of insulin and develop symptoms of diabetes, while single nucleotide polymorphisms of the IDE gene are associated with type 2 diabetes in humans^{10; 11}. IDE also plays a role in preventing formation of amyloid deposits by degrading amyloid- β (A β), a key peptide for the development of Alzheimer's disease^{1; 10; 12; 13; 14}. Outside of insulin and A β , several short peptides with molecular weights of 3–10 kDa have been shown to serve as substrates of IDE, such as insulin-like growth factor-II (IGF-II)^{15; 16}, amylin³, glucagon, transforming growth factor- α (TGF- α)¹⁷, and atrial natriuretic peptide (ANP)¹⁸.

The structural analysis of substrate-bound IDE has revealed several unique features of how IDE selectively degrades certain bioactive peptides^{1; 2; 3; 19; 20; 21}. IDE has a catalytic chamber with a total volume of approximately 16,000 Å³. The unique size of this chamber allows the enzyme to preferentially cleave peptides smaller than 80-amino acids long. In addition, the complementarity in charge and shape of this chamber with the substrate also determines the substrate selectivity as exemplified by the insulin-bound IDE structure²¹. Apart from these unique properties of the IDE catalytic chamber, IDE also has a highly conserved exosite located approximately 30 Å away from the zinc ion bound at the catalytic center¹⁹. The structures of IDE in complex with A β , glucagon, insulin, and bradykinin reveal that this exosite serves as an anchoring site for the N-terminals of IDE substrates^{3; 20; 21}. Such an interaction is hypothesized to serve as a molecular ruler, directing IDE to perform the initial cleavage at least 10 amino acids away from the N-terminal end of its longer substrates¹. In addition, the binding of the exosite with a shorter peptide substrate could reduce the apparent size of the catalytic chamber²⁰. This is postulated to facilitate the cleavage of short substrates of IDE such as 9 amino acid-long bradykinin. However, it remains unclear how IDE utilizes the properties of the catalytic chamber and exosite to recognize and selectively degrade substrates such as IGF-II, TGF- α , and amylin.

IDE exhibits preferential cleavage of the structurally related IGF and EGF proteins, peptide hormones that contain three disulfide bonds each^{1; 15; 16; 17; 22}. IGF-I and IGF-II are structurally and functionally related to insulin^{23; 24}. Together with insulin, this IGF family is involved in regulation of multiple biological processes including carbohydrate metabolism, growth, lifespan, development, and neoplasia^{25; 26}. NMR analysis reveals that the structure of IGF-II is similar to insulin and IGF-I²³. Similar to insulin, IGF-II is also a high affinity (~100 nM) substrate of IDE and is rapidly degraded by this enzyme¹. Paradoxically, IDE binds IGF-I with reduced affinity compared to IGF-II and does not effectively degrade this hormone. Epidermal growth factor (EGF) and TGF- α are structurally related growth factors that work in the endocrine, paracrine, and autocrine systems for various biological activities^{27; 28; 29}. Similar to the IGF family, IDE also selectively degrades TGF- α over EGF¹⁷. The sites where IDE cleaves IGF-II and TGF- α are mostly unknown, as is the molecular basis of substrate selectivity among members within the IGF family and the EGF family.

IDE prefers to degrade amyloidogenic peptides such as amylin, or islet amyloid polypeptide¹³, a 37 amino acid peptide containing a disulfide bond near the N-terminus, between cysteine 2 and cysteine 7. Amylin is normally produced by pancreatic β cells along

with insulin to control glucose homeostasis³⁰. Similar to A β , amylin can induce cell death of pancreatic β cells and may thus be involved in the development of diabetes^{30; 31}. We have previously identified the cleavage sites of amylin by IDE, as well as solved the structure of human IDE in complex with amylin in the presence of a reducing agent³. The reducing agent was required for the crystallization of human IDE protein. However, since a reduced environment breaks disulfide bonds, the molecular basis for the interaction of IDE with amylin that retains the disulfide bond remains elusive.

IDE's ability to degrade a wide variety of substrates that differ in length, structure, and biological properties is of great interest in the fields of diabetes and Alzheimer's research. Determining the mechanism of how IDE recognizes and degrades certain substrates over others as well as how it degrades amyloidogenic peptides is a crucial step in understanding how IDE affects the homeostasis of these peptides in the human body. In order to understand how IDE differentiates and degrades IGF-II, TGF- α , and amylin without breaking their disulfide bonds, we performed structural and biochemical studies on these IDE substrates. We took advantage of the high mass accuracy mass spectrometry to identify the products of IGF-II, TGF- α , and amylin digestion by IDE. To eliminate the need for a reducing agent during the crystallization of IDE, all 13 cysteines in human IDE were mutated to construct a fully functional, cysteine-free IDE^{20; 21}. Using this construct, we determined the structures of human IDE in complex with three disulfide-containing substrates: IGF-II, TGF- α , and amylin. Together with the NMR structures of these three substrates and other peptides within the same families, new insights emerge regarding how IDE uses its exosite and catalytic chamber to bind, unfold, and degrade these disulfide bond-containing substrates.

Results

Mass spectroscopy analysis of the degradation of IGF-II and TGF- α by IDE

IGF-II is a 67-amino acid growth-promoting hormone that plays a vital role in embryo development and epigenetic imprinting. TGF- α is a 50-amino acid long peptide that can induce epithelial cell development. These two peptides differ significantly in their primary sequences, secondary structure, shape, and surface charge distribution (Figure 1A, 1B)^{32; 33; 34}. However, like insulin, these two peptides are high potency inhibitors (60-180 nM, Figure 1C) for the degradation of a fluorogenic bradykinin-mimetic peptide, substrate V by human IDE. The IC₅₀ values are in agreement with the reported K_i values^{4; 16; 17; 22}. Thus, IDE binds IGF-II and TGF- α , two structurally diverse disulfide-bond containing peptides, with high affinity comparable to that of insulin.

IDE can effectively cleave both IGF-II and TGF- α but little is known regarding cleavage sites for each substrate. Both IGF-II and TGF- α are stabilized by three intra-molecular disulfide bonds. IDE is known to degrade its substrates without the assistance of disulfide bond isomerase activity and its proteolytic activity does not require the breakage of any disulfide bonds. To decipher the cleavage sites of IGF-II and TGF- α , we used liquid chromatography coupled with quadruple time-of-flight (LC-Q-TOF) MS. We identified 14 IGF-II fragments and 8 TGF- α fragments that were generated by 5-minute incubations with IDE followed by treatment with a reducing agent, DTT (Table 1). The identification of these fragments is based on the accurate match of the observed mass with the predicted mass of the precursor and b/y product ions. Figures 1D and 1E show sample mass spectra obtained for IGF-II and TGF- α , respectively. We also performed matrix assisted laser desorption/ionization-time of flight tandem mass spectrometry to gain better MS/MS coverage of these precursor ions and confirm the identity of these fragments (Table S1).

Similar to the cleavage pattern of insulin by IDE, we found that IDE preferentially cleaves several discrete regions of IGF-II and TGF- α (Figure 1A). Several of these sites are clustered

together. These include three pairs of cleavage sites on IGF-II, aa 36-37/37-38, aa 47-48/48-49, aa 63-64/64-65 and three pairs of cut sites on TGF- α , aa 14-15/15-16, aa 20-21/21-22, and aa 34-35/35-36. In one case, we also observed three cleavage sites that are adjacent to each other (aa 26-27, 27-28, 28-29) on IGF-II. This is consistent with the probabilistic mode of binding of these regions to the catalytic cleft for cleavage. Such biased stochastic mode is a commonly found feature of IDE substrate cleavage sites^{1; 3; 4; 21}. We did not observe cleavages near the N-terminus of either IGF-II or TGF- α (Figure 1A). This is consistent with the notion that IDE uses the exosite to anchor the N-terminals of both substrates; thus, no cleavage can occur at the first 10 amino acid residues of the N-terminal end. Interestingly, the cleavage sites of TGF- α defined using the accurate mass determination differ significantly from those determined by the blockage of Edman degradation of rat IDE-degraded [¹²⁵I]-TGF- α ³⁵. Such a discrepancy may be due to the methodology and/or source of IDE.

Mass spectrometry analysis of the degradation of amylin and A β (1-40) by IDE

Previously, we used MALDI-TOF mass spectrometry to assess the cleavage sites of amylin and A β (1-40) by IDE³. To more accurately determine the cleavage sites, we used Fourier Transform ion cyclotron resonance (FT-ICR) mass spectrometry for its high mass accuracy and sensitivity (Table 2 and Table S2). Based on the excellent match of the precursor ions to the predicted mass as well as the extensive coverage of b and y product ions from each precursor ion, we identified seven major cleavage sites when the non-reduced amylin was used (Table 2, Figure 2C). We also performed the cleavage reaction in the presence of a reducing agent, TCEP to assess whether the presence of disulfide bond in amylin affects the cleavage site of amylin by IDE. While six out of seven amylin cleavage sites were also cut by IDE when amylin was reduced by TCEP, we did not observe the cleavage between residue 18 and 19, which is located in the middle of amylin (Table 2, Table S2, Figure 2C). This indicates that the presence of disulfide bond can alter the cleavage site of amylin by IDE.

We have analyzed the initial cleavage sites of insulin by IDE to gain the mechanistic understanding of insulin degradation by IDE²¹. While IDE is known to cleave A β (1-40) at multiple sites, the preferred initial cleavage sites have not been defined yet^{3; 36}. To address this, we performed the digestion of A β (1-40) by IDE by varying the time of incubation and then assessed the cleavage products by FT-ICR MS/MS (Table 2). As expected, IDE cleaves A β (1-40) at multiple sites even only brief incubation (1-5 sec). Our mass spectrometry analysis reveals the preferred initial cleavage sites of A β (1-40) by IDE to be in the middle of A β (1-40) (aa 18-19, 19-20, and 20-21). The cleavages at aa14-15, aa15-16, and aa28-29 occurred less frequently (Table 2; Figure 2C). This data is consistent with our proposed yet unproven model that, upon the anchoring of N-terminus of A β to the IDE exosite, the middle segment of A β is preferably cut in a probabilistic manner for its initial cleavage, which would prevent the amyloid fiber formation by A β ¹.

Structures of amylin-, A β (1-42)-, IGF-II-, and TGF- α -bound IDE

Previous structural studies of IDE with various substrates have required the use of a reducing agent in the crystallization conditions, due to the presence of 13 cysteine residues in human IDE³. This reduced environment also breaks any disulfide bond(s) in the substrates, resulting in IDE-substrate complexes that may not reflect the native enzyme-substrate binding conformation. We have recently constructed a catalytically inactive, cysteine-free IDE (IDE-CF-E111Q) that can be crystallized without the reducing agent^{1; 21}. Using this mutant, we reexamined the structural basis of the interactions of IDE with amylin, a substrate containing one disulfide bond. We have solved the crystal structure of IDE-CF-E111Q in complex with amylin at 2.9Å resolution (Figure 2A; table 2). While we were unable to observe the disulfide bond in the previous IDE-amylin structure³, the current structure shows a density that fits well with residues K1-N3, C7-A8, and L12-L16, with a disulfide bond present between C2 and C7

(Figure 2B). However, we could not see density for the majority of the loop formed by aa 4-6 of amylin. Thus, we do observe two discrete segments of amylin, an N-terminal portion in the exosite and another segment in catalytic site of the enzyme; this spatial separation of the substrate is a common feature in IDE-substrate structures. It is worth noting that, similar to the structures of previous IDE-substrate complex, the averaged thermal B-factors of the amylin is higher than that of IDE (Table 3)^{3, 21}. This is presumably due to the structural heterogeneity of the substrates and/or less than 100% occupancy (despite the extensive effort in loading amylin to IDE in order to ensure the high occupancy, see method section). The same phenomenon was observed in the structures of IDE bound to A β (1-42), IGF-II, and TGF- α described below (Table 3).

Amylin and A β , another IDE substrate, are both amyloidogenic peptides and both can be effectively cleaved at multiple locations (Figure 2C). The structure of A β (1-40) in complex with IDE has previously been solved, as have the cleavage sites for amylin and A β by IDE³. A β (1-42) is a form of A β that forms amyloid plaque much faster than A β (1-40) and serves as a better marker for the progression of Alzheimer's disease. To learn whether IDE recognizes these two peptides differently, we also solved the structure of IDE in complex with A β (1-42) (Table 2). We found the binding of A β (1-42) to the exosite and catalytic site of IDE in this structure to be virtually identical to that of A β (1-40) in our A β (1-40)-bound IDE structure. Compared to the A β -bound IDE structure, we found a novel interaction of amylin with the exosite of IDE (Figure 2D). The disulfide bond formed by amylin C2 and C7 changes the local hydrogen bond network to the IDE β 12-strand compared to A β (Figure 2D). Most noticeably, the side chain of the N-terminus of amylin (K1) forms a salt bridge with that of IDE E341. This is different from other substrates of IDE, where the amino terminus of the substrates (i.e. A β) forms hydrogen bonds with the side chain of E341 and the main chain of L359.

The structure of amylin at the catalytic site of IDE shows that amylin is embedded into several hydrophobic pockets and amylin residues F15 and L16, positioned nearby the catalytic metal ion, are coordinated by H108, H112, and E189 of IDE (Figure 2E). These hydrophobic interactions include the contacts of amylin L12 with IDE F202 and W199, that of amylin F15 with IDE F141, and amylin L16 with IDE F115. This corresponds to the biochemical data available for amylin, which indicates that the cleavage between residues 15 and 16 is indeed one of the major cleavage sites (Figure 2C, 2E)³.

To further understand how IDE binds different substrates, the crystal structures of IDE-CF-E111Q complexed with IGF-II and TGF- α were determined at 2.3 Å resolution (Table 2). The overall fold of IDE in both cases is nearly identical to amylin-bound IDE (Figure 3A), with two discrete substrate segments clearly visible; one at the exosite and the other at the catalytic site of the respective structures (Figure 3B). Both main and side chains of the first 5 aa of IGF-II are visible while only the main chains of the first 3 aa of TGF- α are visible (Figure 3C). At the catalytic site, several residues of IDE located in domains 1 and 4 form a largely polar cavity with hydrophobic patches that interact with cleavage sites in both substrates (Figure 3D). At the catalytic site of IDE, IGF-II aa 13-18 and TGF- α aa 10-14 fit nicely into the electron density map, and the observed primary cleavage sites of these two substrates (T16-L17 of IGF-II and S11-H12 of TGF- α) appear poised for cleavage (Figure 3D).

Discussion

IDE cleaves multiple substrates that have various biological functions, including amylin, A β , IGF-II, and TGF- α . These substrates are diverse in their sequence, size, and structure. Based on accumulated structural and biochemical evidence, it has been proposed that the catalytic cycle and substrate specificity of IDE revolve around a conformational switch between the open and closed states of this enzyme, with interactions between potential substrates and the

IDE catalytic chamber stabilized by complementary electrostatic properties (Figure 4)^{1, 3}. We propose that IDE normally exists in an equilibrium between an open (IDE^O) and a closed (IDE^C) state³. In the open state, the negatively charged interior of the IDE-N domain and the positively charged interior of the IDE-C domain result in selective binding of ligands with charge distributions complementary to the electrostatic properties of the chamber (Figure 4A). IDE substrates IGF-II, TGF- α , and amylin also exhibit such a charge distribution (Figure 4B). Modeling the N-terminal end of IGF-II, TGF- α , or amylin in the exosite of IDE reveals charge complementarity between the substrate and the IDE chamber, as the negatively charged surfaces of IGF-II, TGF- α , and amylin fit well with the positively charged chamber wall of IDE-C.

The interactions between the substrate and the IDE chamber would appropriately position the flexible N-terminus of the substrate to bind at the exosite (Figure 4A). The binding of the substrate N-terminus to the IDE exosite and/or the charge complementarity of the catalytic chamber of IDE with the substrate would facilitate the switch of the open conformation to the closed conformation, which is the preferred state of IDE^{1, 3}. By comparing the native NMR structures of IGF-II, TGF- α , and amylin with those in the IDE-bound structures, we find that IDE binding also results in a substantial substrate conformational change (Figure 4C, 4D). The native conformation of the substrates does not position the putative cleavage sites close enough to the zinc-coordinated catalytic center of IDE, suggesting that a change in secondary structure must occur prior to catalysis. For three peptides, the most noticeable change was the conversion of IGF-II aa 13-18, TGF- α aa 10-14, and amylin aa 16-23 from α -helices to β -strands. These required conformational changes for IGF-II, TGF- α , and amylin are similar to those found in IDE in complex with A β and glucagon³. In addition, while IGF-II and amylin fit well in the catalytic cavity, the native conformation of TGF- α has an elongated shape and would extend beyond the walls of the chamber, potentially interfering with the switch from IDE^O to IDE^C (Figure 4B). Since IDE does cleave TGF- α , this offers further evidence that an induced-fit structural rearrangement of substrates would be required for binding and cleavage within the catalytic chamber of IDE. While speculative, structural rearrangement of the substrate, at least in some cases, must occur prior to the formation of IDE^C.

The structural rearrangement would allow certain sections of IDE substrates to fit into the catalytic cleft of IDE for cleavage in a biased stochastic manner. This is consistent with the multiple cleavage sites found by our mass spectrometry analysis on the degradation products of IGF-II, TGF- α , and amylin as well as the previous analysis of cleavage sites and products of IDE substrates. With the anchoring of the N-terminus of substrates to the exosite that is 30Å away from the catalytic center, the given section would like to be at least 10 residues away from its N-terminus for the initial cleavage. Indeed, we found that the preferred initial cleavages of A β (1-40) occurs in the middle of the peptide (aa18-21). In addition, this anchor-dependent cleavage also could explain our structural and mass spectrometry observations that the presence of a disulfide bond in amylin could constrain and alter the binding of amylin to the catalytic chamber of IDE so that an additional cleavage site between residues 18 and 19 of amylin can be cut. The initial cleavage can induce further conformational changes in the substrate, allowing for further cleavage inside the catalytic chamber. Such processive cleavages for the initial digestion of insulin allows IDE to cut it into two pieces and inactivate its function without breaking the disulfide bonds of insulin²¹. Ultimately, the cleavage could induce IDE to switch to the open conformation, leading to release of the proteolytic fragments and allowing for incorporation of other substrates or intermediate degraded fragments to start a new degradation cycle.

Our structures, together with the secondary structures and amino acid composition of the IGF and EGF family hormones, reveal that charge complementarity and exosite anchoring are used by IDE to selectively degrade certain members of those hormone families. IGF-II and IGF-I

share 64% sequence identity while TGF- α and EGF share 36% sequence identity (Figure 5A). Accordingly, their structures are highly homologous within each family (Figure 5B). The r.m.s.d of TGF/EGF is 2.536Å, while that of IGF-I/IGF-II is 1.298Å. However, the surface charge distributions between peptides in the same family differ. TGF- α has a much higher dipole moment (200 Debyes) than that of EGF (90 Debyes) and, compared to EGF, has better charge complementarity for the catalytic chamber of IDE (Figure 5B). IGF-II has a clear dipole charge distribution (330 Debyes) that could complement the charged catalytic chamber of IDE (Figure 5B)³⁷. On the other hand, despite having only a slightly lower dipole moment than IGF-II (300 Debyes, Figure 5C) the surface charge distribution of IGF-I does not coordinate well with the IDE chamber. Thus, within this growth family, the dipole moment and charge complementarity could be used as the parameters to predict susceptibility to IDE.

Currently, the role played by the specific substrate sequences in determining IDE selectivity is unclear. However, it is worth noting that IGF-I residues R55 and R56 would form a positively charged region that would contact the positively charged chamber of IDE-C, decreasing the binding affinity of IGF-I. The corresponding residues in IGF-II, A55 and L56, are non-polar. The same situation can be observed from the comparison of TGF- α and EGF. EGF contains positively charged R45 and R48, which would contact IDE-C and can interfere with the first step in IDE-substrate binding as outlined in our model.

Amylin and A β are both amyloidogenic peptides, exhibit bipolar surface charge distributions and share 36% sequence similarity. Comparison of NMR structures of these peptides reveals that amylin has a high dipole moment (500 Debyes), allowing it to complement well with the charge distribution of the catalytic chamber of IDE (Figure 5B,C). However, such charge complementarity is not observed for A β . Furthermore, these two peptides are much smaller compared to the IGF and EGF family peptides, so that they are likely to have more flexibility in their interaction with the IDE chamber. Thus, it is difficult to assess the relative contribution of charge distribution and conformational flexibility for the binding and degradation of A β and amylin by IDE.

A common feature between the amylin-, IGF-II-, and TGF- α -bound IDE structures is the interaction of the substrate's N-terminus with the IDE exosite. Indeed, this feature has also been observed in our previously reported IDE-substrate structures, indicating that this is an important interaction for IDE-substrate binding. In comparing the NMR structures of IGF-II and IGF-I, we see that both have a relatively stable helix domain in the middle, while the conformations of the N- and C-terminals vary greatly (Figure 5C). A significant difference can be seen at the N-terminus of IGF-II, which is longer and more flexible than that of IGF-I. A longer and more flexible N-terminus can also be observed for TGF- α compared to EGF and A β compared to amylin. We hypothesize that a longer and more flexible N-terminal increases the possibility that the substrate will make the appropriate contacts for anchoring to the IDE exosite. In addition to the charge repulsion from the positive patches found in IGF-I and EGF, the failure of the N-termini of these growth factors to reach the exosite of IDE would also help to explain why IDE preferentially degrades IGF-II over IGF-I and TGF- α over EGF. Differences in the N-terminal may also explain why A β has a higher binding affinity than amylin. However, their structural dissimilarity and the bulkiness of the loop formed by the disulfide bond of amylin may also play a role in determining binding affinity.

Our biochemical assays and structures expand the repertoire of substrates that IDE is known to degrade and our knowledge of the IDE-substrate binding mechanism. Our comparative analyses of the IGF and EGF hormone families revealed that charge distribution and flexibility of the substrate N-terminus are important factors in IDE-substrate recognition and cleavage. Furthermore, proper placement of substrates in relation to the IDE catalytic site requires the requisite structural change of the substrates. Additional work, such as mutagenesis studies and

N-terminal truncation/elongation, will be needed to verify our hypotheses. However, this study provides key information for the engineering of substrates with altered binding affinities to IDE for therapeutic applications.

Methods

Protein expression and purification

The expression vectors for cysteine free human IDE (IDE-CF; C110L, C171S, C178A, C257V, C414L, C573N, C590S, C789S, C812A, C819A, C904S, C966N, C974A) and the catalytically inactive IDE-CF-E111Q mutants were created as described²¹. Wild type human IDE and IDE mutants were expressed in *E. coli* Rosetta (DE3) cells (at 25 °C and 19 hours, IPTG induction) and purified by Ni-NTA, Source-Q, and Superdex S-200 columns as described²¹. Insulin was purchased from RayBiotech, IGF-II and TGF- α were from Peprotech, and amylin amide was from Bachem.

Mass Spectrometry analysis for TGF- α and IGF-II

Enzyme reactions were carried out at 37 °C by mixing 5 μ l of 1 mg/ml TGF- α , and IGF-II in 20 mM HEPES buffer (pH 7.2) with 5 μ l IDE protein in an enzyme to substrate molar ratio of 1:50. Reactions were stopped by the addition of 30 μ l stop solution (170 mM EDTA, 0.07% TFA). For MALDI-TOF-MS, mass spectra were obtained either in linear or positive reflector mode using a Voyager 4700 MALDI/TOF mass spectrometer (Applied Biosystems).

For QTOF-MS2 a 0.3 μ L aliquot of IDE-digested IGF-II and TGF- α were first incubated with 300 mM DTT for 30 minutes and then injected onto an Agilent 1100 LC system attached to an Agilent 6520 QTOF-MS equipped with a Chip-Cube nano-flow interface. A chip packed with Zorbax C18SB (75 μ m id $\times$ 43 mm length, Agilent Technologies G4240-62001) was used to trap and elute the peptides, which were run using a gradient as follows: 0.1% formic acid in water is held at 100% for 5 minutes and then changed to 45% of a 10:90 mix of 0.1% formic acid in Water:Acetonitrile over 20 minutes, then changed to 75% of this mix over 10 minutes. The flow rate of the chromatography segment of the chip is 200 nL/min and that of the enrichment (trapping) segment is 3 μ L/min. Enrichment is conducted for 5 minutes at 100% 0.1% formic acid in water. The QTOF-MS is run with the following parameters: MS1 scan range 300-2000 m/z, MS/MS scan range 50-3000 m/z., MS1 scan rate 8.4 scan per second, MS/MS scan rate 2 scans per second. Positive ion mode was used throughout the run with source Vcap of -1875 V, fragmentor voltage of 175 V, drying gas temperature of 325 °C, and a gas flow rate of 5 L/min. For MS/MS an isolation width of 4 amu was used to filter selected precursor ions into the collision cell, which had a ramped collision energy using a slope of 3 V/100 amu and an offset of 2V. All data was collected as centroided to conserve disk space, and a reference compound simultaneously infused into the spray chamber with m/z of 1221.99 was used to automatically recalibrate each spectrum as it was recorded.

The data acquired this way was subsequently qualitatively analyzed by using the molecular feature extractor (MFE) tool built into the MassHunter (Agilent Technologies version B.03) resulting in the detection of all chromatographed peptides listed by monoisotopic mass. The results of these extractions were exported to Microsoft Excel. Masses detected were compared with lists of possible peptides derived from subsequences of the original protein subject to a minimum mass agreement of 5 ppm between detected peptides and predicted peptides.

Mass Spectrometry Analysis for amylin and reduced amylin

Enzyme reactions were carried out at 37 °C by mixing 5 μ l of 1 mg/ml amylin in 20 mM HEPES buffer (pH 7.2) with 5 μ l IDE protein in an enzyme to substrate molar ratio of 1:50 with or without 10mM TCEP added. Reactions were stopped after 5 minute incubation by the addition

of 30 μ l stop solution (170 mM EDTA, 0.07% TFA). The digested samples were then analyzed using FTICR mass spectrometry. To do so, amylin samples (12 μ l) were injected into a nano RP-HPLC system (Dionex), with a C18 analytical column (Agilent). Peptides were eluted from the nano column with a linear gradient of 5-95% acetonitrile in 0.1% formic acid and sprayed into a LTQ-FT tandem MS instrument (Thermo Scientific). Spectra were acquired using positive ion nano ESI mode with the FTICR acquiring precursor spectra from 200-2000 m/z. For tandem MS, precursor ions were fragmented by collision-induced dissociation (CID). MS/MS spectra were acquired in a data dependent manner from the 5 most intense precursor ions of each FTICR MS scan. The RAW data files are processed by Xtract™ function in Xcalibur™ (Thermo Fisher Scientific) to generate reduced data files containing the deconvoluted masses and intensities for MS spectra.

Protein crystallization and structure determination

IDE-CF-E111Q in complex with IGF-II, TGF- α , amylin, or A β (1-42) was made after 5 cycles of protein-substrate complex formation and separation by gel filtration to ensure the high occupancy as described^{3, 21}. The substrate bound IDE was crystallized by hanging drop vapor diffusion at 18 °C, using 1 μ l of protein (16-20 mg/ml) and 1 μ l of mother liquor (10-13% PEG MME 5000, 100 mM HEPES pH 7.0, 4-14% Tacsimate, 10% dioxane). Clusters of needle crystals appeared in 3 to 5 days and were equilibrated in cryo-protective buffer containing 30% glycerol and mother liquor and were flash frozen in liquid nitrogen. Diffraction data were collected at 100K at the Advance Photon Source 14-BM-C and 19-ID beamlines at Argonne National Laboratory. The data sets were processed using HKL2000³⁸. The structures were solved by molecular replacement (Phaser³⁹) using the IDE portion of A β -bound IDE-E111Q structure as a search model (PDB: 2G47³). Structure refinement and rebuilding were performed using REFMAC and Coot^{38, 40}. The extra electron density at the catalytic chamber of IDE in the structures of IDE in complex with TGF- α , IGF-II, amylin, and A β (1-42) were clearly visible based on σ_A -weighted Fo-Fc map calculated by CNS⁴¹ and manually built. The refinement statistics are summarized in Table 2. Figures were generated using PyMol⁴² and CCP4MG³⁹. The volumes of the catalytic chamber of IDE and that of insulin were calculated using VOIDOO⁴³ and shape complementarity was calculated as described⁴⁴.

Enzymatic Competition Assay

Enzyme activities were assayed using a fluorogenic bradykinin-mimetic substrate of IDE, substrate V (7-Methoxycoumarin-4-yl-acetyl-RPPGFSAFK-2,4-dinitrophenyl, R&D Systems) ^{3, 19, 20, 21}. Competition reactions were carried out at 37 °C by mixing 90 μ l of 0.5 μ M substrate V in 50 mM potassium phosphate buffer (pH 7.3) and 10 μ l of human IGF-II or TGF- α (Peprotech). The reactions were initiated by the addition of 5 μ l of 0.2 mg/ml IDE protein. The substrate V degradation was assessed by monitoring fluorescence intensity for 10 min every 20 sec on a Tecan Safire² microplate reader (I_{ex} = 327 nm, I_{em} = 395 nm).

Accession codes

The coordinates for IGF-II-, TGF- α -, amylin-, and A β (1-42)-bound IDE have been deposited in RCSB protein data bank under accession codes 3E4Z, 3E50, 3HGZ, and 2WK3, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the staff of SBC and BioCars at the Advanced Photon Source, Argonne National Laboratory for help in data collection, Erik Johnson for the synthetic A β (1-42) peptide, and Ray Hulse for the technical assistance.

This work was supported by NIH GM81539 to W.-J. Tang. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under contract No. W-31-109-ENG-38. Use of the proteomics and informatics services facility was supported by the Chicago Biomedical Consortium.

Abbreviations

IDE	insulin degrading enzyme
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
TGF- α	tumor growth factor- α
EGF	epidermal growth factor
A β	amyloid- β

References

1. Malito E, Hulse RE, Tang WJ. Amyloid beta-degrading cryptidases: insulin degrading enzyme, presequence peptidase, and neprilysin. *Cell Mol Life Sci* 2008;65:2574–85. [PubMed: 18470479]
2. Hulse RE, Ralat LA, Tang WJ. Structure, function, and regulation of insulin-degrading enzyme. *Vitam Horm* 2009;80:635–48. [PubMed: 19251053]
3. Shen Y, Joachimiak A, Rosner MR, Tang WJ. Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature* 2006;443:870–4. [PubMed: 17051221]
4. Duckworth WC, Bennett RG, Hamel FG. Insulin degradation: progress and potential. *Endocr Rev* 1998;19:608–24. [PubMed: 9793760]
5. Johnson KA, Bhushan S, Stahl A, Hallberg BM, Frohn A, Glaser E, Eneqvist T. The closed structure of presequence protease PreP forms a unique 10,000 Angstroms³ chamber for proteolysis. *Embo J* 2006;25:1977–86. [PubMed: 16601675]
6. Taylor AB, Smith BS, Kitada S, Kojima K, Miyaura H, Otwinowski Z, Ito A, Deisenhofer J. Crystal structures of mitochondrial processing peptidase reveal the mode for specific cleavage of import signal sequences. *Structure* 2001;9:615–25. [PubMed: 11470436]
7. Maskos, K. pitrilysins/inverzincins.. In: Messerschmidt, A.; Dode, W.; Cygler, M., editors. *Handbook of metalloproteins*. Vol. 3. John Wiley & Sons; 2004. p. 190-198.
8. Fawcett J, Permana PA, Levy JL, Duckworth WC. Regulation of protein degradation by insulin-degrading enzyme: analysis by small interfering RNA-mediated gene silencing. *Arch Biochem Biophys* 2007;468:128–33. [PubMed: 17964527]
9. Mirsky IA, Broh-Kahn RH. The inactivation of insulin by tissue extracts; the distribution and properties of insulin inactivating extracts. *Arch Biochem* 1949;20:1–9. [PubMed: 18104389]
10. Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci U S A* 2003;100:4162–7. [PubMed: 12634421]
11. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, Balkau B, Heude B, Charpentier G, Hudson TJ, Montpetit A, Pshezhetsky AV, Prentki M, Posner BI, Balding DJ, Meyre D, Polychronakos C, Froguel P. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 2007;445:881–5. [PubMed: 17293876]
12. Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP, Selkoe DJ. Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron* 2003;40:1087–93. [PubMed: 14687544]
13. Kurochkin IV. Insulin-degrading enzyme: embarking on amyloid destruction. *Trends Biochem Sci* 2001;26:421–5. [PubMed: 11440853]
14. Kim M, Hersh LB, Leissring MA, Ingelsson M, Matsui T, Farris W, Lu A, Hyman BT, Selkoe DJ, Bertram L, Tanzi RE. Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families. *J Biol Chem* 2007;282:7825–32. [PubMed: 17244626]

15. Keller S, Schmid C, Zapf J, Froesch ER. Inhibition of insulin degradation by insulin-like growth factors I and II in human hepatoma (HepG2) cells. *Acta Endocrinol (Copenh)* 1989;121:279–85. [PubMed: 2549756]
16. Misbin RI, Almira EC, Duckworth WC, Mehl TD. Inhibition of insulin degradation by insulin-like growth factors. *Endocrinology* 1983;113:1525–7. [PubMed: 6352249]
17. Gehm BD, Rosner MR. Regulation of insulin, epidermal growth factor, and transforming growth factor-alpha levels by growth factor-degrading enzymes. *Endocrinology* 1991;128:1603–10. [PubMed: 1847863]
18. Muller D, Baumeister H, Buck F, Richter D. Atrial natriuretic peptide (ANP) is a high-affinity substrate for rat insulin-degrading enzyme. *Eur J Biochem* 1991;202:285–92. [PubMed: 1836994]
19. Im H, Manolopoulou M, Malito E, Shen Y, Zhao J, Neant-Fery M, Sun CY, Meredith SC, Sisodia SS, Leissring MA, Tang WJ. Structure of substrate-free human insulin-degrading enzyme (IDE) and biophysical analysis of ATP-induced conformational switch of IDE. *J Biol Chem* 2007;282:25453–63. [PubMed: 17613531]
20. Malito E, Ralat LA, Manolopoulou M, Tsay JL, Wadlington NL, Tang WJ. Molecular bases for the recognition of short peptide substrates and cysteine-directed modifications of human insulin-degrading enzyme. *Biochemistry* 2008;47:12822–34. [PubMed: 18986166]
21. Manolopoulou M, Guo Q, Malito E, Schilling AB, Tang WJ. Molecular basis of catalytic chamber-assisted unfolding and cleavage of human insulin by human insulin degrading enzyme. *J Biol Chem* 2009;284:14177–88. [PubMed: 19321446]
22. Roth RA, Mesirow ML, Yokono K, Baba S. Degradation of insulin-like growth factors I and II by a human insulin degrading enzyme. *Endocr Res* 1984;10:101–12. [PubMed: 6389104]
23. Terasawa H, Kohda D, Hatanaka H, Nagata K, Higashihashi N, Fujiwara H, Sakano K, Inagaki F. Solution structure of human insulin-like growth factor II; recognition sites for receptors and binding proteins. *EMBO J* 1994;13:5590–7. [PubMed: 7527339]
24. Cooke RM, Harvey TS, Campbell ID. Solution structure of human insulin-like growth factor 1: a nuclear magnetic resonance and restrained molecular dynamics study. *Biochemistry* 1991;30:5484–91. [PubMed: 2036417]
25. Broughton S, Partridge L. Insulin/IGF-like signalling, the central nervous system and aging. *Biochem. J* 2009;418:1–12. [PubMed: 19159343]
26. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008;8:915–28. [PubMed: 19029956]
27. Harvey TS, Wilkinson AJ, Tappin MJ, Cooke RM, Campbell ID. The solution structure of human transforming growth factor alpha. *Eur J Biochem* 1991;198:555–62. [PubMed: 2050136]
28. Kohda D, Inagaki F. Three-dimensional nuclear magnetic resonance structures of mouse epidermal growth factor in acidic and physiological pH solutions. *Biochemistry* 1992;31:11928–39. [PubMed: 1445923]
29. Schneider M, Wolf E. The epidermal growth factor receptor ligands at a glance. *J. Cell. Physiol* 2009;218:460–6. [PubMed: 19006176]
30. Haataja L, Gurlo T, Huang CJ, Butler PC. Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis. *Endocr Rev* 2008;29:303–16. [PubMed: 18314421]
31. Höppener JW, Ahrén B, Lips CJ. Islet amyloid and type 2 diabetes mellitus. *N Engl J Med* 2000;343:411–9. [PubMed: 10933741]
32. Chothia C, Lesk AM, Dodson GG, Hodgkin DC. Transmission of conformational change in insulin. *Nature* 1983;302:500–5. [PubMed: 6339948]
33. Moy FJ, Li YC, Rauenbuehler P, Winkler ME, Scheraga HA, Montelione GT. Solution structure of human type-alpha transforming growth factor determined by heteronuclear NMR spectroscopy and refined by energy minimization with restraints. *Biochemistry* 1993;32:7334–53. [PubMed: 8338831]
34. Torres AM, Forbes BE, Aplin SE, Wallace JC, Francis GL, Norton RS. Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. *J Mol Biol* 1995;248:385–401. [PubMed: 7739048]
35. Hamel FG, Gehm BD, Rosner MR, Duckworth WC. Identification of the cleavage sites of transforming growth factor alpha by insulin-degrading enzymes. *Biochim Biophys Acta* 1997;1338:207–14. [PubMed: 9128138]

36. Leissring MA, Lu A, Condrón MM, Teplow DB, Stein RL, Farris W, Selkoe DJ. Kinetics of amyloid beta-protein degradation determined by novel fluorescence- and fluorescence polarization-based assays. *J Biol Chem* 2003;278:37314–20. [PubMed: 12867419]
37. Felder CE, Prilusky J, Silman I, Sussman JL. A server and database for dipole moments of proteins. *Nucleic Acids Res* 2007;35:W512–21. [PubMed: 17526523]
38. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 1997;53:240–55. [PubMed: 15299926]
39. Potterton E, McNicholas S, Krissinel E, Cowtan K, Noble M. The CCP4 molecular-graphics project. *Acta Crystallogr D Biol Crystallogr* 2002;58:1955–7. [PubMed: 12393928]
40. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004;60:2126–32. [PubMed: 15572765]
41. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 1998;54(Pt 5):905–21. [PubMed: 9757107]
42. Delano, WL. Pymol. 2002. Pymol.sourceforge.net
43. Kleywegt GJ, Jones TA. Detection, delineation, measurement and display of cavities in macromolecular structures. *Acta Crystallogr D Biol Crystallogr* 1994;50:178–85. [PubMed: 15299456]
44. Lawrence MC, Colman PM. Shape complementarity at protein/protein interfaces. *J Mol Biol* 1993;234:946–50. [PubMed: 8263940]
45. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A* 2001;98:10037–41. [PubMed: 11517324]

Figure 1. Characterization of the degradation of IGF-II and TGF- α by IDE

(A) Primary sequences of human IGF-II, TGF- α and insulin. Disulfide bonds are shown by a line connecting two cysteines. Arrows on IGF-II and TGF- α depict the cleavage sites by IDE from this analysis while the cleavage sites of insulin are as reported²¹. The underlined amino acids are observed in the crystal structures of substrate-bound IDE. (B) Comparison of secondary structure (left) and electrostatic surface representation (right) of IGF-II, TGF- α , and insulin. PDB accession codes of IGF-II, TGF- α , and insulin are 1IGL, 1YUF, and 1G7A, respectively. The molecular surface is colored as calculated by APBS45 (<-6 kT in red, 0 kT in white, and > +6 kT in blue). (C) Inhibition of the IDE-mediated degradation of the fluorogenic substrate V (a bradykinin-mimetic) by insulin, TGF- α and IGF-II. IDE (1 μ g) was

mixed with substrate V (450 nM) in the presence of indicated concentrations of insulin, TGF- α , and IGF-II and fluorescence intensity was monitored for 10 minutes at 37°C. Results (means $\pm$ S.D.) are representative of three independent experiments performed in duplicate. (D) and (E) show the representative MS/MS spectrum for N-terminal regions of IGF-II and TGF- α , respectively. The ESI-tandem mass spectrum of the 529.229 ion from IDE-digested TGF- α at m/z 1584.668 (left) and the 976.469 ion from IDE-digested IGF-II at m/z 1950.936 (right); The amino acid sequence and ion identification of the tandem mass spectrum is shown and experimentally observed ions are labeled in the sequence.

Figure 2. Structures of IDE in complex with amylin

(A) Global view of the structure of amylin-bound IDECF-E111Q monomer. IDE-N and IDE-C are colored green and cyan, respectively. Amylin is in the stick representation colored in orange. (B) Stereo view of composite omit map (purple) of the IDE-bound amylin is contoured at 1.5 σ . Oxygen, nitrogen, and carbon atoms of amylin are shown in red, blue, and orange, respectively. (C) Primary sequences of amylin, reduced amylin and A β (1-40). The arrows on the amylin depict the cleavage sites by IDE as experimented and the arrows on A β (1-40) depict the cleavage sites by IDE after short (1-5 second) incubation. The minor cleavage sites on A β (1-40) are marked with the shorter arrows. The underlined amino acids with the scissor bond marked by a red arrow are observed in the crystal structures of substrate-bound IDE. (D) Detailed interaction of the N-terminus of non-reduced amylin (left), reduced amylin (middle) and A β (1-42) (right) with the exosite of IDE. (E) Detailed interaction of amylin with the IDE catalytic site. The color of IDE residues corresponds to the respective color of IDE domain in figure 2A. The PDB codes for structures of amylin-bound IDE-CF-E111Q, reduced amylin-bound IDE-E111Q, and A β (1-42)-bound IDE-CF-E111Q are 3HGZ, 2G48, and 2WK3, respectively.

Figure 3. Structures of IDE in complex with IGF-II and TGF- α

(A) Global view of the structure of IGF-II bound (top) and TGF- α bound (bottom) IDE-CF-E111Q monomer. IDE-N and IDE-C are colored green and cyan as Figure 1. IGF-II and TGF- α are colored orange. (B) Composite omit maps (blue) of IGF-II and TGF- α are contoured at 1.5σ . The substrates are colored orange. (C) The detailed interaction of the N-terminus of IGF-II and TGF- α with the exosite site of IDE. (D) Detail interaction of IDE catalytic chamber with IGF-II and TGF- α . Atoms oxygen, nitrogen, and carbon of substrates are shown in red, blue, and orange, respectively.

Figure 4.

Analysis of IDE's binding with IGF-II, TGF- α , and amylin. (A) A model of how IDE binds, unfolds, and degrades its substrate. IDE has two conformational states: the open state, IDE^o and the closed state IDE^c. The IDE-N and IDE-C are depicted as green and cyan, respectively. IGF-II (PDB code 1IGL) is depicted as red cartoon. IDE^o is theoretically modeled based on the substrate free *E. coli* pitrilysin structure (1Q21), IDE^c corresponds to the atomic coordinate of IDE-CF-E111Q-IGFII (3E4Z). The detailed description of the mechanism is in the discussion. (B) Shape and surface charge distribution of IGF-II (left), TGF- α (middle) and amylin (right) modeled in the catalytic chamber of IDE. (C) Comparison of IGF-II (left), TGF- α (middle) and amylin (right) in their free forms (transparent grey) with IDE-bound forms

(red). The segments in the free forms of IGF-II and TGF- α corresponding to IDE-bound forms are colored in transparent red. The arrows indicate the cleavage sites and the disulfide bonds are colored in yellow. (D) Comparison of IDE-bounded IGF-II (red), TGF- α (green), amylin (blue) and A β (1-42) (orange) in stick model.

Figure 5.

Structural comparison of IGF, EGF, and amyloidogenic peptides. (A) Sequence alignment of the IGF-II/IGF-I and TGF- α /EGF. (B) Comparison of the electrostatic surface of IGF-II (1IIGL), IGF-I (3GF1), TGF- α (1YUF), EGF (1EPH), amylin (2KB8) and A β (1-40) (1AML). The molecular surface is colored as calculated by APBS⁴⁵ (<-6 kT in red, 0 kT in white, and $>+6$ kT in blue). The dipole moment of these peptides was the average from distinct NMR solution structures (10 for IGF-I, 20 for IGF-II, 16 for TGF- α , 10 for EGF, 20 for A β (1-40), and 30 for amylin)³⁷. (C) NMR solution structures of IGF-II, IGF-I, TGF- α , EGF, amylin and A β (1-40).

Table 1Summary of IDE-degraded fragments of IGF-II and TGF- α ^a.

IGF-II	Observed (M+H)	Calculated (M+H)	Δ ppm
1-16	1709.796	1709.793	1.67
1-18	1950.936	1950.935	0.42
17-26	1140.543	1140.539	3.64
17-27	1303.602	1303.603	0.51
17-28	1450.670	1450.675	3.49
19-28	1209.528	1209.533	1.65
37-48	1453.692	1453.690	1.28
37-67	3505.657	3505.659	0.57
38-48	1297.590	1297.591	0.19
38-67	3349.556	3349.573	5.07
49-63	1654.769	1654.770	0.11
49-64	1725.807	1725.809	1.40
48-67	2217.045	2217.052	3.36
49-67	2069.976	2069.980	2.13
TGF- α	Observed (M+H)	Calculated (M+H)	Δ ppm
1-11	1218.497	1218.498	0.53
1-14	1584.663	1584.668	3.64
1-15	1731.731	1731.738	4.04
1-17	1981.809	1981.811	0.94
12-21	1179.459	1179.458	0.85
35-50	1697.794	1697.795	0.59
36-50	1560.735	1560.739	2.56
41-50	1097.529	1097.534	4.56

^a Assignment of the most prominent peaks generated after 5-minute incubation with IDE using Q-TOF MS/MS.

Table 2Ion identification of major IDE-degraded fragments of amylin and A β (1–40).

Amylin ^a	Observed (M+H)	Calculated (M+H)	Δ ppm	Observed b/y Ions ^b
1-15 (C-C) ^c	1639.7677	1639.7873	13.2	b7-b13, y5-6
16-27	1271.6722	1271.6936	16.9	b3-b5, b7-b11, y7-y9
16-28	1358.7054	1358.7257	14.9	b3, b5-b11, y6-y7, y9-y10
19-34	1554.7352	1154.7588	20.5	b4-14, y6, y11-y13
23-34	1152.6000	1152.6089	7.7	b2-b11, y4-y10
23-36	1367.6867	1367.6995	9.3	b3-b6, b8-b13, y2-y11

Amylin ^a (+TCEP)	Observed (M+H)	Calculated (M+H)	Δ ppm	Observed b/y Ions ^b
16-27	1271.6741	1271.6936	15.3	b2-b5, b7-b11, y5, y7-y9
16-28	1358.7127	1358.7257	9.6	b3-b11, y6-y7, y11
16-37	2281.1128	2281.1207	3.5	b5, b7-b10, y5-y10, y12-y17
23-34	1152.6000	1152.6089	7.7	b3-b11, y6-y7, y9
23-36	1367.6866	1367.6995	9.4	b3-b13, y2-y11

A β (1-40) ^d	Observed (M+H)	Calculated (M+H)	Δ ppm	Observed b/y Ions ^b
19-40	2180.1456	2180.1420	1.6	b5-b6, b9, b11-18, y6-y10, y16-18
21-40	1886.0096	1886.0052	2.3	b9-b17, y5-y9, y13, y16
20-40	2033.0727	2033.0736	0.4	b9, b11-b17, y5-y10, y12-14, y16-17
1-28	3261.5354	3261.5328	0.8	b5-b8, b10-b13, y4-y6, y8-y13
15-40	2648.4593	2648.4480	4.3	b6, b8-b10, b17-18, y7-y11, y13, y16-y18
14-40	2785.5235	2785.5069	6.0	b5-b12, b14-15, b17-18, y8-y10, y13, y16-y20

^a 5 minute incubation of amylin with IDE in 50:1 ratio.^b The b and y product ions of the most prominent fragment peaks of IDE degraded Amylin, reduced Amylin and A β 4₂₇ are generated by LC-ESI-FTICR-MS-CID-MS/MS and their identity is analyzed using MassMatrix webserver (http://searcher.rrc.uic.edu/cgi-bin/mm-cgi/search_form.py).^c This fragment has an intact disulfide bond.^d 1-5 second incubation of A β (1-40) with IDE.

Table 3

Data collection and structure refinement statistics of substrate-bound IDE

	IDE-IGF-II	IDE-TGF- α	IDE-amylin	IDE-A β (1-42) ^f
Data Collection				
Beamline	APS 19ID	APS 19ID	APS 19ID	APS 19ID
Space group	P6 ₅	P6 ₅	P6 ₅	P6 ₅
Cell dimension(Å)				
a	263.0	262.2	262.9	261.6
b	263.0	262.2	262.9	261.6
c	90.8	90.5	90.9	90.7
Resolution (Å)	50-2.28	50-2.3	50-2.9	50-2.6
R _{sym} (%) ^a	13.4(50.7)	9.4(52.0)	8.0(33.2)	9.1(45.3)
I/sigma	20.5(2.1)	23.6(3.6)	25.1(3.8)	17.6(3.0)
Redundancy ^b	5.4(3.1)	7.1(6.8)	5.7(4.3)	3.6(3.4)
Completeness (%)	99.7(96.9)	99.8(100.0)	99.9(99.2)	99.8(99.9)
FOM(Figure of Merit)				
Unique reflections	162414	157230	79043	109059
Refinement				
R _{work} ^c	0.206	0.191	0.178	0.186
R _{free}	0.239	0.231	0.223	0.232
No. atoms				
Protein	15710	15644	15818	15590
Water	598	653	346	233
B-factors				
IDE	35.5	28.6	38.6	35.2
Substrate	54.2	52.6	67.8	71.2
Water	44.5	46.5	48.5	36.5
r.m.s. deviations				
Bond lengths (Å)	0.015	0.016	0.012	0.022
Bond angles (°)	1.363	1.463	1.406	1.959
Ramachandran plot (%) ^g				
Favorable region	91.9/91.6	92.3/91.2	89.6/90.0	90.7/100.0
Allowed region	8.1/8.4	7.3/8.8	10.4/10.0	9.3/0.0
Generously allowed region	0 /0	0 /0	0 /0	0 /0
Disallowed region	0/0	0 /0	0 /0	0 /0
PDB code	3E4Z	3E50	3HGZ	2wk3

$$^a R_{\text{merge}} = \Sigma (I - \langle I \rangle) / \Sigma \langle I \rangle$$

$$^b N_{\text{obs}}/N_{\text{unique}}$$

$$^c R_{\text{work}} = \Sigma hkl ||F_{\text{obs}}| - k |F_{\text{calc}}|| / \Sigma hkl |F_{\text{obs}}|$$

$$^d R_{\text{free}}, \text{calculated the same as for } R_{\text{work}} \text{ but on the 5\% data excluded from the refinement calculation.}$$

^e the outer resolution shell. Values in parentheses indicate the highest resolution shell.

^f the backbone nitrogen of Gly²⁹ of A β (1-42) is substituted with 2-Nitrobenzyl group to improve the solubility of A β (1-42) (Erik Johnson, U of Chicago PhD thesis).

^g values for IDE protein/substrate.