Substrate Activation of Insulin-degrading Enzyme (Insulysin)

A POTENTIAL TARGET FOR DRUG DEVELOPMENT*

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The rate of the insulin-degrading enzyme (IDE)-catalyzed hydrolysis of the fluorogenic substrate 2-amino-benzoyl-GGFLRKHGQ-ethylenediamine-2,4-dinitrophenyl is increased 2–7-fold by other peptide substrates but not by peptide non-substrates. This increased rate is attributed to a decrease in K_m with little effect on V_max. An ~2.5-fold increase in the rate of amyloid β peptide hydrolysis is produced by dynorphin B-9. However, with insulin as substrate, dynorphin B-9 is inhibitory. Immunoprecipitation of differentially tagged IDE and gel filtration analysis were used to show that IDE exists as a mixture of dimers and tetramers. The equilibrium between dimer and tetramer is concentration-dependent, with the dimer the more active form. Bradykinin shifted the equilibrium toward dimer. Activation of substrate hydrolysis is not seen with a mixed dimer of IDE containing one active subunit and one subunit that is catalytically inactive and deficient in substrate binding. On the other hand, a mixed dimer containing one active subunit and one subunit that is catalytically inactive but binds substrate with normal affinity is activated by peptides. These findings suggest that peptides bind to one subunit of IDE and induce a conformational change that shifts the equilibrium to the more active dimer as well as activates the adjacent subunit. The selective activation of IDE toward amyloid β peptide relative to insulin suggests the potential for development of compounds that increase IDE activity toward amyloid β peptide as a therapeutic intervention for the treatment of Alzheimer’s disease.

Insulysin, insulin-degrading enzyme (IDE); EC 3.4.24.56 is a zinc metallopeptidase and a member of the inverzin family of proteases. Although IDE is primarily located in the cytosol (1), it is also found in peroxisomes (1), whereas a small fraction of the enzyme can be found on the plasma membrane (2, 3) as well as secreted (4). The substrate specificity of IDE is complex and not well understood. It has been suggested that the enzyme exhibits a preference for P_1 basic (arginine, lysine) or bulky hydrophobic (phenylalanine, leucine, tyrosine) residues (5). Studies from this laboratory confirmed the preference, but not requirement, for cleavage at hydrophobic and basic residues (6). It has also been proposed that the specificity of the enzyme is governed by the three-dimensional structure of the substrate (1), and this is likely to be a contributing factor with larger substrates. A variety of physiological peptides has been shown to be in vitro substrates for IDE including insulin (7), glucagon (7), atrial natriuretic factor (8), transforming growth factor α (9, 10), atrial natriuretic peptide (11), β-endorphin and dynorphins (6), and amylin (12). Although insulin is considered a physiological substrate and IDE is believed to be involved in the regulation of insulin levels, the presence of the enzyme in high levels in insulin-insensitive cells suggests that it has a variety of physiological functions.

Interest in IDE has recently emerged based on its potential involvement in the catabolism of amyloid β peptides in the brain. A number of studies demonstrated that IDE can degrade Aβ_{40} and Aβ_{42} (4, 13–15). Genetic linkage studies indicate that a region of chromosome 10q, which is within 195 kilobases of the IDE gene, is genetically linked to late-onset Alzheimer’s disease (16–18). However, the linkage of this region of chromosome 10q and IDE has yet to be firmly established and has been questioned (19). Perhaps the most convincing evidence that IDE contributes to amyloid β peptide catabolism in vivo comes from the finding that Aβ peptide levels are elevated in the brains of IDE-deficient mice (20, 21).

During the course of our studies of the reaction of IDE with various peptide substrates we observed that several of these peptides, when tested as alternate substrate inhibitors, actually increased enzyme activity. We report here the results of these studies, which suggest that IDE can be a therapeutic target for the prevention and treatment of Alzheimer’s disease.

MATERIALS AND METHODS

The fluorogenic substrates Abz-GGFLRKHGQ-EDDnp, Abz-GGFLRKAGQ-EDDnp, and Abz-GGFLRKMQG-EDDnp were synthesized and purified as previously described (22). Recombinant nardilysin IDE was produced in SF-9 cells as a fusion protein containing an N-terminal hexahistidine tag attached to the enzyme through a linker region containing a tobacco etch virus protease site (23). The enzyme was purified to homogeneity by chromatography on a His-Select HC nickel affinity gel column (Sigma). In some experiments the hexahistidine affinity tag was removed by treatment with tobacco etch virus protease and confirmed by Western blot analysis with an anti-hexahistidine antisera (Clontech). Recombinant nardilysin was produced in SF-9 cells and purified in a similar manner (24).

Dynorphin B-9, dynorphin A-17, bradykinin, γ-endorphin, insulin B-chain, insulin, Leu-Arg, and Leu-enkephalin were obtained from Bachem. β-Endorphin was obtained from Multiple Peptide Systems through the National Institute on Drug Abuse Research Tools program. Iodinated-Aβ_{40} and iodinated insulin were purchased from Amersham Biosciences.

The hydrolysis of the fluorogenic peptides was measured by following
the increase in fluorescence (excitation at 318 nm and emission at 419 nm) that occurred upon peptide bond cleavage (22). Alternatively, Abz-GGFLRKHGQ-EDDnp hydrolysis was followed by measuring the disappearance of the substrate by HPLC. A linear gradient of 5% acetonitrile in 0.1% aqueous trifluoroacetic acid to 50% acetonitrile in 0.1% aqueous trifluoroacetic acid was used. Peptide peaks were monitored by absorbance at 214 nm. The same HPLC system was used to measure the hydrolysis of other peptides.

The hydrolysis of 125I-labeled Aβ1-40 was determined in 15-μl reaction mixtures containing 20 μM potassium phosphate, pH 7.3, 10.7 nM 125I-labeled Aβ1-40 (specific activity 2 × 106 cpm/nmol), and 0.25 ng of IDE. After incubation at 37 °C for 15 min the reaction was stopped by the addition of 15 μl of SDS sample buffer. An aliquot was electrophoresed on a 15% Bis/tris/urea polyacrylamide gel as described by Klafki et al. (25). After drying the gel, band intensities were then quantitated with a storm PhosphorImager (Molecular Dynamics).

The hydrolysis of 125I-labeled insulin was assayed by a modification of the method of Garcia et al. (26). Reaction mixtures (50 μl) containing 0.25 ng of enzyme, 50 nM 125I-labeled insulin (specific activity 2 × 105 cpm/nmol), 0.15% bovine serum albumin, and 100 μM potassium phosphate buffer, pH 7.3, were incubated at 37 °C for 30 min. The reaction was terminated by the addition of an equal volume of 15% trichloroacetic acid. After allowing the sample to sit on ice for 15 min to precipitate unreacted insulin, the reaction was centrifuged for 5 min at 2,000 × g, and the supernatant was counted on a γ counter.

Gel filtration was performed on a Superdex-200 column (Amersham Biosciences) equilibrated with 100 mM sodium phosphate buffer, pH 7.3, and 50 μM bradykinin when added. When bradykinin was included in the gel filtration buffer, the eluted fractions containing IDE were immediately adjusted to 0.5% trifluoroacetic acid and analyzed by HPLC for bradykinin hydrolysis.

Isolation of dimers between His6-IDE and FLAG-IDE was accomplished by co-expression in COS-7 cells. The mixed dimers were precipitated with anti-His antisera and protein G Sepharose beads and analyzed by SDS-PAGE and Western blot analysis with anti-FLAG antisera. The formation of dimers between His6-IDE221232 and wild type IDE and between His6-IDE221131 and wild type IDE was accomplished by co-expression of baculovirus expressing the appropriate form of IDE in Sf-9 cells. Cell extracts were prepared and dimers isolated by chromatography on columns of His-Select HC nickel affinity gel (Sigma).

RESULTS

In an attempt to determine the relative affinity of a number of peptides for IDE we tested them as alternate substrate inhibitors. Initially we assayed IDE activity with the fluorogenic substrate Abz-GGFLRKHGQ-EDDnp as substrate. As shown in Fig. 2, A–H, some but not all of the peptides examined increased IDE activity. Those that increased IDE activity (Fig. 2, A–E) included β-endorphin, bradykinin, dynorphin B-13, dynorphin A-17, and insulin B chain. However, there was considerable variation in the extent of activation observed and the shape of the curves. Activation at low concentrations followed by inhibition at higher concentrations was seen for β-endorphin, dynorphin B-13, dynorphin A-17, and insulin B chain, whereas bradykinin produced a hyperbolic response with a 7-fold increase in activity. At the other extreme the peptides Leu-Arg, Leu-enkephalin, and γ-endorphin were without significant effect, Fig. 2, F–H.

The peptides shown in Fig. 2 were tested as substrates for IDE by following their hydrolysis by HPLC. We found that those peptides that activated IDE, namely dynorphin B-9, β-endorphin, bradykinin, dynorphin B-13, dynorphin A-17, and insulin B-chain, were all substrates. Those peptides that had a minimal effect on IDE activity, γ-endorphin, Leu-Arg, and Leu-enkephalin were not significantly cleaved.

To provide insight into the kinetic mechanism by which the peptides increased IDE activity we measured the effect of dynorphin B-9 on the kinetics of Abz-GGFLRKHGQ-EDDnp hydrolysis. Abz-GGFLRKHGQ-EDDnp was measured as a function of the indicated concentration of dynorphin B-9 by following the reaction by the increase in fluorescence upon peptide bond cleavage (●) or by following the disappearance of the peptide by HPLC (○).

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hydrolysis. As shown in Fig. 3, the effect of dynorphin B-9 was primarily to decrease the $K_m$ for the substrate, with a relatively small effect on $V_{max}$. Attempts to show substrate activation with Abz-GGFLRKHGQ-EDDnp were suggestive that this phenomena occurred. However, sigmoidicity in the substrate versus rate curve was difficult to ascertain with certainty since rate measurements were less reliable at the low substrate concentrations where deviation from a hyperbolic response appeared to occur.

We next determined whether the peptide-dependent increase in IDE activity could be extended to two physiological substrates for the enzyme, $\alpha_\beta_{1-40}$ and insulin. As shown in Fig. 4A, dynorphin B-9 at low concentrations produced an $\sim 2.5$-fold increase in the rate of $\alpha_\beta_{1-40}$ cleavage. However, as shown in Fig. 4B, dynorphin B-9 did not increase the rate of hydrolysis of insulin and was actually inhibitory.

We previously reported that IDE appeared as a dimer as judged by gel filtration (6). To confirm the oligomeric nature of IDE we co-expressed an N-terminally His$_6$-tagged IDE with an N-terminally FLAG-tagged IDE in COS-7 cells. Immunoprecipitation with anti-His$_6$ antisera co-precipitated FLAG-IDE as determined by Western blot analysis with anti-FLAG antisera, Fig. 5. To look for other oligomeric forms of IDE we conducted gel filtration analysis at a protein load of 9 mg/ml and a protein load of 1 mg/ml, Fig. 6. At the lower protein load, $\sim 80\%$ of the activity migrated as an $\sim 220$-kDa species, corresponding to a dimer with the remainder of the activity migrating as a tetramer. At the higher protein load the activity was distributed more nearly equal between the dimer and tetramer. Thus IDE can undergo a concentration-dependent dimer-tetramer equilibrium but is likely a dimer under assay conditions.

To determine whether the peptide activation affected the dimer-tetramer equilibrium, we took enzyme at a high concentration (9 mg/ml) and diluted it to $1$ mg/ml in the presence and absence of $50 \mu M$ bradykinin. The distribution between tetramer and dimer was then determined on a Superdex col-
umn, which was equilibrated, and run with either 100 mM potassium phosphate buffer, pH 7.3, or this buffer containing bradykinin. In the presence of bradykinin the tetramer/dimer equilibrium was shifted to the more active dimer, Fig. 7. To determine whether the dimer or tetramer exhibited the same activity, we compared the specific activity of the enzyme on the ascending peak of the tetramer and the descending peak of the dimer. It was found that the dimer exhibited a 1.6-fold higher specific activity than the tetramer. This is probably a minimal estimate since it is likely that diluting the tetramer into the assay mix led to dissociation of the tetramer into the more active dimer. We found that the bradykinin in the tetramer-containing fractions was significantly hydrolyzed (20–50%); however, complete hydrolysis was seen in the dimer-containing fractions. This observation further suggests that the dimer is the more active species.

The oligomeric nature of IDE suggested that its observed activation by peptides might also occur via a peptide-induced conformational change. To test this hypothesis we utilized two mutant forms of IDE. One, IDE<sup>H112Q</sup>, exhibits about 1% of the activity of the wild type enzyme and a substrate kinetic constant <i>K<sub>m</sub></i> >10-fold higher than that of the wild type enzyme. The other, IDE<sup>E111V</sup>, also exhibits about 1% activity but binds substrate with virtually the same <i>K<sub>m</sub></i> as the wild type enzyme. Based on the induced conformational change model, a dimer between IDE<sup>H112Q</sup> and wild type IDE should not exhibit significant activation by peptides since only one of its subunits can bind substrate and catalyze the reaction. In contrast, a dimer between wild type IDE and IDE<sup>E111V</sup> would be expected to exhibit peptide-dependent activation since both subunits can bind substrate, and the catalytically inactive subunit should be able to induce the hypothetical conformation change in the active subunit.

To generate these dimers we co-expressed wild type IDE with His<sub>6</sub>-IDE<sup>H112Q</sup> or with His<sub>6</sub>-IDE<sup>E111V</sup> in Sf-9 cells and isolated the enzyme on a His-Select HC nickel affinity gel column, which bound the hexahistidine-containing inactive subunit. In this way the only IDE activity obtained is from a dimer between His<sub>6</sub>-IDE<sup>H112Q</sup> and wild type IDE. The His<sub>6</sub>-tagged IDE forms that were in excess, being inactive, would not contribute to any observed IDE activity. Preliminary experiments showed that only a trace of wild type IDE was non-specifically bound to and subsequently eluted from the His-Select HC nickel affinity gel and, under the conditions of the assays performed, contributed little if at all to the observed activity. Preliminary experiments showed that only a trace of wild type IDE was non-specifically bound to and subsequently eluted from the His-Select HC nickel affinity gel and, under the conditions of the assays performed, contributed little if at all to the observed activity. As shown in Fig. 8, when a dimer between His<sub>6</sub>-IDE<sup>H112Q</sup> and wild type enzyme was tested for activation by dynorphin B-9, little activation was seen. In contrast, when a dimer between His<sub>6</sub>-IDE<sup>E111V</sup> and wild type enzyme was tested for activation by dynorphin B-9, this mixed dimer showed activation similar to the wild type enzyme.

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<sup>2</sup> E.-S. Song and L. B. Hersh, manuscript in preparation.
DISCUSSION

The results reported here demonstrate that peptide substrates can affect the kinetics of the IDE reaction by lowering the $K_m$ for the substrate. Because we have shown that IDE is oligomeric undergoing a concentration-dependent dimer-tetramer formation. Gel filtration of IDE on a Superdex S-200 column loaded at a protein concentration of 1 mg/ml (top) or 9 mg/ml (bottom). Fractions of 1 ml were collected and assayed for IDE activity with 2 $\mu$m Abz-GGFLRKHGQ-EDDnp as substrate.

FIG. 6. Gel filtration shows IDE undergoes concentration-dependent dimer-tetramer formation. Gel filtration of IDE on a Superdex S-200 column loaded at a protein concentration of 1 mg/ml (top) or 9 mg/ml (bottom). Fractions of 1 ml were collected and assayed for IDE activity with 2 $\mu$m Abz-GGFLRKHGQ-EDDnp as substrate.

FIG. 7. Gel filtration shows that bradykinin shifts the dimer-tetramer equilibrium toward dimer. Gel filtration of IDE was conducted on a Superdex S-200 column in which IDE was diluted from $\sim$9 mg/ml to $\sim$1 mg/ml in either phosphate buffer (top) or phosphate buffer containing 50 $\mu$m bradykinin (bottom). Protein was monitored by absorbance at 280 nm. Fractions of 1 ml were collected. The specific activity of fractions 62–68 and 76–80 from the gel filtration run in the absence of bradykinin were used to determined the specific activity of the tetramer and dimer respectively.

FIG. 8. Comparison of the effect of dynorphin B-9 on the activity of the His$_6$-IDE$^{E112Q}$/wild type IDE dimer, the His$_6$-IDE$^{E111V}$/wild type IDE dimer, and wild type IDE. IDE activity was determined using the fluorometric assay as described in Fig. 1. Activity was determined as a function of dynorphin B-9 for wild type enzyme ($\bullet$), the His$_6$-IDE$^{E111V}$/wild type IDE dimer ($\otimes$), and the His$_6$-IDE$^{E112Q}$/wild type IDE dimer ($\Delta$). Activity is expressed relative to that of each enzyme form in the absence of added dynorphin B-9.

DISCUSSION

The results reported here demonstrate that peptide substrates can affect the kinetics of the IDE reaction by lowering the $K_m$ for the substrate. Because we have shown that IDE is oligomeric undergoing a concentration-dependent dimer-tet-
rimer equilibrium, we propose that the activating effect of peptide substrates is a result of their binding to one subunit and inducing a conformational change. We suggest that this conformational change is induced within the dimer, making it more active by increasing the affinity of the adjacent subunit for its substrate and also by shifting the equilibrium from the less active tetramer to the more active dimer.

Several observations support this hypothesis. First, only substrates are able to increase IDE activity. Thus, γ-endorphin and Leu-enkephalin, which are not substrates, were without effect. This would suggest that peptide activation is the result of binding to the active site. This would also mean that both subunits do not have to turn over simultaneously; that is, one subunit can retain bound substrate, whereas the other subunit undergoes catalysis. In such a model only one of the two subunits within the dimeric unit need be catalytically active.

Secondly, activation is observed with the physiological substrate amyloid β-peptide 1–40 but not with another physiological substrate insulin. This can be explained by suggesting that insulin, which is a dimer of A and B chains, can simultaneously bind to both subunits of the IDE dimer, with the A chain of insulin bound to one subunit and the B chain bound to the other subunit. This would preclude other peptide substrates from binding to an “empty” subunit and activating the enzyme. Peptide binding could only displace insulin, leading to inhibition as is observed. The binding of insulin to both subunits of the IDE dimer can also explain why insulin has such a high affinity for IDE. The $k_{cat}$ for insulin cleavage (0.56 min$^{-1}$) is not particularly high in relation to other IDE substrates; the $k_{cat}$ for gastrin-releasing factor 1 is 23 min$^{-1}$, whereas the $k_{cat}$ for β-endorphin is 21 min$^{-1}$ (6). However the $K_m$ of 0.13 mM for insulin is considerably lower than that for GRF 1–29 (12 μM) and β-endorphin (13 μM) (6).

Last, we show that a mixed dimer containing an active IDE subunit and an inactive low affinity subunit is not activated to nearly the same extent as the wild type enzyme. This can be accounted for by the inability of the inactive subunit to bind substrate. In contrast, a mixed dimer containing an active IDE subunit and an inactive subunit that binds substrate with approximately the same affinity as the wild type enzyme is activated by peptide to nearly the same extent as the wild type enzyme. This can best be explained by the catalytically inactive subunit binding peptide and inducing a conformational change that affects the active subunit.

Direct measure of the specificity of the tetrameric and dimeric forms of IDE show that the dimer is the more active form. This is further supported by the finding that during gel filtration in the presence of bradykinin, the tetramer-containing fractions only partially hydrolyzed bradykinin, whereas in the dimer-containing fractions bradykinin was completely hydrolyzed. Although peptide binding can shift the equilibrium from the less active tetramer to the more active dimer, this effect would only be significant at higher enzyme concentrations where the tetramer predominates.

Camberos et al. (27) recently report ATP inhibition of insulin cleavage by IDE. Preliminary studies suggest that ATP binds at a distal site that is distinct from the peptide binding site observed in this study.

The finding of selective substrate activation of IDE toward amyloid β-peptide cleavage, but not toward insulin cleavage, suggests the opportunity to develop small molecule peptide analogs that increase IDE activity toward amyloid β-peptide cleavage in vivo. Because IDE has been implicated in the clearance of amyloid β-peptides in the brain (20, 21), elevating its activity has the potential to decrease amyloid burden without affecting insulin levels and, thus, possibly delay or prevent the onset of Alzheimer’s disease. Thus IDE represents a target for the development of a new class of drugs for the treatment of Alzheimer’s disease that lowers amyloid β-peptide levels by increasing its rate of catabolism.

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