Degradation of Alzheimer's Amyloid- β by a Catalytically Inactive Insulin Degrading Enzyme

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Abstract

Insulin-degrading-enzyme (IDE) is a key target to treat type-2 diabetes, and also known to clear Alzheimer's amyloid- β (A β). However, the development of catalytically inactive IDE mutant (E_{111Q} IDE) could risk A β clearance. Here, we demonstrate A β degradation by E_{111Q} IDE and the removal of zinc from the toxic A β -Zn complex enabling proteolysis by IDE. Fluorescence and NMR results show delays in A β aggregation by both wild-type and E_{111Q} IDE in their zinc-bound and unbound states. Diffusion NMR and LC-MS revealed the delayed kinetics is due to A β degradation. Remarkably, IDEs exhibited no proteolysis against zinc bound A β species as evidenced from high-speed AFM, electron microscopy, chromatography and NMR. On the other hand, zinc removal from the Zn-A β complex enabled the proteolysis by IDEs. These findings highlight the role of zinc in switching on/off the proteolysis of A β and urge the development of potent zinc chelators as a strategic alternative therapeutic for AD.

Amyloid- β (A β) aggregation is a key molecular factor contributing to Alzheimer's disease (AD) by impairing synaptic function via neuronal cell death.^[1,2] Two major A β isoforms A β (1-40) and A β (1-42) are targeted in controlling AD progression.^[3] The anti-amyloid immunotherapy is a promising strategy to control AD progression that includes the inhibition of A β aggregation, reduction of the formation of soluble neurotoxic oligomers, rapid fibrillation, and generation of amorphous aggregates.^[4,5] Unfortunately, till date, there is no success in the therapeutic development.^[6] The failure in targeting A β could be due to several factors that are poorly understood at the molecular level.^[6] While A β plaque deposition is moderately correlated to AD, plaque deposition as such has been identified in healthy brains without dementia.^[7,8] Also, the involvement of other cellular components (such as membrane, metal ions,

enzymes, and apolipoproteins) during A β aggregation restricts the strategy for a successful therapeutic development.^[9-11] For example, the interaction of water-soluble A β with membrane or metal ions (like Zn²⁺ and Cu²⁺) generates amorphous or low-molecular weight oligomers that are highly polymorphic and vary in neurotoxicity.^[12-16] Recent studies have identified the metal bound A β species to be highly stable and toxic and pathologically crucial targets.^[17,18] Metal ion binding to amyloid peptides can affect their enzymatic degradation.^[19-21] Reduction in A β accumulation is evidenced by enzymes such as insulin-degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme, and matrix metalloproteinase-9.^[22-27] IDE and NEP are reported to clear soluble A β , whereas matrix metalloproteinase-9 enzyme was found to degrade both soluble and A β fibers.^[28] IDE, a conserved Zn²⁺ metalloproteinase, selectively interacts with A β monomers^[29], and its activity is mediated by the dynamic equilibrium between soluble A β monomers and aggregates.^[30] The IDE-bound A β 40 structure reveals a catalytic chamber of size ~35 Å suitable to degrade short peptides.^[31] Importantly, the inhibition of IDE's activity by small molecule inhibitors^[32] or by the development of catalytically inactive mutant (E_{111Q} IDE)^[33] could suppress A β degradation associated with AD.^[34] Here, we demonstrate A β degradation by E_{111Q} IDE, an important targeted enzyme to treat type-2 diabetes, and the role of zinc in the dysfunction of IDEs.

The catalytic rates of w_t IDE and E_{111Q} IDE were tested on the rate of hydrolysis of a bradykinin-mimetic fluorogenic peptide, substrate V.^[35] Results showed that w_t IDE is active on substrate V hydrolysis whereas E_{111Q} IDE mutant and IDE variants co-incubated with EDTA (w_t IDE^{EDTA} and E_{111Q} IDE^{EDTA}) are not (Fig. S1). Enzymatic degradation activities of w_t IDE and an inactive E_{111Q} IDE mutant were tested, in the absence and presence of EDTA, on A β 40 using thioflavin-T (ThT) fluorescence assay. At sub-nanomolar IDE concentrations (enzyme:A β =1:1000 or 1:250), E_{111Q} IDE exhibited a small increase in the lag-time of A β aggregation as compared to w_t IDE that showed no ThT fluorescence (Fig. S2). When mixed with EDTA, w_t IDE showed substantial delay, whereas E_{111Q} IDE^{EDTA} showed a small delay, on the aggregation kinetics (Fig. S2). Remarkably, at superstoichiometric concentration

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(enzyme:A β =1:10), all IDE variants showed substantial enzymatic degradation activities illustrating no A β 40 aggregation up to day 12 (Fig. S3).^[30,36] Zinc chelated IDE variants pre-incubated with EDTA also showed substantial catalytic activity and no A β aggregation (Fig. S3). These results suggest that IDE variants are active against A β 40 aggregation despite selective mutation or zinc chelation. Although the concentration of IDE is higher than A β in brain, the threshold E111Q IDE concentration used in this study (0.5 μ M) is ten times lower than A β 40 indicating the efficiency of the enzyme to treat type-2 diabetes and AD.^[37] It should be noted that, the ThT fluorescence quenching in the presence of IDE mutant could also be due to a possible chaperonin activity of IDE.^[36,38] To address this, we hypothesize that under a catalytic environment, IDE and its variants will generate fragments of A β 40.^[30] In contrast, under a chaperonin environment, there will be an increase in A β 40 size due to aggregation or no change due to inhibition.^[36]

Strikingly, HPLC-mass spectrometry (LC-MS) analysis identified A β 40 fragments in all three IDE samples (wtIDE, E111Q IDE and E111Q IDE^{EDTA}) with molecular weights varying from >200 to <800 Da (Figs. 1 and S4-S6). It should be noted that although IDE cleaved A β fragments can be larger or smaller than 1-kDa (see Fig. S7), the large size fragments (typically >1-kDa) have a tendency to

aggregate^[30] and are removed using a 10-kDa filter prior to LS-MS measurement in this study. The findings from LC-MS were further confirmed using diffusion ordered NMR spectroscopy (DOSY) in real time. DOSY results revealed ~3 hours incubation of 1:10 wtIDE:A β presented small (diffusion constant "D"= 2.2×10^{-6} cm²/s) and large (6.5×10^{-7} cm²/s) size species (Figs. 1D and S8). In addition, similar to wtIDE, several small A β 40 fragments were identified in the presence of E111Q IDE with the diffusion constant varying between 0.6×10^{-6} and 3×10^{-6} cm²/s (Fig. 1C,D); based on the DOSY experiments on known molecular weight short peptides (Fig. S9), the observed small A β 40 fragments are expected to be ~300 Da in size. This observation is remarkable as it rules out non-chaperone activity for both IDEs. Thus, these results from LC-MS and NMR confirm the catalytic activities of wtIDE, E111Q IDE and zinc-chelated E111Q IDE^{EDTA} against A β 40. Unlike A β 40 that formed fibrils after ~24 hours of incubation (Fig. S10), A β incubated with wtIDE, E111Q IDE and E111Q IDE^{EDTA} generated globular species (Fig. 1H-J). The presence of morphologically similar A β species in both wild-type and mutant IDE mixture suggests a similar proteolytic activity. This further correlates to the LC-MS and NMR observations. Although, fluorescence, TEM and LC-MS results correlate with each other, none of these provides substantial information about the aggregation of the degraded A β 40 fragments. To test this, we

Figure 1. Characterization of A β 40 species in the presence of IDE. (A-B) HPLC-MS spectra revealing the formation of A β fragments by wtIDE and E111Q IDE. 50 μ M of A β monomers were incubated with 5 μ M of IDE for ~12 hours at room temperature prior to HPLC-MS sample preparation. Figs. S4-S6 show the original HPLC-MS spectra. (C) Characterization of E111Q IDE cleaved A β fragments (see Fig. S8 for wtIDE) DOSY). (D) 3D plot showing the diffusion constant (cm²/s) of 25 μ M A β fragments cleaved by 5 μ M E111Q IDE. (E-F) 2D ¹⁵N/¹H SOFAST-HMQC spectra of 25 μ M A β 40 dissolved in 10 mM sodium phosphate buffer, pH 7.4, 10% D₂O with and without 2.5 μ M E111Q IDE. (G) Mapping the depletion in residue peak volume and appearance of new peaks as a function of time in A β 40 in the absence or presence of E111Q IDE enzyme, zinc and EDTA. NMR spectra are shown in Figs. 1(E, F), 3A, 4B and S13. The peak volume ratio obtained at ~24 h (star) or 7 days (rectangle) is normalized with respect to the corresponding spectrum obtained at ~1 h (circle). New peaks are marked with ' and ', and the unassigned peaks with "na" in (G). (H-J) Negatively stained TEM images of 5 μ M A β 40 in the presence of 0.5 μ M wtIDE, E111Q IDE and E111Q IDE+5 μ M EDTA incubated ~24 hours at room temperature prior to imaging show globular A β 40 species with size $\geq$ 20 nm. The scale bar is 200 nm.

Figure 2. Catalytic effect of E_{111Q} IDE on A β 40 in the presence of zinc and EDTA. (A) ThT curve (average from triplicate is shown in Figure S12) representing the aggregation kinetics of 5 μ M A β 40 in the absence or presence of 5 μ M zinc, EDTA and 0.5 μ M E_{111Q} IDE as indicated in colors at 25 $^{\circ}$ C. (B) 1H NMR spectra of 25 μ M A β 40 showing a reversible change in signal intensity upon zinc binding and chelation using EDTA at the indicated stoichiometry. The change in signal intensities are normalized with respect to A β 40 in the absence of any additives. (C-D) Size-profile analysis of 25 μ M A β 40 using SEC. (C) A β 40 oligomers were prepared in F-12 media (see methods) and characterized in reference to commercially available BioRad protein ladder. (D) SEC of NMR samples used for $^{15}N/^1H$ SOFAST-HMQC (Figs. 1, 3 and 4) on day-7. The highlighted colors corresponds to the size distribution indicated in (C).

used NMR to evaluate A β 40 fragmentation by E_{111Q} IDE.

NMR spectra indicate an unstructured conformation incubated for $\sim$ 1 hour at room temperature, whereas after $\sim$ 24 hours and 7-days incubations, showed line-broadening indicating a structural rearrangement and aggregation (Fig. 1E). Remarkably, incubation of 1:10 E_{111Q} IDE:A β 40 showed no major changes at $\sim$ 1 hour, but several new peaks appeared at $\sim$ 24 hours with substantial line-broadening (Fig. 1F,G). Peak analysis for selected residues showed a ratio of $\sim$ 10-20% for the new peaks (red stars in Fig. 1G). Strikingly, most of the peaks disappear on day-7 suggesting the E_{111Q} IDE-cleaved A β 40 fragments form large-size aggregates that are not detected by solution NMR (Fig. 1E, green). The size-dependent NMR invisibility can also be correlated to the TEM observations that showed particles of size $\sim$ 32 nm (Fig. 1I). Next, we monitored the A β 40 cleavage by E_{111Q} IDE at sub-stoichiometric concentration (enzyme:substrate=1:100) to ensure the concentration dependent proteolytic activity as evidenced from ThT fluorescence (Fig. S2). NMR spectra showed A β 40 fragmentation with the appearance of several new peaks, but a comparatively slow degradation was identified at

$\sim$ 8 hours (Fig. S11). Importantly, unlike the observation at a superstoichiometric concentration of E_{111Q} IDE (Fig. 1F), we did not observe severe line-broadening at substoichiometric concentration on day-7. This is most likely due to the difference in the relative kinetics of A β 40 self-assembly and fragmentation that vary at least in several orders of magnitude. Considering the proteolytic cavity of IDE (volume $\sim$ 1.3 $\times$ 10 4 $\text{\AA}^3$) as evidenced from the X-ray structure,^[31] the low-ordered NMR visible aggregates that do not fit the active site are not substrates of E_{111Q} IDE and are more likely to appear in the $^{15}N/^1H$ spectrum.

Following the NMR observation, that showed size-dependent proteolytic activity of E_{111Q} IDE, we next investigated the effect of E_{111Q} IDE on the zinc-A β 40 complex that has been shown to be toxic and prevents degradation by NEP, IDE and matrix metalloprotease.^[14,39,40] ThT fluorescence showed retardation and acceleration in A β 40 aggregation by zinc and EDTA, respectively (Figs. 2A and S12). Notably, while zinc-chelated-IDE abolished A β 40 fibrillation, zinc-chelated-A β 40 showed fibrillation in the absence of E_{111Q} IDE (but not in the presence) indicating zinc-chelated-A β 40 is a substrate of E_{111Q} IDE. To probe this, 1H NMR experiments were carried out. A substantial decay in signal intensity

of the A β 40 fingerprint region (6.5 to 8.5 ppm) was observed when mixed with equimolar zinc indicating the formation of zinc-peptide aggregates (Fig. 2B). Remarkably, zinc chelation by EDTA (1:1) recovered the loss of ^1H signal intensity suggesting dissociation of the Zn-A β 40 complex to form monomer-like A β 40 species. This is further verified by size profiling using size-exclusion chromatography (SEC). The A β 40 oligomers prepared using F-12 media showed an elution profile with a triplet near ~18-19 mL and a singlet near ~21 mL. The SEC peaks are referenced with respect to Bio-Rad's gel filtration standard mixture (Fig. 2C, black) containing thyroglobulin, γ -globulin, ovalbumin, myoglobin, and vitamin B12 (MW 1.35 to 670 kDa). The triplet and singlet observed for A β 40 oligomers correspond to oligomers and monomers, respectively (Fig. 2C, red). 7-days incubation of A β 40 monomers showed major populated fibers eluting at the dead volume (~7 mL), small amount of oligomers (~15 mL) and monomers (~21 mL). $\text{E}_{111\text{Q}}$ IDE was observed to elute near ~8 mL, and Zn-A β 40 complexes depicted monomers, oligomers and larger aggregates (Fig. 2D, black and red). As anticipated, A β 40 mixed with $\text{E}_{111\text{Q}}$ IDE presented monomers and large aggregates, which agree with TEM observations. Notably, A β 40 incubated with zinc/EDTA/IDE or EDTA/IDE showed monomers as major population and no oligomer peak (Fig. 2D, green and purple). Taken together, the SEC, ThT fluorescence and NMR results reveal (i) zinc-chelated- $\text{E}_{111\text{Q}}$ IDE is proteolytically degrade A β 40,

and (ii) removal of zinc by EDTA resulted in Zn-A β 40 complex dissociation and the formation of A β 40 monomers or low-molecular weight aggregates.

The effect of $\text{E}_{111\text{Q}}$ IDE on the Zn-A β 40 complex was next monitored using NMR. The 1:1 Zn-A β 40 complex incubated for ~1 hour showed substantial line-broadening for A2, E3, Y10, E11, V12 and K16 (Fig. S13A), as compared to the A β 40-alone spectrum (Fig. 1E), in agreement with the reported zinc binding sites.^[15,41] Further line-broadening observed for several residues (Fig. 1G, blue squares) on day-7 indicate an increase in the size of Zn-A β 40 aggregates.^[16] Surprisingly, the Zn-A β 40 complex (25 μM) incubated with 2.5 μM $\text{E}_{111\text{Q}}$ IDE showed no substantial change in the spectrum even after 7 days (Fig. 3A). Unlike the observation for A β 40 proteolysis by $\text{E}_{111\text{Q}}$ IDE in the absence of zinc, the appearance of new peaks (Fig. 1G, green squares) and the disappearance of existing peaks until day-7 (Fig. 3A) was not observed. These results suggest that zinc bound A β 40 species are not substrates of $\text{E}_{111\text{Q}}$ IDE due to their large size that cannot be accommodated on IDE's catalytic cavity. No significant changes in signal intensities for A β 40 mixed with zinc, or zinc and $\text{E}_{111\text{Q}}$ IDE, (Figs. 3B and S13B, 7.5-8.5 ppm) were observed ruling out the possibility of monomer dissociation, proteolysis and fibrillation. Considering the crucial role of zinc in AD pathology,^[39] the reported NMR results highlight a dual function for zinc that has been poorly explored.

Figure 3. Monitoring the proteolytic activity of $\text{E}_{111\text{Q}}$ IDE on A β 40 in the presence of zinc. (A) Time-lapse NMR spectra of 25 μM A β 40 co-incubated with 25 μM zinc in the presence of 2.5 μM $\text{E}_{111\text{Q}}$ IDE as indicated in colors. NMR samples were prepared using NaPi buffer, pH=7.4 containing 10% D $_2\text{O}$. (B) Decay in ^1H NMR signal of 25 μM A β 40 co-incubated with 25 μM zinc in the absence or presence of 2.5 μM $\text{E}_{111\text{Q}}$ IDE as a function of time (see Fig. S13B). Real-time monitoring of 5 μM zinc-A β (1:1) complex incubated with 0.5 μM $\text{E}_{111\text{Q}}$ IDE in the absence (C) or presence of 5 μM EDTA (D) using HS-AFM. (E) Quantitative analysis of the particle size obtained from the HS-AFM images frame-5 and frame-21 (C-D) using ImageJ. The particles are shown in Figs. S14, S15.

Figure 4. Zinc chelation resumes the proteolytic activity of E111Q IDE. (A) SOFAST-HMQC spectra of 25 μM ^{15}N labelled A β 40 co-incubated with 25 μM EDTA and 2.5 μM E111Q IDE. (B) Titration of 25 μM EDTA to pre-incubated 25 μM A β 40, 25 μM zinc and 2.5 μM E111Q IDE. (C) Representation of how zinc impairs IDE's catalytic activity by forming Zn-A β complex that does not fit IDE's ≈ 35 Å active site. Zinc removal from Zn-A β complex by EDTA enables the degradation of A β by IDE.

Zinc binding enhances the toxic activity of A β ,^[14,39] and as shown here, zinc could also impair IDE's proteolytic activity. To this extent, we next probed the possibility of reversing IDE's activity via zinc removal by chelation with EDTA. HS-AFM was used to probe the efficacy of EDTA to reverse the morphology of Zn-A β 40 aggregates and generate E111Q IDE preferred small aggregates or monomers. Size of zinc-bound-A β 40 was found to vary from ~ 5 nm to several hundreds of nm in diameter (Figs. 3C,D and S14). Analysis of particle size (Fig. S14) presented a relatively high number of small particles (area 50-200 nm²); an increase to 500-1000 nm² was observed highlighting the assembling of small Zn-A β 40 particles over time (Fig. 3E, black bar), whereas EDTA reduced the number of small particles (Fig. S15) as illustrated in Fig. 3E (green bars) and restricted particle fusion and the formation of large aggregates (Figs. 3E and S15). Thus, HS-AFM data revealed the dissociation of small aggregates (area 50-200 nm²) to invisible species, which could be a preferable substrate for IDE.

Time-lapse SOFAST-HMQC experiments were performed to test if the A β 40 species formed from the A β 40-Zn complex, due to zinc removal by EDTA-Zn chelation, are substrates of E111Q IDE. E111Q IDE showed activity when co-incubated with EDTA followed by titration to A β 40 (Fig. 4A). Appearance of several new peaks at ~ 24 hours resembled the proteolytic activity of zinc-chelated-

E111Q IDE (Fig. 4A). Strikingly, when the pre-incubated Zn-A β 40 aggregates that showed resistance to enzymatic proteolysis (Fig. 3A) titrated with EDTA, several new peaks appeared (Fig. 1G, purple stars) within ~ 24 hours indicating A β 40 fragmentation (Fig. 4B). This NMR observation correlates to ThT fluorescence quenching (Fig. 2A, yellow trace) suggesting that zinc-A β 40 are proteolytically cleaved by E111Q IDE in the presence of EDTA.

In conclusion, in this study we have demonstrated the proteolytic activity of an IDE mutant (E111Q) on A β 40, which has been developed to treat type-2 diabetes, indicating its dual action on type-2 diabetes and AD. Both wild-type and IDE mutant are catalytically active and able to cleave A β 40 monomers to small fragments. The cleaved A β fragments by wild-type IDE or E111Q mutant self-assemble to form morphologically similar globular structures of size ~ 20 -30 nm that are tested to be nontoxic.^[30] A key finding in this study is the ability of zinc to generate proteolytic resistant A β 40 species that are pathologically relevant (Fig. 4C). Zinc binding generates off-pathway A β aggregates that grow in size and do not fit to the catalytic cavity (≈ 35 Å) of IDE (Fig. 4C). Whereas, removal of zinc by EDTA from the Zn-A β complex was shown to resume the proteolytic activity of IDE and A β fragmentation. This highlights the key role of zinc in AD in

incapacitating the enzymatic degradation of soluble A β monomers. Thus, these findings urge the development of potent therapeutic zinc chelating agents to resume the enzymatic degradation of soluble A β and prevention of AD progression.

Experimental Section

Experimental Details are provided in the supporting information.

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Graphical abstract