# Exchange Broadening Underlies the Enhancement of IDE-Dependent Degradation of Insulin by Anionic Membranes

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## ABSTRACT:
Insulin-degrading enzyme (IDE) is an evolutionarily conserved ubiquitous zinc metalloprotease implicated in the efficient degradation of insulin monomer. However, IDE also degrades monomers of amyloidogenic peptides associated with disease, complicating the development of IDE inhibitors. In this work, we investigated the effects of the lipid composition of membranes on the IDE-dependent degradation of insulin. Kinetic analysis based on chromatography and insulin’s helical circular dichroic signal showed that the presence of anionic lipids in membranes enhances IDE’s activity toward insulin. Using NMR spectroscopy, we discovered that exchange broadening underlies the enhancement of IDE’s activity. These findings, together with the adverse effects of anionic membranes in the self-assembly of IDE’s amyloidogenic substrates, suggest that the lipid composition of membranes is a key determinant of IDE’s ability to balance the levels of its physiologically and pathologically relevant substrates and achieve proteostasis.

## INTRODUCTION
Insulin-degrading enzyme (IDE) is a ubiquitous zinc metalloprotease that has been implicated in the efficient degradation of insulin monomer. However, IDE also degrades monomers of amyloidogenic peptides associated with disease, complicating the development of IDE inhibitors. In this work, we investigated the effects of the lipid composition of membranes on the IDE-dependent degradation of insulin. Kinetic analysis based on chromatography and insulin’s helical circular dichroic signal showed that the presence of anionic lipids in membranes enhances IDE’s activity toward insulin. Using NMR spectroscopy, we discovered that exchange broadening underlies the enhancement of IDE’s activity. These findings, together with the adverse effects of anionic membranes in the self-assembly of IDE’s amyloidogenic substrates, suggest that the lipid composition of membranes is a key determinant of IDE’s ability to balance the levels of its physiologically and pathologically relevant substrates and achieve proteostasis.
Recombinant human IDE was expressed and purified in-house, as previously described. Cells relevant to metabolism including insulin-producing β cells were also prepared. We use SUVs as model membranes in our in vitro studies of the biophysical and biochemical properties of amyloidogenic proteins such as insulin and Aβ because of their high membrane curvatures that facilitate biologically relevant membrane-induced protein conformational transitions. Insulin solutions were prepared at pH 7.4. At this pH and in the presence of Zn²⁺, it is well known that insulin self-assembles to hexamers. In our hands, we found that the dominant oligomeric state of insulin depends on its concentration. Analytical gel filtration indicates that as the concentration of insulin was increased from 15 to 170 μM, the dominant oligomeric state of insulin changes from dimers to hexamers.

The IDE-dependent degradation of insulin was carried out at 37 °C. Figure S2 presents time-dependent circular dichroic spectra showing that IDE remains active at 37 °C for 48 h. To quantitatively assess the effect of SUVs on the IDE-dependent degradation of insulin, we used a combination of chromatographic and spectroscopic techniques. Figure S3 presents representative time-dependent chromatograms of quenched insulin digestion reactions in the absence or presence of SUVs. Each data set shows the gradual loss of the insulin peak accompanied by the appearance of peaks at short retention times, consistent with proteolysis of the substrate. The amount of undigested insulin was determined using eq 1 and plotted against digestion time, as shown in Figure S4. We noted that the loss of insulin with digestion time in samples that contain SUVs composed of 100% DOPC is similar to that in control samples which do not contain SUVs. In contrast, insulin in the presence of SUVs containing anionic DOPS was degraded more rapidly in a manner that correlates with the anionic lipid content of the vesicles. Figure S4 shows that after 1 h of digestion, <10% and ~45% of insulin remains in digests that contain SUVs composed of 100% DOPS and DOPC/DOPS (7:3 mol/mol), respectively. In contrast, almost 60% of insulin remain in samples that contain SUVs composed of 100% DOPC and in samples that do not contain SUVs.

Next, we determined the Michaelis–Menten kinetic constants for the IDE-dependent degradation of insulin using circular dichroism (CD) spectroscopy. Recently, we demonstrated that the dichroic spectrum of insulin is sensitive to the kinetics of its proteolysis by IDE. As degradation proceeded, the intensity of insulin’s ellipticity at 222 nm, widely used as a measure of the helical content of proteins, decreased with an increase in digestion time. We calculated the amount of digested insulin using eq 2, as seen in Figure 2.
concentration resulted in a hyperbolic plot fitted by the Michaelis–Menten equation. Table 1 presents the steady-state kinetic constants $K_M$ (Michaelis constant), $k_{cat}$ (turnover number), and $k_{cat}/K_M$ obtained from the Michaelis–Menten plots. IDE’s catalytic efficiency, indicated by $k_{cat}/K_M$, in the presence of SUVs composed of 100% DOPC was similar to the control. However, IDE’s efficiency increased by 67 and 94% in the presence of SUVs composed of DOPC/DOPS (7:3 mol/mol) and 100% DOPS, respectively. Together, our kinetic analysis based on chromatography (Figure S3) and insulin’s helical circular dichroic signal (Figure 2) show that membranes containing anionic lipids enhance the IDE-dependent degradation of insulin.

To explain the increased rate of insulin degradation in the presence of anionic lipids, we used solution-state $^1$H NMR spectroscopy. Insulin solutions at a concentration of 100 $\mu$M were prepared in deuterated buffer to prevent H$_2$O-induced distortion of the peaks from insulin. We noted that the aromatic region (i.e., 6.6–7.4 ppm) of the $^1$H NMR spectra of the insulin digests is most sensitive to degradation. First, we determined the sensitivity of $^1$H NMR to the kinetics of degradation by IDE. Figure 3 presents portions of the $^1$H NMR spectra of insulin digests in the absence and presence of SUVs. In the absence of IDE, the peaks in the aromatic regions of the spectra are broad. In the presence of IDE, the broad peaks are increasingly replaced by sharp peaks with digestion time, consistent with the production of insulin fragments that tumble rapidly in solution. The spectra of the digests in the 3-h spectra increase in the order of increasing amounts of DOPS, i.e., DOPC < DOPC/DOPS < DOPS, indicating that the degradation of insulin is enhanced by the presence of anionic lipids. Together, our NMR results are consistent with the kinetic results obtained by chromatographic (Figure S3) and circular dichroic spectroscopic methods (Figure 2 and Table 1).

Next, we analyzed the spectra of insulin in the absence of IDE to decipher the mechanism for the enhancement of degradation by anionic lipids. At a concentration of 100 $\mu$M, insulin in the presence of Zn$^{2+}$ and at neutral pH exists mainly as a mixture of monomers and oligomers that are predominantly hexamers (Figure S1), consistent with results obtained by others using dynamic light scattering, size-exclusion chromatography, and analytical ultracentrifugation. Because insulin at a concentration of 100 $\mu$M is degradable by IDE (Figure 3) and the enzyme only degrades concentration in the absence or presence of SUVs follows Michaelis–Menten kinetics. Plots of $V_0$ against insulin concentration are hyperbolic. Each data point represents the mean from three kinetic trials, and the error bars are standard deviations. The lines are fits to the Michaelis–Menten equation.

Table 1. Steady-State Kinetic Parameters for the IDE-Dependent Degradation of Insulin in the Absence and Presence of SUVs

| SUVs                  | $K_M$ (M)       | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$) |
|-----------------------|-----------------|----------------------|-----------------------------------|
| none                  | $3.8 \pm 0.3 \times 10^{-5}$ | $0.027 \pm 0.0006$ | $7.2 \pm 0.5 \times 10^{2}$     |
| 100% DOPC             | $3.2 \pm 0.5 \times 10^{-5}$ | $0.027 \pm 0.003$ | $8.5 \pm 0.5 \times 10^{2}$     |
| 100% DOPS             | $2.1 \pm 0.4 \times 10^{-5}$ | $0.028 \pm 0.001$ | $1.4 \pm 0.3 \times 10^{3}$     |
| DOPC/DOPS (7:3 mol/mol) | $2.1 \pm 0.03 \times 10^{-5}$ | $0.026 \pm 0.0009$ | $1.2 \pm 0.05 \times 10^{3}$     |
monomeric substrates,\textsuperscript{9,10} insulin monomer must be in dynamic exchange with insulin oligomers (Figure 4). Insulin monomer thus samples two environments: one in which it is in association with other molecules of itself and the other in which it is by itself. In NMR spectroscopy, this is known as chemical exchange,\textsuperscript{66} also known as magnetic site exchange.\textsuperscript{67} Chemical exchange is characterized by an exchange rate, denoted by $k_{\text{ex}}$ (Figure 4). The relative values of $k_{\text{ex}}$ and $\Delta \omega$, where $\Delta \omega$ is the difference in frequency between the two sites, define the slow and fast exchange limits of chemical exchange:

$$k_{\text{ex}} \ll \Delta \omega \quad \text{slow exchange}$$

$$k_{\text{ex}} \approx \Delta \omega \quad \text{intermediate exchange}$$

$$k_{\text{ex}} \gg \Delta \omega \quad \text{fast exchange}$$

Because the peaks in the aromatic region of the 1D $^1$H NMR spectra of insulin in the absence of IDE are broad (Figure 3),

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Figure 3. IDE-dependent degradation of insulin monitored by NMR. One-dimensional $^1$H NMR spectra of insulin digestion reactions in (A) the absence of SUVs, and the presence of SUVs composed of (B) 100% DOPC, (C) DOPC/DOPS (7:3 mol/mol), and (D) 100% DOPS. In the absence of IDE, the broadening of the peaks in the aromatic region ($6.6−7.4$ ppm) in samples that contain membranes with anionic DOPS increased. In the presence of IDE, the broad peaks in the aromatic region are replaced by sharp peaks due to insulin fragments that tumble rapidly in solution. All spectra were recorded at 37 °C. The NMR samples were incubated at 37 °C in between the acquisition of spectra. The concentration of insulin in all samples was set at 100 μM. At this concentration, insulin exists mainly as hexamers. The insulin-to-lipid and the substrate-to-enzyme molar ratios were set at 1:50 and 100:1, respectively.

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Figure 4. Chemical exchange in insulin. Oligomeric insulin and insulin monomer are in dynamic equilibrium with one another. This equilibrium is characterized by the exchange rate $k_{\text{ex}}$. The distribution of oligomers is indicated by $n$, which ranges from 2 to 6. At the concentration of insulin used in the NMR studies, hexamers are the dominant oligomers.
and sharp peaks expected for a protein of the size of the insulin monomer, i.e., ∼5.8 kDa were not detected, the exchange between insulin oligomers and insulin monomers must be in the intermediate timescale. This conclusion is also supported by 2D NOESY NMR of insulin in the absence of IDE (Figure 5S), which shows the absence of cross peaks due to insulin, in sharp contrast to NOESY spectra we reported for insulin at pH 2 in the absence of SUVs. Additionally, our NOESY data (Figure 5S) indicate that multidimensional relaxation-based NMR methods that were recently used to investigate the interaction of Aβ/42 with IDE do not apply. In the presence of SUVs containing anionic lipids and in the absence of IDE, the broadening of the peaks in the aromatic regions of the spectra shown in Figure 3C, D increased. This is exchange broadening that results from an increase in the exchange rate between insulin oligomers and insulin monomer. In turn, the increase in exchange rate leads to increased IDE-dependent degradation of insulin monomer.

Our work shows that the lipid composition of membranes is an efficient regulator of the dissociation of insulin monomer from oligomeric insulin and, thus, of the ensuing IDE-dependent degradation of insulin monomer. When anionic lipids are present, the exchange rate between insulin oligomer and insulin monomer increases, leading to increased availability of insulin monomer for degradation by IDE. Interestingly, levels of anionic phospholipids in islet cells increase significantly after glucose stimulation. We speculate that glucose stimulation in vivo also leads to an increase in anionic-phospholipid levels in β-cell membranes, resulting in increased release of insulin monomer.

Finally, in light of IDE’s ability to degrade insulin, Aβ/42, and IAPP, it is no surprise that there has been a strong interest in the development of small molecules that modulate IDE’s activity. Several inhibitors have been reported to elevate insulin levels in cells or mice, including BMD44768, B35, 6bk, I1, and P12-3A. In spite of these significant advances, outstanding challenges remain. Most importantly, the inhibition of IDE may lead to increased levels of Aβ/42 and IAPP. Attractive strategies in response to this challenge include development of substrate-selective IDE inhibitors, identification of allosteric ligands, and allosteric mutagenesis of IDE. These strategies require consideration of the mechanisms that regulate the levels of the monomeric states of IDE’s substrates. In the case of insulin, we have shown here that anionic lipids in membranes increase the levels of IDE-degradable insulin monomer. However, this finding does not apply to all of IDE’s substrates. The self-assembly of IDE’s amyloidogenic substrates has been shown to be enhanced by anionic lipids in membranes. Zhang et al. showed that even low levels of anionic lipids promote the aggregation of cationic IAPP and facilitate IAPP-induced leakage of membranes. Clusters of anionic GM1 gangliosides in membranes accelerate Aβ aggregation to form assemblies with increased cytotoxicity. Aggregation is preceded by binding of Aβ to anionic lipids, which is mediated by cationic lysine residues (K16 and K28). The differential effects of anionic lipids in membranes in modulating the levels of the monomeric states of IDE’s substrates indicate that the lipid composition of membranes is a key determinant of IDE’s ability to balance the levels of its physiologically and pathologically relevant substrates. This finding should be considered in the development of IDE-centric therapeutic strategies for T2D and AD.

### Experimental Methods

**Materials.** Phospholipids, including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (>99% pure) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) (>99% pure) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Human insulin (99% pure, 0.4% zinc) was purchased from Sigma-Aldrich (St. Louis). Stock solutions of insulin were prepared in 10 mM phosphate buffer (pH 7.4). To overcome the low solubility of insulin at pH 7.4, the solutions were incubated overnight at 37 °C, followed by centrifugation for 1 min at 16 000g at room temperature to separate undissolved insulin. The concentration of insulin was determined by UV absorbance at 275 nm using a molar extinction coefficient of 6190 M⁻¹ cm⁻¹.

**Analytical Gel Filtration Chromatography.** Five insulin solutions with concentrations of 500, 300, 200, 100, 50, and 25 μM in 50 mM Tris at pH 7.4 were prepared. To perform the chromatography, a precalibrated Superdex 75 HR 10/300 GL column connected to an AKTA pure FPLC system was used. The concentration of the fractions containing the sample that yielded maximum absorbance at 275 nm was determined by UV spectroscopy, as described above.

**Preparation of Small Unilamellar Vesicles.** SUVs containing 100% DOPC, 100% DOPS, or DOPC/DOPS (7:3 mol/mol) were prepared as previously described. Briefly, lipids were dissolved in methanol/chloroform (1:1, v/v). After drying, the lipid films were hydrated with 1 mL of 10 mM phosphate buffer (pH 7.4). The resulting suspension of multilamellar vesicles was then subjected to 10 freeze–thaw cycles to increase their size homogeneity. This was followed by sonication using a VCX750 Vibra-Cell ultrasonic liquid processor equipped with a tapered microtip (Sonics and Materials, Inc., Newtown, CT) to produce SUVs. The SUVs were separated from titanium particles by centrifugation for 15 min at 16 000g at room temperature.

**IDE Overexpression and Purification.** Recombinant human IDE was expressed and purified as previously described. Briefly, glutathione-S-transferase tagged IDE (GST-IDE) was overexpressed in E. coli BL21-CodonPlus RIL cells. After cell lysis, GST-IDE was separated using a GSTrap Fast Flow column connected to an AKTA pure FPLC system and eluted with phosphate-buffered saline containing 10 mM glutathione. GST PreScission protease was then used to cleave the GST tag. IDE was further purified using standard gel filtration. The concentration of IDE was determined by UV absorbance at 280 nm using its molar extinction coefficient of 113 570 M⁻¹ cm⁻¹.

**IDE-Dependent Degradation of Insulin Monitored by Reversed-Phase HPLC.** Four proteolysis samples, each with a total volume of 300 μL, were prepared in triplicates. These samples included (1) insulin in the absence of SUVs; (2) insulin in the presence of 100% DOPC SUVs; (3) insulin in the presence of 100% DOPS SUVs; and (4) insulin in the presence of DOPC/DOPS (7:3 mol/mol) SUVs. The concentration of insulin was set at 40 μM. The ratio of insulin to lipid was set at 1:50 (mol/mol). The substrate-to-enzyme ratio was set at 100:1 (mol/mol). IDE was added last to initiate the reaction, followed by incubation of the reaction solutions at 37 °C. At the 1-min, 1-h, 3-h, 6-h, 24-h, and 48-h time points of proteolysis, 50 μL of the reaction solution was removed and transferred into an Eppendorf tube containing 23
μL of 1% (v/v) trifluoroacetic acid in water to quench the reaction.

All quenched samples were analyzed using a Varian ProStar 210 HPLC system equipped with a ProStar 325 Variable Wavelength UV–Visible Detector. Fractionation of insulin digests was carried out at room temperature using an Agilent AdvanceBio mAb C4 column. Solvent A was 0.1% (v/v) formic acid in H$_2$O, whereas solvent B was 0.1% (v/v) formic acid in acetonitrile. Water used for the mobile phase was obtained from a Millipore Milli-Q 185 Plus system (Millipore, Bedford, MA). Acetonitrile of chromatographic grade was supplied by Fisher Scientific. Aliquots (20 μL) of solutions of quenched insulin digests were injected into the HPLC manually and eluted with a 15-min linear gradient of 0–100% B at a flow rate of 1 mL/min. Elution of analytes was monitored by UV absorbance at wavelengths 214 and 254 nm. By utilizing the integrals of the insulin peak, the amount of undigested insulin remaining at a specific time point of the proteolysis reaction was calculated using eq 1 and plotted against digestion time.

Kinetics of IDE-Dependent Degradation of Insulin by Circular Dichroism Spectroscopy. To obtain the steady-state kinetic parameters for the IDE-dependent degradation of insulin in the presence of SUVs, we used a kinetic assay that takes advantage of the loss of insulin’s helical circular dichroic signal at 222 nm with digestion time. Briefly, seven insulin solutions in 10 mM phosphate buffer (pH 7.4) with concentrations ranging from 15 to 110 μM (i.e., 15, 20, 25, 30, 50, 80, and 110 μM) were prepared. The insulin solutions were prepared in the absence and presence of SUVs with the insulin/lipid ratio set at 1:50 (mol/mol). After mixing, the solution was transferred into a quartz cuvette with a path length of 1 mm and loaded into the sample holder of our JASCO J-815 spectropolarimeter set at 37 °C. The ellipticity at 222 nm ([θ]$_{222}$ (nm)) was then recorded for 5 min. The real-time ([θ]$_{222}$ (nm)) data were then used to calculate the amount of digested insulin ([DI]) using eq 2. Linear regression analysis of plots of [DI], against digestion time (up to 5 min) yielded $V_0$, the initial rate or velocity of the IDE-catalyzed degradation of insulin. Michaelis–Menten plots were then generated, from which the kinetic constants $K_M$, $V_{max}$, $k_{cat}$, and $k_{cat}/K_M$ were determined by curve-fitting to the Michaelis–Menten equation (eq 3)

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}$$

IDE-Dependent Degradation of Insulin Monitored by $^1$H NMR Spectroscopy. Stock solutions of insulin and SUVs in 10 mM phosphate buffer were prepared using 99.9% D$_2$O (Sigma-Aldrich). The concentration of insulin in all NMR samples was set at 100 μM. In samples that contain SUVs, the substrate/enzyme ratio was set at 1:50 (mol/mol). The substrate/enzyme ratio was set at 100:1 (mol/mol). All 1D and 2D $^1$H NMR spectra were recorded at 37 °C using a Varian INOVA spectrometer operating at 400 MHz. For chemical shift referencing, the methyl peak of the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate was set to 0 ppm. NOESY spectra were recorded in phase-sensitive mode using mixing times ranging from 100 to 400 ms. All samples were incubated at 37 °C in between spectral acquisitions.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02747.

Analytical gel filtration of insulin at pH 7.4 (Figure S1); far UV circular dichroic spectra of insulin as a function of digestion time at 37 °C (Figure S2); IDE-dependent degradation of insulin monitored by reversed-phase HPLC (Figure S3); dependence of the amount of undigested insulin on digestion time (Figure S4); and 2D NOESY spectra of insulin in the presence of SUVs composed of 100% DOPS (Figure S5) (PDF)

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