Islet amyloid polypeptide demonstrates a persistent capacity to disrupt membrane integrity

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Amyloid fiber formation is correlated with pathology in many diseases, including Alzheimer’s, Parkinson’s, and type II diabetes. Although β-sheet–rich fibrillar protein deposits define this class of disorder, increasing evidence points toward small oligomeric species as being responsible for cell dysfunction and death. The molecular mechanism by which this occurs is unknown, but likely involves the interaction of these species with biological membranes, with a subsequent loss of integrity. Here, we investigate islet amyloid polypeptide, which is implicated in the loss of insulin-secreting cells in type II diabetics. We report the discovery of oligomeric species that arise through stochastic nucleation on membranes and result in disruption of the lipid bilayer. These species are stable, result in all-or-none leakage, and represent a definable protein/lipid phase that equilibrates over time. We characterize the reaction pathway of assembly through the use of an experimental design that includes both ensemble and single-particle evaluations. Complexity in the reaction pathway could not be satisfied using a two-state description of membrane-bound monomer and oligomeric species. We therefore put forward a three-state kinetic framework, one of which we conjecture represents a non-amyloid, non-β-sheet intermediate previously shown to be a candidate therapeutic target.

Amyloidogenic β-sheets, Parkinson’s, type II diabetes, and other epide-
imologically important diseases are characterized, in part, by the deposition of proteinaceous plaques termed amyloid (1,2). For each disease, a specific protein is involved in amyloid as-
sebling in a process that is associated with cell dysfunction and death. The energetic and structural basis by which these states mediate toxicity, however, is unclear.

Islet amyloid polypeptide (IAPP or amylin) is a 37-residue peptide hormone cosecreted with insulin by pancreatic β-cells. Unmodified, wild-type IAPP self-assembles to form amyloid in type II diabetic patients in a process that is associated with β-cell dysfunction (2,5). Indeed, the capacity of species-based sequence variants to form amyloid is correlated with the observation of metazoic disease (5). Thus, whereas primates and cats readily form amyloid and acquire diabetes, rats and mice do not spontaneously develop diseases, and rodent IAPP does not form amyloid or other β-sheet aggregates. Diabetic symptoms can be induced in model rodents either by using toxins or by using rodent trans-

genic for human IAPP (7).

Previous in vitro studies have shown that the binding of human and rat IAPP to lipid bilayers is cooperative (8), an observation accounted for by the presence of oligomeric species. The populations of membrane-bound oligomers are correlated with the capacity of IAPP to permeabilize model membranes (8, 9) and induce cell toxicity (10). Importantly, what distinguishes human from rat protein in such assays is not amyloid formation. Rather, it is the fact that significantly more rat than human protein is required for binding cooperativity and leakage to be observed (8).

More recently, the membrane interface of IAPP has been shown to be mediated by an α-helical structure comprised of the first 20 residues of IAPP (11–13), which, apart from a conservative change (H18R) is identical between the two variants. Furthermore, structure-based small-molecule targeting of this α-helix is protective of human IAPP toxicity in cell culture (14). Thus, rodent IAPP provides an excellent opportunity to study relevant aspects of preamyloidogenic assemblies of IAPP without the practical restrictions of losing protein to amyloid formation itself.

In the current study, we sought to determine the mechanism of membrane integrity loss using the binding of IAPP to a model membrane. Our strategy was to treat the evolution of membrane-bound states of IAPP as a chemical reaction characterized by conformation and oligomeric changes. Leakage in these experiments, although of potential pathological relevance, is simply the experimental readout used to study the protein’s kinetics. A com-
bination of ensemble and single-molecule based methods enable us to delineate between alternative origins of membrane integrity loss. We interpret our results by developing a minimal model that is relevant not only to IAPP but may be extended to other systems where membrane leakage occurs in response to a membrane-mediated disorder-to-order transition.

Results

In order to elucidate the molecular mechanism by which mem-
brane-bound states of IAPP disrupt lipid bilayers, we observed the evolution over time of leakage of unilamellar 1,2-dioleoyl-sn-
glycero-3-phospho-(1′-rac-glycerol) (DOPG) liposomes induced by rat IAPP. All experiments, unless otherwise stated, were con-
ducted at 25 °C in 50 mM MOPS, 125 mM KCl, 10 μM EDTA, 200 μM lipid (in monomer units), and pH 7.4. Previous measure-
ments of IAPP by our lab and others have shown rat IAPP to
be monodisperse and monomeric under similar conditions prior to interaction with bilayers (11, 15). Insights are gained in this work through the use of independent reporters of leakage com-
bined with ensemble and single-molecule measurements of fluorescence.

IAPP induces leakage in liposomes with rates that are de-
pendent on protein concentration. Liposomes were prepared in the presence of 440 μM Ca2+ and 500 μM of Fluo-8, a Ca2+
sensitive fluorophore. Extraluminal Ca2+ and fluorophore were then removed by gel filtration into an EDTA-containing buffer. The kinetics of leakage were monitored as a decrease in fluorescence upon addition of varying amounts of IAPP to a fixed amount of liposomes. At 10 μM IAPP a single exponential decay to approxi-
mately 2% of the initial intensity is observed (Fig. L4 and Fig. S1, circles), and fits to a rate constant of 6.3 × 10−4 ± 0.1 × 10−4 s−1.

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Fig. 1. IAPP-induced leakage from liposomes. (A) Liposomes were prepared with encapsulated dye (Fluo-8 and Ca\(^{2+}\)) and extraluminal EDTA. Representative leakage profiles at 10 \(\mu M\) (circles) and 20 \(\mu M\) (squares) IAPP are shown with solid lines representing single exponential fits. (B) Liposomes were prepared with membrane-associated Oregon Green DHPE. Representative leakage profiles are observed by addition of the fluorescence quencher, DPX, immediately \((t_{\text{DPX}} = 0 \; \text{h}, \text{filled circles})\) or 2 d \((t_{\text{DPX}} = 48 \; \text{h}, \text{unfilled circles})\) after addition of 8 \(\mu M\) IAPP to liposomes. (C) Semilog plot of average \((N \geq 8)\) leakage rate constant versus protein concentration for initial \((t_{\text{DPX}} = 0 \; \text{h}, \text{filled circles})\) and delayed leakage \((t_{\text{DPX}} = 48 \; \text{h}, \text{unfilled circles})\). Solid line shows parameters derived from global fit of heterogeneous oligomer model (B) (see Discussion) to 50 kinetic profiles collected at equilibrium \((t_{\text{DPX}} = 48 \; \text{h}; \text{see Fig. S5})\). (Inset) Repploting of data taken at \(t_{\text{DPX}} = 0 \; \text{h}\) showing broader concentration range.

At 20 \(\mu M\), the rate constant increases to \(21.0 \times 10^{-4} \pm 0.5 \times 10^{-4} \; \text{s}^{-1}\) (Fig. 1A, squares). Experiments conducted over a decade of protein concentrations \((4.5-50 \; \mu M)\) give a profile of rate constants suggestive of a change in mechanism (Fig. 1C, filled circles). The apparent reaction order changes from approximately 3.8 for 4–10 \(\mu M\) IAPP to approximately 1.7 for 12–20 \(\mu M\) IAPP, implying a change in leakage mechanism. This change is not a consequence of IAPP-induced liposome aggregation because fluorescence correlation spectroscopy (FCS) shows unchanged liposome diffusion rates at concentrations less than 30 \(\mu M\) IAPP (Fig. S2).

Single-particle analysis can delineate between alternative origins of membrane integrity loss. For example, consider a point in time where a population-averaged measure of leakage (e.g., Fig. 1A, circles) is 50% complete. Typical leakage measures cannot directly distinguish between a process that results in 100% exchange of 50% of the liposomes and a process in which there is 50% exchange of 100% of the liposomes. To address this, liposomes were prepared as above, with the addition of a spectrally separable Ca\(^{2+}\)-insensitive membrane-bound fluorophore Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylenammonium salt (Texas Red DHPE). This approach, akin to one applied to melittin (16), permits single liposomes to be measured as two-color fluorescent bursts in the red (Texas Red) and green (Fluo-8) channels of a confocal microscope. The ratio of green to red fluorescence counts in a burst of photons reflects the extent of leakage of an individual liposome. Unleaked (Fig. 2A) and fully leaked control liposomes (Fig. 2B) are readily distinguished from background and from one another. Importantly, artificial samples in which 50% of liposomes are 100% exchanged (Fig. 2C), are readily distinguished from samples in which 100% of liposomes are 50% exchanged (Fig. 2D). Clearly, this approach can directly distinguish fully leaked from partially leaked states.

Single-particle measurements establish that the initial phase of IAPP-induced membrane leakage follows an all-or-none mechanism. For 5 \(\mu M\) IAPP, the half-life of leakage is approximately 20 h (Fig. 1C). Single-particle analysis under these conditions typically records approximately 1,000 events over a 2-h period beginning 12 to 24 h after initiation of the reaction. Importantly, only leaked and unsealed liposomes are apparent in the analysis (Fig. 2E). Statistical assessment of this observation was made by performing repeated assays over a concentration range of 4–7 \(\mu M\) IAPP and making observations at time points near the half-life of the ensemble leakage. For each assessment, the ensemble leakage was determined from the average green/red fluorescence ratio measured across all liposomes. Using controls (Fig. 2C and D), we established a cutoff for this ratio below which liposomes could be unequivocally characterized as fully leaked. A comparison of the ensemble average leakage with the fraction of fully leaked liposomes shows a one-to-one correspondence \((R^2 = 0.73, \text{Fig. 2F})\). In order for the former measure to be fully accounted for by the latter, leakage must follow an all-or-none mechanism.

The extent of membrane integrity loss increases with prolonged exposure. In order to make assessments of membrane
integrity at time points after protein exposure, a leakage-resistant reporter was used in place of Fluo-8. Specifically, liposomes were either doped (1:1,000) with Oregon Green–labeled DHPE or prepared so as to encapsulate 70-kDa fluorescein-labeled dextran. The large size of the dextran ensures that the majority of it is retained in the liposome lumen, even when smaller molecules leak out upon exposure to IAPP (Fig. S3). For both reporters, leakage was then measured at time points after protein exposure by addition of the soluble quencher p-xylene-bis-pyridinium bromide (DPX). This approach generates two time-based frames of reference. First, the protein:liposome interaction is initiated at $t = 0$. Second, kinetic profiles of leakage are initiated at the time of DPX addition, $t_{\text{DPX}}$. Direct comparison of Ca$^{2+}$-based protocols to DPX measurements at $t_{\text{DPX}} = 0$ show Fluo-8 and DPX assays to have comparable kinetic profiles (Fig. S4). In marked contrast, when leakage is observed with the addition of DPX 48 h after addition of protein ($t_{\text{DPX}} = 48$ h), an increase in the leakage rate constant is observed (Fig. 1B). Importantly, this difference occurs only above a critical point of approximately 8 μM IAPP. For concentrations >10 μM, this increase exceeds an order of magnitude (Fig. 1C, unfilled circles) limiting the practical concentration range for observable kinetics to 4.5–14 μM IAPP. Over this range, rate constants are observed with a profile that is asymptotic to the Fluo-8 detection of leakage measured immediately upon protein addition (i.e., equivalent to $t_{\text{DPX}} = 0$; Fig. 1C, filled circles).

The presence of a persistent capacity to leak requires that IAPP stabilize defects in the membrane. Our observations eliminate leakage mechanisms predicated solely on the active re-equilibration of bilayer stress caused by binding. For example, carpeting and detergent models propose that stress is caused by an initially unequal distribution of peptide (carpeting) or lipid (detergent) between leaflets of the membrane (17, 18). Transient rifts associated with relaxation of this stress are accompanied by leakage. Importantly, leakage ceases once equilibrium is regained. In this work, the capacity of the liposomes to leak is retained at equilibrium (Fig. 1B and C), indicating the formation of a stable carpeting or lipid/protein complex or phase. This state must represent some form of protein-stabilized hole, as liposomes are neither dispersed nor aggregated under these conditions (Fig. S2). The kinetic profiles we observe here report on the nature of these stabilized holes and the processes by which they are created and evolve.

Leakage kinetic profiles suggest the adoption of leakage competence is a consequence of a multistep process. To probe the evolution of leakage competence evident at $t_{\text{DPX}} = 48$ h (Fig. 1C), we first evaluated a progression of short $t_{\text{DPX}}$ values. For example, a representative assay conducted at 8 μM IAPP and $t_{\text{DPX}} = 0$ has a half-life of approximately 1,500 s. The same reaction conducted with $t_{\text{DPX}} = 950$ s demonstrates quenching of fluorescence within the dead-time of this experiment (approximately 20 s) (Fig. 3A). Importantly, the magnitude of the dead-time change corresponds to the level of fluorescence observed for the $t_{\text{DPX}} = 0$ at $t = 950$ s. This is followed by exponential decay that mirrors the profile of the $t_{\text{DPX}} = 0$ reaction. Similar results were apparent for other values of $t_{\text{DPX}}$, such as 4,000 s (Fig. 3A), assayed across the relaxation profile of the $t_{\text{DPX}} = 0$ reaction. For comparison, consider the expected behavior if IAPP rapidly equilibrated to a partially leaking state present across all liposomes. Such behavior would be characterized by identical profiles regardless of the value of $t_{\text{DPX}}$ (e.g., dashed line at $t = 4,000$ s). Instead, it is clear that once an individual liposome has been induced to leak, its capacity to leak is rapid and persistent.

An additional relaxation process reduces, but does not eliminate, the degree of lipid permeability. Kinetic profiles observed at short $t_{\text{DPX}}$ show a dead-time event (Fig. 3A), whereas those at equilibrium do not (Fig. 1B and C). To observe the transition between these behaviors, we measured liposome leakage rates over a range of $t_{\text{DPX}}$ (Fig. 3B). In a representative experiment at 7 μM IAPP, the leakage rate constant at $t_{\text{DPX}} = 2.4$ h is 0.057 ± 0.009 s$^{-1}$, whereas at 27 h it is more than 10-fold smaller, 0.0037 ± 0.0001 s$^{-1}$. Statistical examination of these decreasing rate constants as a function of $t_{\text{DPX}}$ shows relaxation behavior (Fig. 3B, Inset) that fits to a single exponential decay

\[ k(t) = k_0 \exp\left(-\frac{t}{\tau}\right) \]

with a characteristic time constant $\tau$ that is a function of $t_{\text{DPX}}$.

**Fig. 3.** Measurement of leakage at time points after exposure to protein. Liposomes were prepared with encapsulated 70-kDa fluorescein dextran or membrane-associated Oregon Green DHPE. (A) Leakage profiles are observed by addition of the fluorescence quencher DPX at the indicated ($t_{\text{DPX}}$) time points after addition of 8 μM IAPP. Dashed line shows predicted behavior at $t_{\text{DPX}} = 4,000$ s for a model in which IAPP rapidly creates semi-permeable pores (see main text). (B) Representative leakage profiles observed by addition of the fluorescence quencher, DPX, at the indicated ($t_{\text{DPX}}$) time points after addition of 7 μM IAPP. (Inset) Statistical assessment ($N = 3$) of kinetics represented by (B) fitting single exponents. Result is a plot of rate constants versus $t_{\text{DPX}}$ with solid line showing a single exponential fit to this data. (C) Acceleration of leakage rate upon secondary addition of protein. Liposomes were preincubated for 48 h with 7 μM IAPP. Leakage was measured via addition of DPX with (green) or without (violet) the further secondary addition of 2 μM IAPP 30 s prior to measurement. (Inset) Statistics ($N = 4$) of the change in apparent leakage rate constant at time points, $t_{\text{DPX}}$, after secondary addition of 2 μM protein. Starred point indicates the leakage rate constant prior to secondary protein addition. Dashed line shows the midpoint for initial equilibration of 7 μM IAPP (as determined in B). Note, rates observed in this figure are slightly elevated compared to Fig. 1a as a result of protocol-specific sample manipulation effects on protein concentration (see SI Materials and Methods).
with a constant of \(2.0 \times 10^{-4} \pm 0.4 \times 10^{-4} \text{s}^{-1}\). This behavior can be extrapolated to shorter \(t_{\text{DPX}}\) time scales. At \(t_{\text{DPX}} = 950\) s, for example, we estimate the leakage half-life to be approximately 6 s. This is shorter than the dead-time of measurement (approximately 20 s). Thus, the very rapid, dead-time leakage behavior observed at short \(t_{\text{DPX}}\) is part of the same process that gives measurable leakage profiles at later time points. Of mechanistic importance is the fact that this process completes its equilibration on the hours timescale and yields permeable liposomes.

Formation of the leakage-competent state is a nucleation-dependent process. Initial leakage profiles are exponential (Fig. 1A), result in all-or-none leakage (Figs. 2 and 3A), and lead to persistent leakage competence at equilibrium (Fig. 1B and C). The apparent reaction order is 1 at all concentrations, indicative of the requirement of multiple IAPP molecules for the stabilization of a pore. This behavior is suggestive of a stochastically initiated event such as a phase transition between leaking and unleaking states and/or formation of definable protein/lipid structure. For an assembly reaction to be characterized as nucleation dependent, it must further be shown that preexisting product can catalyze (or seed) formation of new product from precursor (19).

Consider that after 48 h, for example, 7 \(\mu\)M IAPP fully equilibrates its binding with DOPG liposomes. Leakage observed at this time point occurs with a rate constant of \(7.1 \times 10^{-4} \text{s}^{-1}\) (Fig. 3C). If a further 2 \(\mu\)M of fresh IAPP is added to these liposomes, bringing the total IAPP concentration to 9 \(\mu\)M, leakage measured approximately 30 s after protein addition occurs with a rate constant of \(4.2 \times 10^{-3} \text{s}^{-1}\) (Fig. 3C). This represents an acceleration of approximately 6-fold. The expected rate constant (Fig. 1C) for 2 \(\mu\)M IAPP at \(t_{\text{DPX}} = 0\) is approximately \(1.3 \times 10^{-3} \text{s}^{-1}\). Thus, if the 7 and 2 \(\mu\)M populations of protein were acting independently of one another, the contribution of the 2 \(\mu\)M component would be vanishingly small. Alternatively, the expected rate constant (Fig. 1C) for 9 \(\mu\)M IAPP is \(3.6 \times 10^{-3} \text{s}^{-1}\) at \(t_{\text{DPX}} = 0\), significantly slower than what we measure. Plainly, the preexisting presence of 7 \(\mu\)M equilibrated IAPP greatly accelerates the initial rate of change in the leakage rate constant upon IAPP addition. Statistics for this measurement were collected as function of \(t_{\text{DPX}}\) (Fig. 3C, Insert). Note that here \(t_{\text{DPX}}\) represents the time of DPX addition after addition of 2 \(\mu\)M fresh IAPP. In this situation, the observed rate constant has reached the midpoint of its transition as of the first measured profile at 45 s. This contrasts sharply with the time required for the initial 7 \(\mu\)M protein to reach its equilibrated state (approximately 1 h). This two orders of magnitude increase in equilibration rate constant clearly indicates that new IAPP introduced to the system is rapidly incorporated into preexisting structures. Collectively, these characteristics are hallmarks of a nucleated process (19).

A persistent leakage-competent state is also observed for human IAPP. Experiments with human IAPP are limited by its conversion to amyloid fibers. Fiber formation can be monitored kinetically using unlabeled liposomes and introduction of thioflavin T (ThT), a fluorescent indicator of amyloid conversion (20). At 4 \(\mu\)M protein and 200 \(\mu\)M lipid, human IAPP converts to amyloid with a midpoint, \(t_{\text{DPX}}\), of 3,600 s ± 900 s (Fig. 4). Under the same conditions, but without ThT and using labeled liposomes, leakage was monitored with quencher applied at various \(t_{\text{DPX}}\). At \(t_{\text{DPX}} = 0\), leakage can clearly be seen to occur within the lag time of fiber formation (Fig. 4), consistent with previous reports (8). Importantly, for \(t_{\text{DPX}} > 0\), but within the lag time, we observe dead-time changes in fluorescence. As with rat IAPP (Fig. 3A), the magnitude of these changes are closely similar to the total leakage for the profile at \(t_{\text{DPX}} = 0\). The leakage rate constant is approximately 40-fold faster at \(7.4 \times 10^{-4} \text{s}^{-1} \pm 0.2 \times 10^{-4} \text{s}^{-1}\), compared to an extrapolated rate constant of \(1.7 \times 10^{-5} \text{s}^{-1}\) for rat IAPP at 4 \(\mu\)M. Thus, human IAPP mirrors qualitatively, albeit not quantitatively, the behavior of rat IAPP in its ability to induce a persistent, membrane permeabilized state without conversion to a \(\beta\)-sheet–rich amyloid.

**Discussion**

Our overall goal is a determination of the mechanism by which IAPP disrupts membrane integrity. Toward this end, we have shown the following. (i) The initial disruption of a lipid bilayer upon exposure to IAPP results in all-or-none liposome leakage. (ii) The rate of formation of leakage-competent liposomes is concentration-dependent, with an apparent reaction order that changes with protein concentration. (iii) Two relaxation phases are evident in the leakage. One corresponds to the stochastic formation of leakage-competent states. The second corresponds to evolution to an equilibrium leakage rate. (iv) Below a critical concentration, equilibrium leakage behavior is not distinct from initial leakage behavior. (v) The leakage-competent state acts in a manner akin to seed, capable of rapidly incorporating additional protein precursor. (vi) Leakage-competent states formed on the hour time scale are stable for days.

These characteristics define a leakage mechanism where a porous phase of protein is formed due to stochastic nucleation and subsequently evolves to a final equilibrium state. Discrete pore-like structures of IAPP have been suggested previously from electrophysiological (21) and electron microscopy (22) studies. Other groups (23), including our own (8) have suggested persistent and porous mat-like proteolipid structure on the membrane. Such states have also been characterized as less structurally defined chaotic pores (24, 25), an appealing view given the non-specific and pathological nature of aggregation.

Equilibrium analysis of the current data is consistent with our previous efforts showing that heterogeneously sized, \(\alpha\)-helix–rich oligomers are correlated with leakage (8). In that work, binding isotherms were modeled by adapting the work of Wimley et al. (26). Briefly, a simple partition \((K_c)\) divides IAPP between an aqueous and monomeric lipid-bound phase, and a single nucleation \((\sigma)\) and propagation \((\lambda)\) parameter define the relative distributions of monomer- and oligomer-bound fractions. Using the 50 equilibrium \((t_{\text{DPX}} = 48 \text{ h})\) leakage profiles as a function of protein concentration, this same model and global analysis results in parameters consistent with published values from equilibrium-binding isotherms (8), and FCS (27) (Fig. 1C, Table S1, and Fig. S3). The results of this model are also qualitatively similar to a simple partitioning of protein above a critical concentration into an oligomeric state, as is seen with many antimicrobial peptides such as melittin and alamethicin (28). A similar critical concentration is seen in the current model at approximately 8 \(\mu\)M IAPP, above which the fraction of protein in an oligomeric state increases dramatically. An additional parameter of the present analysis is \(\lambda\), defined as the leakage rate constant per unit protein in the oligomeric fraction. Using this parameter, we can estimate...
that the fastest leakage rates observed in this work (i.e., those 
<20 s; Fig. 3A) correspond to the presence of approximately 
10,000 molecules of IAPP in the oligomeric form per liposome. 
The consistency of the heterogeneous aggregation model with 
two broadly different classes of data strongly supports the idea 
that an oligomeric, protein–lipid state is responsible for loss of 
membrane integrity.

This nucleation-dependent model is insufficient to account for 
the two phases of leakage evident at protein/lipid ratios above a 
critical concentration. Consider that above the critical concentra-
tion the rate of apparent leakage evolves from faster than dead-
time (<20 s) to the measurable rates observed at equilibrium 
(Fig. 3B). This indicates that the behavior of the protein and lipid 
involved in leakage competence is evolving. Many models can 
be envisioned to capture such behavior, but these fall within two 
limiting possibilities: (i) Oligomers of different size have leakage 
rates that vary with their size. The evolution of leakage behavior 
reflects equilibration of the population and size distribution of 
the oligomers. (ii) Equilibration of the oligomer distribution oc-
curs within the dead-time of measurement, but a further change 
in state, such as a conformational change, results in a diminished 
capacity to leak. Although either scenario could explain our 
observations, we favor the first possibility because it provides 
a framework that more readily accounts for behavior below 
the critical concentration. Below the critical concentration, popu-
lations of leakage-competent oligomers are vanishingly small. 
Thus, reaching the equilibrium distribution is trivial and rapid. 
Small oligomers nevertheless form under this regime, but they 
dissociate faster than any processes that might give rise to an 
increase in size. By contrast, for the second limiting possibility, 
oligomers formed at low protein/lipid ratios would be required 
to bypass the initial conformation assembled at higher concen-
trations and assemble directly to the equilibrium conformation. 
Importantly, we note that for either limiting case, a simple 
phase-transition model between monomer (non-leaking) and 
oligomer (leaking) is insufficient, which would not allow 
for the two observed phases of pre-equilibrium leakage. Graded 
leakage caused by semipermeable holes, although possible, is not 
evident here as we observe all-or-none leakage below (Fig. 2) and 
above (Fig. 3A) the critical concentration. Any leakage events, 
regardless of the size of oligomers present on the membrane, 
leads to complete leakage of vesicle contents. We therefore in-
volve a third state, which, given the small size of IAPP, is most 
likely to be oligomeric.

Our observations are satisfied by a model (Fig. 5) with two 
oligomeric classes: those that leak and those that do not. In 
addition, initially formed species are leakage incompetent, and 
these are overall more stable and therefore more highly popu-
lated than leakage-competent states. Conversion of leakage 
incompetence to competence can be a consequence of confor-
mational change. Alternatively, it can be regarded as the result 
of an oligomer nucleating and/or stabilizing a surface tension-
induced membrane defect (29). In either case, this formulation 
allows for slow apparent leakage rates because leakage is limited 
by the forward rate of conversion from the non-leaking to leak-
age-competent oligomeric state. That we observe only all-or-none 
leakage simply requires that the reverse rate (rate of closure) be 
slow compared to the time required to equilibrate the lumen of 
the liposome with external buffer (18). Two oligomer classes also 
allow us to reconcile the approximately 10,000 IAPP oligomers 
in monomer units per liposome needed to obtain dead-time 
leakage rates (see above). The high number reflects a leakage 
mechanism that is instead mediated by a subset of the total 
protein in the oligomeric fraction. The exact nature of the hole 
is not accessible in this work. However, because carpeting and 
detergent models can be ruled out (see Results above), the leaking 
oligomer is some form of pore, the possibilities of which include 
barrel-stave, toroidal, or chaotic models.

Before equilibration and above the critical concentration, we 
believe formation of oligomeric species to be followed by seeded 
expansion (Fig. 5A). In this case, rapid expansion subsequent to 
an initial nucleation event will result in the formation of a single 
large oligomer. This is the origin for the generation of nonequi-
librium distributions of oligomeric species and is supported by 
the capacity of oligomers to act as seed to further addition of 
IAPP (Fig. 3C). There is a subsequent relaxation to the final 
distribution of smaller oligomers. However, in order to reconcile 
the evolving leakage behavior evident under such conditions 
(Fig. 3B), there must be size dependence to the rate of hole for-
formation by oligomers. One possibility we suggest is that larger 
oligomers more readily sample and persist in the leakage-compe-
tent form. Thus, we are able to accommodate a very complex 
set of new kinetic observations by adding only a single state to 
our established model used for equilibrium binding studies of 
α-helical aggregated states of IAPP.

The conformations and membrane interactions of IAPP evi-
dent here are likely sampled in vivo. We have previously noted 
that the binding affinity of IAPP for lipid bilayers is strongly 
dependent on charge interactions (30). It would therefore be 
reasonable to expect that with diminished charge density present 
on biological membranes, the observations reported here might
diminish in magnitude. However, as a secreted protein, IAPP is normally present at concentrations that may rise as high as 4 mM after packaging in the granule (6, 31). Our experiments here are 100- to 1,000-fold lower in concentration. It would therefore be reasonable to expect that with increased protein concentrations, a greater sampling of membrane-bound states would be evident. Other relevant physiological elements include the effects of binding partners, such as insulin, and increased temperature. We have previously shown that structure-based small molecules that target the non-amyloid, membrane stabilized α-helical states of IAPP are protective of IAPP-induced toxicity in cell culture (14). This suggests that the leakage properties observed here, mediated by non-amyloid membrane conformers, will serve as important surrogates in other efforts aimed at understanding the in vivo effects of insulin, temperature, protein concentration, and membrane chemistry, and their potential relevance to therapeutic development.

The significance of what we have demonstrated is that non-amyloid IAPP oligomers induce membrane leakage through a mechanism that incorporates both transient and stable characteristics. Elements of this behavior can be seen in functional protein/membrane systems such as antimicrobials (32) and mitochondrial-associated apoptotic factors (33). These proteins, magainin and Bax, respectively, have been shown to follow a kinetic profile similar to our report here in which stochastic nucleation is followed by a subsequent slowdown to a nonzero equilibrium leakage rate. We also note that leakage in the antimicrobial, Cecropin A, has been suggested to be rate limited by changes in state and not pore size (24). Further parallels can be seen in Aβ from Alzheimer’s disease (34), where, for example, the size distribution of oligomers is reported to have an effect on overall leakage rates (35). Thus, the behavior of IAPP and likely other peptide amyloids such as Aβ, represents a generic property of the interaction of amphipathic peptides with biological membranes. The importance of a clear identification of intermediate states, such as described here, holds the promise of illuminating mechanism across both pathological and functional investigations of this phenomenon.

Materials and Methods

Materials. Rat and human IAPP were synthesized using standard t-Boc methods and purified by HPLC. Stock solutions (approximately 1 mM) were prepared in water and DMSO, respectively. Large unilamellar vesicle stock solutions (approximately 5 mM) were prepared in buffer by extrusion of DOPC using 100- or 200-nm-diameter pores. For additional details, see SI Materials and Methods.

Leakage Measurements. Leakage reactions were initiated by first diluting IAPP stocks into buffer. Reactions were considered initiated upon dilution of liposomes into this solution. Leakage and amyloid reactions were monitored using a Quantamaster C-61 spectrophotometer (PTI) to measure the fluorescence of Fluo-8, Oregon Green DHRPE, fluorescein dextrans, and ThT. Single liposome measurement of leakage was performed using a home-built inverted microscope fitted with laser excitation and two-channel detection. Photon traces were collected with 1-ms time bins. For additional details, see SI Materials and Methods.

Analysis. Unless otherwise stated, all points shown in this work represent an average from at least three independent measurements. Displayed confidence intervals represent one standard deviation. All fitting and global analysis performed with MATLAB (MathWorks). For additional details, see SI Materials and Methods.

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