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Helix stabilization precedes aqueous and bilayer catalyzed fiber formation in islet amyloid polypeptide

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Abstract

Islet amyloid polypeptide (IAPP) is an unstructured polypeptide hormone that is cosecreted with insulin. In patients with type 2 diabetes, IAPP undergoes a transition from its natively disordered state to a highly ordered, all β -strand amyloid fiber. Although predominantly disordered, IAPP transiently samples α -helical structure in solution. IAPP adopts a fully helical structure when bound to membrane surfaces in a process associated with catalysis of amyloid formation. Here, we use spectroscopic techniques to study the structure of full-length, monomeric IAPP under amyloidogenic conditions. We observe that the residues with helical propensity in solution (1-22) also form the membrane-associated helix. Additionally, reduction of the N-terminal disulfide bond (Cys2-Cys7) decreases the extent of helix formed throughout this region. Through manipulation of sample conditions to increase or decrease the amount of helix, we show that the degree of helix formed affects the rate of amyloid assembly. Formation of helical structure is directly correlated with enhanced amyloid formation both on the membrane surface and in solution. These observations support suggested mechanisms in which parallel helix associations bring together regions of the peptide that could nucleate β -strand structure. Remarkably, stabilization of non-amyloid structure appears to be a key intermediate in assembly of IAPP amyloid.

Keywords

islet amyloid polypeptide; type 2 diabetes; amylin; intrinsically disordered proteins; amyloid

Introduction

The aberrant conversion of normally soluble proteins into fibrillar assemblies is a hallmark of amyloid disease.^{1,2} These diseases have diverse phenotypes depending on the identity of the protein and the location of deposition *in vivo*. Examples include deposition of the protein A β in the brains Alzheimer's patients, β -2 microglobulin in the joints of end-stage renal disease patients, and islet amyloid polypeptide (IAPP) in the endocrine pancreas of type 2 diabetics.² The proteins associated with such disorders have diverse native structures, yet all assemble into a common, highly ordered filamentous structure. Amyloid fiber structure is

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cross- β defined as a β -sheet assembly comprised of two or more sheets in which the β -strands run perpendicular to the long axis of the fiber.³ Amyloid fibers form via a nucleation dependent mechanism in which intermediate states are transiently populated.⁴⁻⁶

One common feature of amyloid assembly intermediates is the transient adoption of partially structured states. For amyloid precursors that are natively folded, destabilizing mutations and/or changes in solution conditions are frequently associated with accelerated fibrillogenesis. For example, familial mutations in lysozyme are a prerequisite to amyloid disease and give rise to a functional enzyme that more readily populates partially folded states.⁷ In other amyloid systems, the precursor proteins are intrinsically disordered. Such proteins include A β , IAPP, and α -synuclein.^{2,8} For these proteins, it is the sampling of partially structured states that is correlated with the rate of conversion to amyloid. For fibrillogenesis of α -synuclein, which is associated with Parkinson's disease, particular natural sequence variants are associated both with accelerated assembly and specific adoption of transiently structured states.⁹ Study of pre-amyloid order-disorder transitions is therefore central to understanding both assembly mechanism and disease pathology in all amyloid systems.

IAPP (also known as amylin) is an unstructured peptide hormone, cosecreted with insulin by the islet β -cells of the pancreas.¹⁰ In type 2 diabetics, IAPP undergoes conformational changes that result in self-assembly into amyloid fibers. These fibers are associated with β -cell depletion that leads to insulin replacement therapy.¹¹ The IAPP found in amyloid plaques is wild type and unmodified,^{12,13} suggesting it is cellular changes associated with obesity and disease state that facilitate fiber formation.¹⁴ IAPP is 37 residues long, contains a disulfide bond between Cys2 and Cys7 and is amidated at its C-terminus (Fig. 1).^{12,13} The proposed native hormonal activities of IAPP are diverse.¹⁵ These include activities distal from the β -cell, such as gastric emptying and bone resorption, as well as local paracrine and autocrine regulation of insulin secretion.¹⁵⁻¹⁷ Both the disulfide bond and the terminal amidation are required for full IAPP hormonal activity.¹⁵

The intermediates of amyloid assembly have been generally implicated as the toxic species. Oligomer specific antibodies have been used to suggest that amyloid oligomers localize to cell membranes.^{18,19} For IAPP, exogenous addition to cell culture has been shown to induce abnormal morphology in the plasma membrane¹⁹ and to trigger disruption of cell-cell adhesion, improper insulin secretion, and apoptosis.²⁰ Furthermore, *in vitro* experiments have demonstrated that IAPP pre-amyloid oligomers permeabilize synthetic vesicles.²¹ Such permeabilization may be an intrinsic property of a membrane bound oligomer, or may be associated with the conformational transitions from oligomer to the amyloid fiber state.²² Thus, while the mechanism of IAPP toxicity is not yet known, the importance of the amyloid intermediate assembly process at membrane surfaces is clearly established.

IAPP has been shown to spontaneously bind anionic lipid bilayers *in vitro*. This binding both accelerates fiber formation and distorts the membrane.^{23,24} The membrane bound species are predominantly α -helical in structure²⁵ suggesting that stabilization of α -helical forms catalyzes β -sheet formation. Helix-to-sheet transitions are evident in other amyloid systems. This includes predominantly helical globular proteins such as insulin²⁷ and natively unstructured systems such as A β .^{26,27} Additionally, IAPP binds cooperatively to the membrane surface, suggesting the presence of at least two bound forms that differ in oligomeric size.²⁴ The larger states are most strongly associated with acceleration of amyloid formation and membrane leakage. Our working model suggests that oligomerization is mediated by parallel helix-helix interactions that lead to a high local concentration of non-helical, but amyloidogenic, regions of the peptide (Fig. 1).^{8,24,28} Mature IAPP fibers are composed of parallel β -sheets,^{29,30} therefore surface binding in such

a parallel arrangement can be conjectured to serve as catalyst. A structural understanding of the basis of helix-sheet transitions in IAPP both in solution and on lipid bilayers is clearly required.

We have previously reported that IAPP from rat (rIAPP) adopts a transiently helical structure in solution between residues 5-22.³¹ rIAPP differs from human IAPP (hIAPP) at five residues outside the helical region (Fig. 1a) resulting in a system that is indefinitely soluble under physiological conditions. This facilitates structural studies of preamyloidogenic membrane-bound states. In this work, we use NMR spectroscopy to interrogate the structure of pre-fibrillar hIAPP and rIAPP under fiber forming conditions. The role of observed structures in subsequent amyloid formation is qualitatively assessed by monitoring effects of altered conditions on both assembly kinetics and identified structures. We test the hypothesis that helical structures transiently sampled in solution are essential to nucleation and that lipid bilayer catalysis is mediated by stabilization of these same states

Results

The goal of this study is to determine IAPP structural states formed prior to amyloid formation and their relevance to assembly. IAPP is studied under standardized solution conditions (50 mM potassium phosphate, 100 mM potassium chloride, and 10% D₂O at 4°C and pH 5.5, unless otherwise noted). We first examine the solution and bilayer-bound properties of the non-amyloidogenic IAPP sequence variant from rat (rIAPP). Next, we extend and relate our study to the solution properties of the amyloidogenic human IAPP variant (hIAPP). NMR spectroscopy is the primary tool used for structural assessment, as it is ideal for characterizing dynamic and partially ordered conformations. Far-UV CD is used to complement the NMR data and to calculate thermodynamic parameters. Lastly, fluorescence spectroscopy is used to characterize kinetics of amyloid assembly by hIAPP. Perturbations to structure and kinetics are made through alteration of temperature, addition of denaturant, fluorinated alcohol or membranes, and reduction of the intrinsic disulfide bond.

Rat IAPP in solution

rIAPP under our standard conditions is predominantly monomeric. Pulsed field gradient NMR (PFG-NMR) was used to measure translational diffusion constants for rIAPP at 25°C to more accurately determine the dominant oligomeric state.³² The diffusion constants of rIAPP at 1.5 mM and 100 μM are $1.50 (\pm 0.05) \times 10^{-6} \text{ m}^2/\text{s}$ and $1.75 (\pm 0.07) \times 10^{-6} \text{ m}^2/\text{s}$, respectively. The predicted translational diffusion constants for monomeric and dimeric rIAPP are $1.59 (+0.24, -0.28) \times 10^{-6} \text{ m}^2/\text{s}$ and $1.17 (+0.19, -0.23) \times 10^{-6} \text{ m}^2/\text{s}$, respectively. The diffusion constants of rIAPP at both concentrations are within error of the predicted monomer value. These results agree with a sedimentation velocity analytical ultracentrifugation report of soluble IAPP under conditions similar to ours that was unable to detect small ($n < 100$) oligomers.³³ Our NMR approach is particularly sensitive to the presence of low-order oligomers, even if such states have no single dominant size. This is noteworthy as we observe diffusion at 1.5 mM to be measurably smaller than at 100 μM. Both specific and non-specific self-associations may contribute to this difference. However, the decrease we observe is consistent with concentration effects measured for other disordered peptides.³⁴ Furthermore, we see no evidence of changes to chemical shifts with changes in protein concentration (not shown). We therefore assert that under the conditions used in this work, soluble rIAPP is predominantly monomeric.

The rIAPP structured helical population is small and is not affected by the intrinsic disulfide bond. We previously reported rIAPP is transiently structured as an α -helix between residues 5-22.³¹ Secondary shifts indicate some individual residues spend as much as 50% of their

time in a helix, yet the far-UV CD spectrum of rIAPP under comparable conditions shows the peptide to be predominantly random coil (Fig. 2a). We chose to characterize the thermodynamics of the helix-coil transition to further investigate the helical state. Here, helical structure was stabilized by addition of trifluoroethanol (TFE) (Fig. 2a). Measured by CD at 222 nm, rIAPP undergoes a cooperative transition to a highly helical state between 0-40% TFE (Fig. 2b). This transition was fit to a two-state model³⁵ to give an apparent free energy for the native state to helix transition of $\Delta G_{\text{TFE}} = 3.3 \pm 0.7$ kcal/mol. This is similar to what was observed for peptide variants of the major α -helix of barnase.³⁶ The ΔG_{TFE} value suggests rIAPP samples a very small population (<1%) of fully structured helix under our standard conditions. Therefore, although individual residues are quite helical in solution, a fully formed helix exists only a small percentage of time. A low helical population is supported by the lack of any reported helical NOEs for IAPP in solution.³⁷ As disulfide bonds have been implicated in helix capping³⁸ and specifically in hIAPP helical stability,³⁷ the analysis was repeated on reduced rIAPP (rIAPP_{red}). Thermodynamic analysis of the rIAPP_{red} transition gives a $\Delta G_{\text{TFE}} = 3.9 \pm 0.6$ kcal/mol (Fig. 2b), which is not significantly different than native rIAPP. The disulfide therefore appears not to affect the stability of helical conformation.

The native disulfide bond affects structure through the entire helical region. A ¹⁵N-HSQC spectrum of rIAPP_{red} was recorded to further determine the role of the disulfide in helix structure (Fig. 3). As expected, the chemical shifts of the residues between and adjacent to the disulfide bond have shifted in the reduced spectrum. Resonances for Cys2-Ala13 are sufficiently perturbed such that their assignment cannot be inferred by simple comparison to the native spectrum. Additionally, Asn14-Asn21 resonances are apparently close to, but shifted downfield (away from helical propensity) compared to their wild type counterparts. Resonances outside the helical region, Asn22-Tyr37, overlay exactly with the spectrum of wild type rIAPP. Far-UV CD scans of rIAPP_{red} show a decrease in signal at 222 nm in both 0% and 40% TFE (Fig. 2a), consistent with a loss in helical structure upon disulfide reduction. The structural restriction of the disulfide bond does not affect the stability of the helical state, but it does appear to influence the dynamic sampling of near isoenergetic structures in the helical region.

NMR spin-relaxation rates in rIAPP indicate motional contributions on the μ s-ms timescale throughout the helical domain. ¹⁵N longitudinal (R_1) and transverse (R_2) relaxation rates were measured for 100 μ M rIAPP under standard conditions (Supplementary Fig. 1). Variations in R_1 reflect contributions from motions on the ps-ns timescale while R_2 is influenced by both fast ns-ps and slow μ s-ms motions.³⁹ The product of R_1 and R_2 (R_1R_2) can give an approximation of the relaxation component of R_2 attributed to conformational exchange (R_{ex}) by removing contributions from fast motions as well as the effects of motional anisotropy.^{39,40} The rIAPP R_1R_2 values (Fig. 4a) are elevated in the N-terminus of the peptide, indicating this region undergoes a conformational exchange process. Interestingly, R_1R_2 decreases from the N- to C-terminus, indicating a steady decrease in structure. Similar behavior has been observed in the basic region of the GCN4 leucine zipper, which undergoes rapid helix-coil exchange when not bound to DNA.⁴¹ In addition, this gradient of increasing flexibility toward the C-terminus is similarly observed in hIAPP when it adopts a helical state in association with SDS micelles.⁴²

Relaxation-compensated Carr Purcell Meiboom Gill (rcCPMG) dispersion experiments⁴³ were performed on rIAPP to directly characterize μ s-ms motions. Residue-specific ¹⁵N R_2 values for rIAPP were measured over a series of time delays (t_{cp}) as described,⁴³ with only one residue, Thr4, demonstrating a measurable dispersion profile (Fig. 4b). The observation of non-flat CPMG dispersion curves for Thr4 is consistent with a conformational exchange process. For simplicity, a two-site exchange model was assumed. Dispersion curves can be

used to find the exchange rate constant, k_{ex} (the sum of the forward and reverse rates). However, given the small magnitude of R_{ex} determined from the CPMG experiment ($R_{ex} \sim 1.6 \text{ s}^{-1}$), fitting with either the fast-limit equation or the general Carver-Richards equation is unreliable.⁴⁴ We therefore did not rigorously quantify the exchange kinetics, although the data suggest $k_{ex} > 4000 \text{ s}^{-1}$. Such a fast rate is consistent with a helix-coil transition, as seen in the 36-residue villin headpiece domain ($k_{ex} \sim 10,000 \text{ s}^{-1}$).⁴⁵ Our inability to measure exchange rates in rIAPP could result from rates outside the detection limit for CPMG, insufficient changes in chemical environment between states or that the minor population is below the sensitivity limit ($<1\%$).⁴⁴ Nevertheless, the combined R_1R_2 and CPMG data indicate structural exchange in the N-terminal regions of rIAPP with an apparent fast rate consistent with helix-coil transitions.

Rat IAPP bound to lipid bilayers

IAPP binds to and adopts a helical conformation on phospholipid bicelles. Liposomes have been used in previous characterizations of membrane bound IAPP,^{24,25} but are not readily amenable to solution NMR studies. Detergent micelles have been used for NMR studies,^{42,46} however, micelles are small, highly curved membrane models that can introduce bias into the structure. Such potential bias may be particularly relevant to the adoption of membrane bound structure by intrinsically disordered states. As a result, we have characterized IAPP bound to phospholipid bicelles. Bicelles are disk-shaped membrane bilayers comprised of long chain lipids surrounded by a ring of short chain lipids that isolate the hydrophobic core of the bilayer from solvent. Bicelle size is dictated by the ratio, q , of long to short chain lipids.⁴⁷ Here, we evaluate DHPC/(DMPC/DMPG) bicelles with $q=0.5$, which tumble isotropically in solution.⁴⁸ Addition of 20 mM bicelles (based on free lipid concentration) to 40 μM rIAPP and hIAPP in standard conditions at 30°C stabilizes helical structure, as observed by far UV-CD (Fig. 2c). These spectra are similar to what we observed for IAPP on completely anionic DOPG liposomes.²⁴ We also reported that IAPP binds cooperatively to DOPG membranes giving a distribution of surface bound oligomers. Here, the proportion of lipid:IAPP is very high (on average one IAPP molecule/bicelle surface) and the charge density is reduced. Under these conditions, at most one IAPP molecule should be associated with any given bicelle surface. It is likely, therefore, that we are observing IAPP molecules that are bound to but not self-associated on the lipid surface.

Rat IAPP forms a structured helix through its N-terminal region when bound to a membrane surface. The amide resonances of 100 μM rIAPP on 50 mM bicelles at 30°C are clearly resolved by ¹⁵N-HSQC (Fig. 5a), despite the overall size of the IAPP bicelle complex. This is commonly observed in studies of protein membrane interactions^{47,49} and is a consequence of membrane dynamics greatly diminishing the overall correlation time of the complex. H_N , N_H , C_α and C' resonances were assigned using standard heteronuclear NMR experiments.³¹ The chemical shifts of the C-terminal residues (Thr30-Tyr37) are nearly identical between bound and unbound rIAPP indicating no interaction with or structural change upon binding the bilayer. In contrast, resonances corresponding to residues 1-20 are both broadened (not shown) and shifted. Binding studies indicate a long-lived association with the membrane surface,²³ therefore broadening is likely a result of association with the large bicelle and not from exchange with free peptide. Consistent stretches of upfield and downfield deviations from empirically derived random coil values, called secondary shifts, are indicative of secondary structure.⁵⁰ We calculated secondary shifts of bicelle bound rIAPP by subtraction of sequence and temperature corrected random coil shift values.³¹ Average secondary shifts for a structured helix are +2.6 ppm for C_α and +1.7 ppm for C' .⁵¹ C_α and C' secondary shifts (δppm) of bound rIAPP (Fig. 5b,c) are clearly consistent with helical structure over residues 5-23. In fact, with only one exception (Leu12 C'), residues 5-15 exceed the average values. EPR measurements of disulfide-less hIAPP on liposomes suggest helical structure is

present across residues 9-22.⁵² Additionally, a NMR structure of non-amidated ¹⁵N-hIAPP in SDS micelles at pH 4.6 indicates helical structure across residues 5-28.⁴² Here, our H_N secondary shifts are less consistently helical than the carbon shifts (Supplementary Fig. 2). Interestingly, residues with reduced helical H_N secondary shifts correspond to those residues the EPR measurements suggest to be buried in the membrane. The H_N secondary shifts have a 3-4 residue periodic trend between residues 7-17, which would result from such a dramatic change in chemical environment. In total, our secondary shift analysis shows that full-length rIAPP on bicelles has helical structure spanning residues 5-23. This result is broadly consistent with published structural measurements, with the distinction that it represents the wildtype sequence on a bilayer surface.

Human IAPP in solution

Soluble human IAPP samples helical conformations between residues 5-19. Addition of 2M urea and filtration (0.2 μm) delayed fibrillogenesis for ~1 week, allowing assignment of H_N, H_α, C_β, C, C' and N_H nuclei. Assignments of H_N and N_H shifts in 2 M urea were transferred to standard conditions (no urea) (Fig. 6) by titration monitored by ¹⁵N-HSQC. In agreement with a ¹H NMR study of IAPP,³⁷ H_N secondary shifts indicate a helical trend for residues 6-17 under native conditions (Fig. 7a). This helical trend is reduced, but partially evident even in 2 M urea. C' and C_α secondary shifts in 2 M urea indicate a helical propensity through residues 5-18 (Fig. 7b,c). H_α secondary shifts in 2 M urea, however, are uncorrelated after residue Gln10, indicating a more disordered structure (Fig. 7d). Far-UV CD measurements of hIAPP in standard conditions show a slight loss of helical structure upon addition of urea (not shown), consistent with published observations.³⁷ Taken together, heteronuclear secondary shift analysis of hIAPP shows helical structure across residues 5-19 that is partially reduced in urea.

Temperature coefficient analysis of hIAPP reveals a urea-sensitive structured region in residues 10-18. The temperature sensitivity of backbone amide proton chemical shifts (expressed as ppb/K) is useful as an indicator of hydrogen bonding as structured protons shift less than exposed protons with changes in temperature.⁵³ These coefficients are expressed relative to empirically derived random coil values as Δppb/K to highlight regions of nonrandom structure.³¹ Temperature coefficient analysis for hIAPP was performed under native conditions and in 2 M urea by ¹⁵N-HSQC measurement over 5-30°C (Fig. 8). In native conditions, these data reveal structure between residues 10-18. Analysis of hIAPP in 2 M urea indicates this region is the most sensitive to denaturation. Urea does not affect the covalent structure induced by the disulfide bond between Cys2 and Cys7. Smaller regions of structure are also apparent in the C-terminal half of hIAPP, particularly at residues 25 and 30-31. Addition of urea also reduces the structure at these positions, but not to the dramatic extent observed in 10-18. Denaturation of the N-terminal helical structure explains the relative weakness of H_N secondary shift helical character observed in 2 M urea. The combination of secondary shifts and temperature coefficients clearly indicate helical structure between residues 5 and 19.

IAPP amyloid assembly kinetics are inhibited by addition of urea. hIAPP fibrillogenesis was initiated by dilution from water stocks to 20 μM protein into our standard NMR solution conditions at 30°C. Amyloid formation over time was detected by the change in fluorescence of 50 μM thioflavin T (ThT), an exogenously introduced dye that binds to IAPP amyloid fibers (Fig. 9 inset).⁵⁴ The characteristic sigmoidal kinetic traces were fit to extract the time at the midpoint of assembly (t₅₀). The t₅₀ of hIAPP is 8.9 ± 0.4 × 10⁴ s under our standard conditions. Addition of urea increases the t₅₀. For example, in the presence of 1 M urea the timescale of assembly is slowed by ~1.5 fold (Fig. 9). Addition of urea will undoubtedly affect many steps in assembly and therefore cannot be used alone to directly assess the role of the helix in assembly. Nevertheless, denaturation of an off-pathway

structure might reasonably have been expected to accelerate the rate of fiber formation. Instead, we observe that the levels of urea that affect the extent of residual helical structure have a significant inhibitory effect on the rate of fiber assembly.

Factors that stabilize the helical character of IAPP result in acceleration of fiber formation. The above standard reaction was modified to contain components that increase the population of helical states. TFE induces helical structure in hIAPP as it does in rIAPP (Fig. 2a,b) with a $\Delta G_{\text{TFE}} = 2.0 \pm 0.4$ kcal/mol. hIAPP fiber formation with 2% TFE is strongly accelerated, with the t_{50} decreased by 230-fold (± 30) compared to reactions conducted in the absence of TFE. Similar results have been observed with the fluorinated alcohol HFIP.⁵⁵ Anionic liposomes also induce helical structure and catalyze fiber formation.^{23,25} Here, addition of 5 mM 1:1 DOPC:DOPG liposomes decreased the t_{50} by 25-fold (± 1). Lastly, we assessed the effect of the bicelles used in our structural measurements. Addition of 20 mM DHPC/(DMPC/DMPG) bicelles to our standard conditions reduced the t_{50} by 5-fold (± 0.5). These kinetic studies therefore show that stabilization of helical structure is correlated with acceleration of fiber formation reactions. As such, these results also compliment the above observation of an inhibitory effect when the helix is denatured. We have demonstrated a correlation between the presence of structure in the helical domain and effects on amyloid assembly.

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Discussion

In this work, we have examined the pre-fibrillar conformations of IAPP under a range of amyloidogenic solution conditions. Previously, we showed that a non-amyloidogenic sequence variant of IAPP from rat is transiently α -helical across residues 5-19 with additional structure evident through residue 22.³¹ We assert that this helix formation is critical in the nucleation of fiber formation by IAPP. Using detailed structural and kinetic measurements under model and biologically relevant solution conditions, our analyses provide the following insights: (i) The native disulfide bond bridging Cys2 and Cys7 is critical to the sampling of helical structure through residue 21. (ii) Dynamic sampling occurs on timescales typical for helix-coil transitions in solution. (iii) Oligomeric states need not be significantly populated under solution conditions that show evidence of helical structure. (iv) Lipid bilayers stabilize IAPP helical structure across residues 5-23. (v) Stabilization of the α -helical conformation is correlated with accelerated formation of β -sheet rich amyloid. Our discussion below focuses on comparisons between assembly mechanisms of IAPP in solution and on membrane surfaces.

Human and rat IAPP transiently sample an α -helical structure in solution that becomes fully stabilized when bound to a membrane surface. The chemical shifts of hIAPP, as shown by ^{15}N -HSQC (Fig. 6), are nearly identical to those of rIAPP, except at and immediately adjacent to positions where the sequences differ (Fig. 1). Additionally, hIAPP secondary shifts and temperature coefficients display the same N-terminal helical trend we previously reported for rat IAPP (Figs. 7,8).^{31,37} Here, we additionally observe that residues 5-23 of rIAPP adopt a fully helical structure when bound to a bicelle surface. The helix formation on bicelles, evident by far-UV CD with both rat and human IAPP (Fig. 2c), can therefore be mapped to the N-terminal region of the peptide. Under these solution conditions, hIAPP aggregates too quickly for a direct NMR comparison to be made. However, given the near perfect sequence identity of rat and human IAPP across residues 1-22, it is reasonable to expect that hIAPP should be similarly structured through this region on the membrane surface. In fact, a recent NMR structure of non-amidated hIAPP in SDS micelles shows the formation of helix in residues 5-28.⁴² The differences in structure between hIAPP and rIAPP evident by far-UV CD are likely attributable to conformational differences past residue 23.

Formation of fiber structure is kinetically limited by nucleation processes that can be mediated by a subset of peptide residues. Nucleation phenomena have many manifestations, including generation of peptide solutions with much longer reaction times after scrupulous removal of preformed aggregates.^{30,37} The central residues of IAPP (20-29) were initially identified as the “amyloidogenic core” as these residues contain the most differences between human and rat IAPP (including three prolines in rIAPP).⁵⁶ However, recent solid-state NMR efforts indicate that IAPP fiber structure includes the majority of the peptide sequence.³⁰ The nucleating structure of IAPP appears to map to residues 1-20 as suggested, in part, by studies that alter the terminal disulfide.

The disulfide and helical region appear to serve a role in amyloid nucleation. Amyloid assembly mechanisms are complex, consisting of primary nucleation (fiber-independent), secondary nucleation (fiber-independent), and elongation events.^{4,55} Reduction or elimination of residues 1-7 affects assembly in several ways dependent on the reaction conditions. In the absence of preformed aggregates, reduced hIAPP polymerizes slower than oxidized IAPP.³⁷ Importantly, reduced IAPP reactions seeded with preformed fibers have an exponential kinetic profile, in contrast to the sigmoidal profile of seeded, wildtype IAPP reactions. Sigmoidal profiles in seeded amyloid reactions reflect the continued presence of nucleation processes. These results were therefore taken to indicate a dominant role for the disulfide in nucleation rather than elongation processes.⁵⁷ A recent structural analysis of hIAPP by ^1H NMR described a distorted twist in the disulfide as evidenced by an H_N - H_N NOE between Thr4 and Ala8.³⁷ Furthermore, analyses of IAPP₁₋₁₉ in detergent micelles show the disulfide region to be distorted and dynamic.⁴⁶ These studies identify the importance of structure and dynamics local to the disulfide. Our work here suggests the disulfide may affect nucleation processes by perturbing the sampling of helical structure throughout the N-terminal domain. Taken together, it is clear the N-terminal domain of IAPP samples specific structures relevant to fiber nucleation and that these are greatly affected by the intrinsic disulfide constraint.

Mutagenesis efforts suggest that the identified α -helical structure is responsible for nucleation. For example, hIAPP variants substituted with the proline residues of rIAPP (positions 25, 28 and 29) are capable of aggregation, albeit to a reduced extent.⁵⁸ In contrast, amyloid formation is effectively eliminated by triple proline substitutions at positions 17, 19 and 30, further illustrating the importance of positions outside residues 20-29. Solid-state NMR study of the IAPP fiber indicates a strand-turn-strand structure with the N-terminal strand ending at residue 17.⁵⁹ Whereas positions in the turn region (residues 18-27) might be expected to tolerate proline substitution, mutation at position 17 would be incompatible with

helical structure and interactions, as well establishment of β -structure in this region. In our own efforts, we showed that mutagenesis of Asn residues within the helical domain were far more deleterious than mutations to Asn residues outside this domain.⁶⁰ Furthermore, we showed that the “swap” mutants (L12N N14L) and (L16N N14L) effectively eliminate fiber formation. Reports in systems such as yeast prion suggest that formation of parallel β -sheets in amyloid is resistant to scrambling of the peptide sequence.⁶¹ The *i, i+2* mutation of L12N N14L should therefore be benign with respect to parallel β -sheet formation yet it is indefinitely soluble. In contrast, such a mutation would be disruptive in an amphipathic helix. In fact, the intrinsic helical propensity of the swap mutant is greatly reduced (Supplementary Fig. 3). It is clear that the sampling of α -helical structure in the N-terminal domain of IAPP is critical to fiber formation.

Helix formation results in parallel self-associations that correlate with membrane toxicity and amyloid structure. In EPR studies of nitroxide labeled, membrane bound hIAPP, the α -helix is clearly identified through residue 22 and is shown to be parallel to the membrane surface.⁵² Solution NMR studies of IAPP₁₋₁₉ in detergent micelles also indicate surface binding for hIAPP at pH 6.0.⁴⁶ In these studies, and ours shown here, solution conditions strongly favor monomeric forms of surface bound IAPP. As the helix is amphipathic (Fig. 1b), it is likely that helix-helix interactions will form at higher protein:lipid ratios. Such structures might occur in the plane of the membrane and could be associated with the transient sampling of transmembrane structures. The dynamic and reversible sampling of transmembrane states by helical oligomers might account for membrane leakage and integrity loss associated with cytotoxicity. This has been suggested as an origin of toxicity by Alzheimer's disease A β membrane interactions.^{8,62} Furthermore, in detergent studies of IAPP₁₋₁₉, the peptide is observed to switch from a buried to surface bound state in a pH dependent manner.⁴⁶ Importantly, these pre-amyloid membrane bound states are helical while IAPP amyloid fibers are comprised of parallel stacks of in-register β -strands.^{29,30} This observation is consistent with membrane binding resulting in a reduction of entropy that accelerates the initiation of amyloid formation at residues outside the helical domain.^{24,28}

Oligomerization and subsequent amyloid formation by membrane associated helical states of hIAPP involves residue contacts outside the helical domain. In the membrane environment, hIAPP₁₋₁₉ adopts a helical structure and does not progress to amyloid⁶³ whereas IAPP₈₋₂₀ is competent to form amyloid in solution.⁶⁴ Our NMR data with monomeric rIAPP indicate the C-terminus remains unstructured when the peptide is bound to the membrane. Even without proline residues the C-terminus is free to self-associate, as hIAPP residues 22-37⁵² or 29-37⁴² are unstructured on the membrane surface. We previously showed that binding of helical forms of IAPP to membrane surfaces is cooperative and therefore includes energetic contributions from protein-protein interactions.²⁴ At neutral pH, human IAPP binds to membranes more tightly than rIAPP. This may result, in part, from the change in charge at residue 18, which is His in hIAPP and Arg in rIAPP. NMR structural studies of the IAPP₁₋₁₉ subfragment at pH 7.3 in detergent micelles show that H18R can introduce a kink in the helix and change detergent-protein interactions. These differences are eliminated at pH 6 where His18 is more likely to be protonated.⁴⁶ In contrast, the full length human peptide binds membranes much more tightly than rat variant even at low pH.²⁴ This suggests that the differences in binding cooperativity between human and rat IAPP is a result of energetic contributions from interactions outside the helical domain. IAPP₂₀₋₂₉ and IAPP₃₀₋₃₇ can self-associate to form amyloid in isolation.⁶⁵ Cooperativity of binding evident at higher protein:lipid ratios may therefore be mediated by residues 20-37 and these interactions may have β -sheet structure. Conversely, the prolines in rIAPP would impose restrictions on the capacity of this region to self-associate. It is therefore reasonable to assert that protein-protein interactions include both helix-helix contacts as well as contacts mediated by residues 20-37.

It is plausible that nucleation in solution is mechanistically distinct from nucleation on membranes. We suggest, however, that membrane catalysis is a consequence of stabilization of nucleation competent helical oligomers also present in solution (Fig. 10a). In such a mechanism, the relative extent of the IAPP helix is correlated with amyloid assembly rate (Fig. 10b). Destabilization of the helix by denaturation or alteration of the sequence inhibits assembly, whereas stabilization by organic solvents or membrane surfaces accelerates assembly. Importantly, overstabilization of helical structure will prevent amyloid formation, as demonstrated by the stability of IAPP in solutions with high proportions of HFIP.⁶⁶ The structural model of the IAPP amyloid fiber places β -strands across residues 8-17 and 28-37³⁰ indicating the N-terminus must undergo a helix to strand transition. On the membrane, charge interactions with the anionic membrane are thought to stabilize the IAPP helix. Optimal acceleration under physiological conditions is sensitive to the percent anionic composition of the membrane.²³ Only a small change in bilayer surface properties, such as might be found along the secretory pathway of a diabetic patient, may be required for acceleration.

Stabilization of non-amyloidogenic domains to facilitate or inhibit amyloid assembly has been observed in other amyloid systems. For example, transition metal ions can bind to the N-terminus of A β ₁₋₄₀ and stabilize parallel inter-peptide association. This in turn leads to structural changes in the amyloid core and subsequent amyloid formation.⁶⁷ Parallels to our work are seen in studies of amyloid formation by α -synuclein from Parkinson's disease.⁸ Fiber formation by α -synuclein can be accelerated by low concentrations of membrane, where a partially folded structure is stabilized. However, at high membrane concentrations, a stable α -helix is formed that prevents amyloid formation.⁶⁸ We and others have shown that helical associations in IAPP are a critical factor affecting the nucleation rate and enabling fiber formation to occur at relatively low concentrations (μ M). IAPP concentrations are exceptionally high (mM) *in vivo* in the secretory granule, which might enable stabilization of helix-helix associations. Thus, it is even possible stronger helix-helix associations must be destabilized for amyloid initiation *in vivo*. Facilitating nucleation via non- β -strand mediated oligomeric associations appears to be a general phenomenon accessible to intrinsically disordered amyloid precursors. The importance of this is clear as it suggests that non-amyloid structures can be targeted for the creation of novel therapeutics.

Materials and Methods

Reagents

All oligonucleotides and synthetic peptides were synthesized in house at the W. M. Keck Foundation Biotechnology Resource Laboratory. Molecular biology reagents were obtained from New England Biolabs. Buffers, salts and solvents were obtained from Macalaster Bicknel, American Bioanalytical, J.T. Baker, or Sigma. Isotopic salts and solvent were obtained from Cambridge Isotope Laboratories. Lipids were obtained from Avanti Polar Lipids, Inc. ThioflavinT (ThT) was obtained from Acros.

Recombinant Protein

Human IAPP was expressed and purified using our published method used to produce recombinant rIAPP,³¹ using the bacterial optimized coding sequence for hIAPP.

Standard Conditions

All experiments were conducted in 50 mM potassium phosphate, 100 mM potassium chloride, and 10% D₂O at 4°C and pH 5.5. IAPP samples were prepared by dissolving lyophilized peptide in water followed by dilution to standard conditions. Water stocks of hIAPP were used within 24 hours and are stable over this time period and longer.⁶⁶

Reduction of the disulfide bond was achieved by incubation with 1 mM TCEP and confirmed by reverse-phase HPLC (not shown).

Liposomes and bicelles

Synthetic phospholipids used were 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(3-lysyl(1-glycerol)))] (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG). As purely anionic bicelles are unstable in solution,⁴⁸ 50% DMPG/DMPC bicelles were used and the liposome composition was matched accordingly. Bicelles were prepared from chloroform stocks of DHPC and 50% mix of DMPC/DMPG dried separately under argon and lyophilized overnight. DMPC/DMPG lipids were dissolved first in buffer and then added to the dried DHPC lipid making a final bicelle stock concentration of 300 mM total lipid with a 0.5 *q* value (ratio of long chain to short chain lipids). Liposome solutions contained a 50/50 mix of DOPC and DOPG from chloroform stocks dried under argon and then lyophilized for one hour before dissolving in standard buffer. Liposomes were prepared by extrusion through a 100 nm Whatman filter at a final liposome stock concentration of ~25 mM.

NMR

Lyophilized peptide was dissolved in water to half the final volume and vortexed, followed by dilution to stated concentrations in standard conditions. All spectra were recorded in house on a 500 MHz Varian Unity Inova with a room temperature or cryogenic probe. The proton chemical shifts were referenced to the water peak and the ¹⁵N and ¹³C shifts were referenced indirectly. The temperature was set to 4°C (unless noted) and calibrated with neat methanol. All NMR data were processed in NMRDraw⁶⁹ using standard window functions, zero-filling and linear prediction. All spectra were analyzed in Sparky (T.D. Goddard and D.G. Kneller, University of California, San Francisco).

H_N, N, C_α and C' resonances of rIAPP on bicelles were assigned using HNCA, HN(CO)CA and HNCO NMR experiments³¹ for 100 μM rat IAPP with 50 mM bicelles at 30°C. A ¹⁵N-HSQC spectrum was recorded with excess bicelles at 4°C, however this spectrum had only a handful of broad peaks corresponding primarily to the C-terminal residues (not shown). Increasing the temperature to 30°C improved the spectra so that all rat IAPP residues could be uniquely identified. Triple resonance backbone assignments of 100 μM hIAPP in standard conditions plus 2 M urea were made using double and triple resonance experiments as described for rat IAPP.³¹ Assignments of native human IAPP H_N and N_H shifts were determined by titration from 2 to 0 M urea using a series of ¹⁵N-HSQC experiments. All residues were assigned using this method except for Ser19 and Ser20, which overlap in 2 M urea and could not be identified as they separated at lower urea concentrations. Ser19 and Ser20 were identified by comparison to the rIAPP assignment.³¹ Ser20 is flanked by the same residues in both rat and human IAPP and was therefore inferred to be the peak that overlaps in both spectra (Fig. 3).

Translational diffusion constants were measured at 25°C as described,³² across a gradient range of 2-48 gauss/cm. Predicted translational diffusion rates were calculated from the molecular weight of the monomer or dimer using an equation empirically derived from diffusion rates of variants of the similarly disordered amyloid peptide, Aβ.⁷⁰ R₁ and R₂ relaxation rates were measured as described.⁷¹ Relaxation compensated Carr-Purcell-Meiboom-Gill (rcCPMG) dispersion experiments were measured on 100 μM ¹⁵N-rat IAPP as described.⁴³ Global fitting of Thr4 dispersion curves was performed using the equation

$$R_2(1/\tau_{cp})=R_2(1/\tau_{cp} \rightarrow \infty)+R_{ex}[12 \tanh(k_{ex}\tau_{cp}/2)/(k_{ex}\tau_{cp})].^{72}$$

Error bars for all relaxation measurements were generated from the single exponential fits calculated from single measurements. Secondary shift and temperature coefficient analyses were performed as previously described for our characterization of rIAPP in solution.³¹

Circular Dichroism

Lyophilized synthetic IAPP was dissolved in water, filtered with a 0.2 μ m HT Tuffryn syringe filter, then diluted with buffer to a final concentration of 40 μ M in standard conditions. Individual samples were made in triplicate at the indicated temperatures. CD measurements were made on an Aviv 215 spectrometer using a 1 mm cuvette. Spectra were recorded across 195-260 nm, in 1 nm steps, with 5 s averaging per step. Matched blanks were subtracted from all IAPP spectra. For the TFE titrations, mean molar residue ellipticity at 222 nm was fit to a two-state model to perform an m-value analysis.³⁵ All fitting was done in Prism (GraphPad Software, Inc.). For display purposes only, some spectra were smoothed using a sliding polynomial function built into the spectrophotometer software.

ThT plate reader kinetics

Filtered hIAPP water stocks (described above) were diluted to 20 μ M in the standard buffer plus 50 μ M thioflavin T (ThT). Trifluoroethanol (TFE), urea, bicelles or liposomes were added at the stated concentrations. Amyloid formation was monitored at 30°C (for *de novo* comparisons) or room temperature (for urea series) over time in a PTI FluoDia fluorescence plate reader with 10 nm bandpass filters for excitation at 450 nm and emission at 485 nm. Timecourses of intensity (I) over time (t) were fit in Prism (GraphPad Software, Inc) to

$$I=(I_2+m_2t+(e^{(t50t)/\tau}(I_1+m_1t)))/(1+e^{(t50t)/\tau}),$$

where t_{50} is the time at half maximum intensity, τ is a constant associated with curvature, I_1 and I_2 are the lower and upper baselines, respectively, and m_1 and m_2 are the slopes of the corresponding baselines. All kinetics were measured in triplicate with t_{50} values reported as the mean $\pm$ one standard deviation.

AGADIR analysis

AGADIR was implemented at <http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html>.⁷³ Helicity was predicted at pH 7.4 and 25°C with the C-terminus amidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Westermark P. Aspects on human amyloid forms and their fibril polypeptides. FEBS J. 2005; 272:5942–5949. [PubMed: 16302959]

2. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem.* 2006; 75:333–366. [PubMed: 16756495]
3. Tycko R. Progress towards a molecular-level structural understanding of amyloid fibrils. *Curr Opin Struct Biol.* 2004; 14:96–103. [PubMed: 15102455]
4. Ruschak AM, Miranker AD. Fiber-dependent amyloid formation as catalysis of an existing reaction pathway. *Proc Natl Acad Sci U S A.* 2007; 104:12341–12346. [PubMed: 17640888]
5. Powers ET, Powers DL. Mechanisms of protein fibril formation: nucleated polymerization with competing off-pathway aggregation. *Biophys J.* 2008; 94:379–391. [PubMed: 17890392]
6. Frieden C. Protein aggregation processes: In search of the mechanism. *Protein Sci.* 2007; 16:2334–2344. [PubMed: 17962399]
7. Chiti F, Dobson CM. Amyloid formation by globular proteins under native conditions. *Nat Chem Biol.* 2009; 5:15–22. [PubMed: 19088715]
8. Hebda JA, Miranker AD. The interplay of catalysis and toxicity by amyloid intermediates on lipid bilayers: insights from type II diabetes. *Annu Rev Biophys.* 2009; 38:125–152. [PubMed: 19416063]
9. Wu KP, Kim S, Fela DA, Baum J. Characterization of conformational and dynamic properties of natively unfolded human and mouse alpha-synuclein ensembles by NMR: implication for aggregation. *J Mol Biol.* 2008; 378:1104–1115. [PubMed: 18423664]
10. Kahn SE, D'Alessio DA, Schwartz MW, Fujimoto WY, Ensink JW, Taborsky JG Jr, Porte D. Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. *Diabetes.* 1990; 39:634–68. [PubMed: 2185112]
11. Kapurniotu A. Amyloidogenicity and cytotoxicity of islet amyloid polypeptide. *Biopolymers.* 2001; 60:438–459. [PubMed: 12209476]
12. Westermark P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, Johnson KH. Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc Natl Acad Sci U S A.* 1987; 84:3881–3885. [PubMed: 3035556]
13. Cooper GJ, Willis AC, Clark A, Turner RC, Sim RB, Reid KB. Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc Natl Acad Sci U S A.* 1987; 84:8628–8632. [PubMed: 3317417]
14. Hayden MR. Islet amyloid, metabolic syndrome, and the natural progressive history of type 2 diabetes mellitus. *Jop.* 2002; 3:126–138. [PubMed: 12221327]
15. Cooper GJ. Amylin compared with calcitonin gene-related peptide: structure, biology, and relevance to metabolic disease. *Endocr Rev.* 1994; 15:163–201. [PubMed: 8026387]
16. Cornish J, Callon KE, Lin CQ, Xiao CL, Mulvey TB, Coy DH, Cooper GJ, Reid IR. Dissociation of the effects of amylin on osteoblast proliferation and bone resorption. *Am J Physiol.* 1998; 274:E827–E833. [PubMed: 9612240]
17. Matveyenko AV, Butler PC. Beta-cell deficit due to increased apoptosis in the human islet amyloid polypeptide transgenic (HIP) rat recapitulates the metabolic defects present in type 2 diabetes. 2006; 55:2106–2114.
18. Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science.* 2003; 300:486–489. [PubMed: 12702875]
19. Janson J, Ashley RH, Harrison D, McIntyre S, Butler PC. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes.* 1999; 48:491–498. [PubMed: 10078548]
20. Ritzel RA, Meier JJ, Lin CY, Veldhuis JD, Butler PC. Human islet amyloid polypeptide oligomers disrupt cell coupling, induce apoptosis, and impair insulin secretion in isolated human islets. *Diabetes.* 2007; 56:65–71. [PubMed: 17192466]
21. Anguiano M, Nowak RJ, Lansbury PT. Protofibrillar Islet Amyloid Polypeptide Permeabilizes Synthetic Vesicles by a Pore-Like Mechanism that may be Relevant to Type II Diabetes. *American Chemical Society.* 2002

22. Engel MF, Khemtémourian L, Kleijer CC, Meeldijk HJ, Jacobs J, Verkleij AJ, de Kruijff B, Killian JA, Höppener JW. Membrane damage by human islet amyloid polypeptide through fibril growth at the membrane. *Proc Natl Acad Sci U S A*. 2008; 105:6033–6038. [PubMed: 18408164]
23. Knight JD, Miranker AD. Phospholipid catalysis of diabetic amyloid assembly. *J Mol Biol*. 2004; 341:1175–1187. [PubMed: 15321714]
24. Knight JD, Hebda JA, Miranker AD. Conserved and Cooperative Assembly of Membrane-Bound alpha-Helical States of Islet Amyloid Polypeptide. *Biochemistry*. 2006; 45:9496–9508. [PubMed: 16878984]
25. Jayasinghe SA, Langen R. Lipid membranes modulate the structure of islet amyloid polypeptide. *Biochemistry*. 2005; 44:12113–12119. [PubMed: 16142909]
26. Shao H, Jao S, Ma K, Zagorski MG. Solution structures of micelle-bound amyloid beta-(1-40) and beta-(1-42) peptides of Alzheimer's disease. *J Mol Biol*. 1999; 285:755–773. [PubMed: 9878442]
27. Kirkitadze MD, Condrón MM, Teplov DB. Identification and characterization of key kinetic intermediates in amyloid beta-protein fibrillogenesis. *J Mol Biol*. 2001; 312:1103–1119. [PubMed: 11580253]
28. Jayasinghe SA, Langen R. Membrane interaction of islet amyloid polypeptide. *Biochim Biophys Acta*. 2007; 1768:2002–2009. [PubMed: 17349968]
29. Jayasinghe SA, Langen R. Identifying structural features of fibrillar islet amyloid polypeptide using site-directed spin labeling. *J Biol Chem*. 2004; 279:48420–48425. [PubMed: 15358791]
30. Luca S, Yau WM, Leapman R, Tycko R. Peptide Conformation and Supramolecular Organization in Amylin Fibrils: Constraints from Solid-State NMR. *Biochemistry*. 2007; 46:13505–13522. [PubMed: 17979302]
31. Williamson JA, Miranker AD. Direct detection of transient alpha-helical states in islet amyloid polypeptide. *Protein Sci*. 2007; 16:110–117. [PubMed: 17123962]
32. Lapham J, Rife JP, Moore PB, Crothers DM. Measurement of diffusion constants for nucleic acids by NMR. *J Biomol NMR*. 1997; 10:255–62. [PubMed: 9390403]
33. Vaiana SM, Ghirlando R, Yau WM, Eaton WA, Hofrichter J. Sedimentation studies on human amylin fail to detect low molecular weight oligomers. *Biophys J*. 2008; 94:L45–L47. [PubMed: 18223003]
34. Mansfield SL, Jayawickrama DA, Timmons JS, Larive CK. Measurement of peptide aggregation with pulsed-field gradient nuclear magnetic resonance spectroscopy. *Biochim Biophys Acta*. 1998; 1382:257–265. [PubMed: 9540797]
35. Santoro MM, Bolen DW. Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry*. 1988; 27:8063–8068. [PubMed: 3233195]
36. Kippen AD, Arcus VL, Fersht AR. Structural studies on peptides corresponding to mutants of the major alpha-helix of barnase. *Biochemistry*. 1994; 33:10013–10021. [PubMed: 8060969]
37. Yonemoto IT, Kroon GJ, Dyson HJ, Balch WE, Kelly JW. Amylin Proprotein Processing Generates Progressively More Amyloidogenic Peptides that Initially Sample the Helical State. *Biochemistry*. 2008; 47:9900–9910. [PubMed: 18710262]
38. Lanigan MD, Tudor JE, Pennington MW, Norton RS. A helical capping motif in ShK toxin and its role in helix stabilization. *Biopolymers*. 2001; 58:422–436. [PubMed: 11180055]
39. Hass MA, Led JJ. Evaluation of two simplified 15N-NMR methods for determining micro-ms dynamics of proteins. *Magn Reson Chem*. 2006; 44:761–769. [PubMed: 16705625]
40. Kneller JM, Lu M, Bracken C. An effective method for the discrimination of motional anisotropy and chemical exchange. *J Am Chem Soc*. 2002; 124:1852–1853. [PubMed: 11866588]
41. Bracken C, Carr PA, Cavanagh J, Palmer AG. Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: implications for the entropy of association with DNA. *J Mol Biol*. 1999; 285:2133–2146. [PubMed: 9925790]
42. Patil SM, Xu S, Sheftic SR, Alexandrescu AT. The dynamic alpha-helix structure of micelle-bound human amylin. *J Biol Chem*. 2009
43. Loria JP, Rance M, Palmer AG. A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *J AM CHEM SOC*. 1999; 121:2331–2332.

44. Kempf JG, Loria JP. Measurement of intermediate exchange phenomena. *Methods Mol Biol.* 2004; 278:185–232. [PubMed: 15317998]
45. Wang M, Tang Y, Sato S, Vugmeyster L, McKnight CJ, Raleigh DP. Dynamic NMR line-shape analysis demonstrates that the villin headpiece subdomain folds on the microsecond time scale. *J Am Chem Soc.* 2003; 125:6032–6033. [PubMed: 12785814]
46. Nanga RP, Brender JR, Xu J, Veglia G, Ramamoorthy A. Structures of Rat and Human Islet Amyloid Polypeptide IAPP1-19 in Micelles by NMR Spectroscopy. *Biochemistry.* 2008
47. Vold RR, Prosser RS, Deese AJ. Isotropic solutions of phospholipid bicelles: a new membrane mimetic for high-resolution NMR studies of polypeptides. *J Biomol NMR.* 1997; 9:329–335. [PubMed: 9229505]
48. Struppe J, Whiles JA, Vold RR. Acidic phospholipid bicelles: a versatile model membrane system. *Biophys J.* 2000; 78:281–289. [PubMed: 10620292]
49. Andersson A, Mäler L. NMR solution structure and dynamics of motilin in isotropic phospholipid bicellar solution. *J Biomol NMR.* 2002; 24:103–112. [PubMed: 12495026]
50. Dyson HJ, Wright PE. Nuclear magnetic resonance methods for elucidation of structure and dynamics in disordered states. *Methods Enzymol.* 2001; 339:258–270. [PubMed: 11462815]
51. Wishart DS, Sykes BD. Chemical shifts as a tool for structure determination. *Methods Enzymol.* 1994; 239:363–392. [PubMed: 7830591]
52. Apostolidou M, Jayasinghe SA, Langen R. Structure of alpha-helical membrane-bound human islet amyloid polypeptide and its implications for membrane-mediated misfolding. *J Biol Chem.* 2008; 283:17205–17210. [PubMed: 18442979]
53. Cierpicki T, Otlewski J. Amide proton temperature coefficients as hydrogen bond indicators in proteins. *J Biomol NMR.* 2001; 21:249–261. [PubMed: 11775741]
54. Padrick SB, Miranker AD. Islet amyloid polypeptide: identification of long-range contacts and local order on the fibrillogenesis pathway. *J Mol Biol.* 2001; 308:783–94. [PubMed: 11350174]
55. Padrick SB, Miranker AD. Islet amyloid: phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis. *Biochemistry.* 2002; 41:4694–703. [PubMed: 11926832]
56. Westermark P, Engstrom U, Johnson KH, Westermark GT, Betsholtz C. Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. *Proc Natl Acad Sci U S A.* 1990; 87:5036–540. [PubMed: 2195544]
57. Koo BW, Miranker AD. Contribution of the intrinsic disulfide to the assembly mechanism of islet amyloid. *Protein Sci.* 2005; 14:231–239. [PubMed: 15576552]
58. Green J, Goldsbury C, Mini T, Sunderji S, Frey P, Kistler J, Cooper G, Aebi U. Full-length Rat Amylin Forms Fibrils Following Substitution of Single Residues from Human Amylin. *J Mol Biol.* 2003; 326:1147–1156. [PubMed: 12589759]
59. Abedini A, Raleigh DP. Destabilization of human IAPP amyloid fibrils by proline mutations outside of the putative amyloidogenic domain: is there a critical amyloidogenic domain in human IAPP? *J Mol Biol.* 2006; 355:274–281. [PubMed: 16303136]
60. Koo BW, Hebda JA, Miranker AD. Amide inequivalence in the fibrillar assembly of islet amyloid polypeptide. *Protein Eng Des Sel.* 2008; 21:147–154. [PubMed: 18299291]
61. Shewmaker F, Ross ED, Tycko R, Wickner RB. Amyloids of shuffled prion domains that form prions have a parallel in-register beta-sheet structure. *Biochemistry.* 2008; 47:4000–4007. [PubMed: 18324784]
62. Bokvist M, Lindström F, Watts A, Gröbner G. Two types of Alzheimer's beta-amyloid (1–40) peptide membrane interactions: aggregation preventing transmembrane anchoring versus accelerated surface fibril formation. *J Mol Biol.* 2004; 335:1039–1049. [PubMed: 14698298]
63. Brender JR, Lee EL, Cavitt MA, Gafni A, Steel DG, Ramamoorthy A. Amyloid fiber formation and membrane disruption are separate processes localized in two distinct regions of IAPP, the type-2-diabetes-related peptide. *J Am Chem Soc.* 2008; 130:6424–6429. [PubMed: 18444645]
64. Jaikaran ET, Higham CE, Serpell LC, Zurdo J, Gross M, Clark A, Fraser PE. Identification of a novel human islet amyloid polypeptide beta-sheet domain and factors influencing fibrillogenesis. *Journal of Molecular Biology.* 2001; 308:515–525. [PubMed: 11327784]
65. Jaikaran ET, Clark A. Islet amyloid and type 2 diabetes: from molecular misfolding to islet pathophysiology. *Biochim Biophys Acta.* 2001; 1537:179–203. [PubMed: 11731221]

66. Higham CE, Jaikaran ET, Fraser PE, Gross M, Clark A. Preparation of synthetic human islet amyloid polypeptide (IAPP) in a stable conformation to enable study of conversion to amyloid-like fibrils. *FEBS Letters*. 2000; 470:55–60. [PubMed: 10722845]
67. Lim KH, Kim YK, Chang YT. Investigations of the molecular mechanism of metal-induced abeta (1-40) amyloidogenesis. *Biochemistry*. 2007; 46:13523–13532. [PubMed: 17973493]
68. Zhu M, Fink AL. Lipid binding inhibits alpha-synuclein fibril formation. *J Biol Chem*. 2003; 278:16873–16877. [PubMed: 12621030]
69. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
70. Danielsson J, Jarvet J, Damberg P, Graslund A. Translational diffusion measured by PFG-NMR on full length and fragments of the Alzheimer A beta(1-40) peptide. Determination of hydrodynamic radii of random coil peptides of varying length. *MAGN RESON CHEM*. 2002; 40:S89–S97.
71. Doan-Nguyen V, Loria JP. The effects of cosolutes on protein dynamics: the reversal of denaturant-induced protein fluctuations by trimethylamine N-oxide. *Protein Sci*. 2007; 16:20–29. [PubMed: 17123958]
72. Millet O, Loria JP, Kroenke CD, Pons M, Palmer AG. The static magnetic field dependence of chemical exchange linebroadening defines the NMR chemical shift time scale. *J AM CHEM SOC*. 2000; 122:2867–2877.
73. Munoz V, Serrano L. Development of the multiple sequence approximation within the AGADIR model of alpha-helix formation: comparison with Zimm-Bragg and Lifson-Roig formalisms. *Biopolymers*. 1997; 41:495–509. [PubMed: 9095674]

Abbreviations used

CD	circular dichroism
DHPC	1,2-dihexanoyl- <i>sn</i> -glycero-3-phosphocholine
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DMPG	1,2-dimyristoyl- <i>sn</i> -glycero-3-[phospho- <i>rac</i> -(1-glycerol)]
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-[phospho- <i>rac</i> -(3-lysyl(1-glycerol))]
EPR	electron paramagnetic resonance
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
IAPP	islet amyloid polypeptide
MRE	mean molar residue ellipticity
NMR	nuclear magnetic resonance
TCEP	tris(2-carboxyethyl)phosphine
ThT	Thioflavin T
TFE	trifluoroethanol

Figure 1.

(a) Amino acid sequences for human and rat IAPP (Swiss-Prot P10997 and P12969, respectively). Sequence differences at six positions are indicated in bold and the disulfide bond between Cys2 and Cys7 is indicated. (b) Helical wheel representation of rIAPP₅₋₂₂ showing cationic residues (grey) and the hydrophobic moment (arrow). The hIAPP variation at position 18 is illustrated with the dashed circle. The helical wheel was generated using MPEx (S. Jaysinghe, K. Hristova, W. Wimley, C. Snider, and S. H. White (2006) <http://blanco.biomol.uci.edu/mpex>).

Figure 2.

Circular dichroism spectra of IAPP. **(a)** Far UV-CD of rIAPP_{red} (green), rIAPP (black) and hIAPP (blue) in 0% and 40% TFE. **(b)** Mean molar residue ellipticity (MRE) at 222 nm is plotted against TFE % for each IAPP variant. Average values from experiments done in triplicate are plotted as points with error bars as one standard deviation. Data were fit to a two-state model and plotted as solid lines. **(c)** Far UV-CD spectra recorded at 30°C of 40 μM rIAPP (black) and hIAPP (blue) in standard conditions and with 20 mM bicelles (red and grey, respectively).

Figure 3. ^{15}N -HSQC spectra of rIAPP under standard conditions (black) and with 1 mM TCEP (green) to reduce the disulfide bond between Cys2 and Cys7. Assignments indicate previously published native rIAPP chemical shifts.³¹

Figure 4.

(a) The product of rIAPP ^{15}N - R_1 and R_2 (R_1R_2) plotted per residue. (b) R_2 dispersion curves of rIAPP Thr4 measured by rcCPMG NMR at two field strengths. R_2 was measured as a function of the time delay, τ_{cp} .

Figure 5.

(a) ^{15}N -HSQC of 100 μM rIAPP in standard conditions (black) and with 50 mM bicelles (red) at 30°C. Labels indicate bicelle assignment. Secondary chemical shifts for C_α (b) and C' (c) for rIAPP in solution (red) and with bicelles (black).

Figure 6.

^{15}N -HSQC spectra of rIAPP (black) and hIAPP (blue) under standard conditions. Assignment labels correspond to hIAPP resonances. The spectra differ at and adjacent to where the sequences differ (indicated with bold sequence annotations).

Figure 7. Heteronuclear secondary shifts for H_N (a), C' (b), C_α (c), and H_α (d) of hIAPP (open), hIAPP in 2 M urea (black) and rIAPP (grey). rIAPP secondary shifts data are from previously reported data.³¹

Figure 8.

Effect of urea on hIAPP structure. **(a)** Deviation of temperature coefficients from random coil values for hIAPP in standard conditions (blue) and with 2 M urea (cyan). The grey box at $\pm 1.12 \Delta \text{ppb/K}$ is twice the reported variance in the random coil coefficients and serves as a threshold for significant structure formation.³¹

Figure 9.

Relative timescales of IAPP assembly kinetics under different solution conditions. IAPP kinetics were monitored over time with the change in fluorescence of the exogenous dye ThT. Kinetic profiles were fit to a sigmoid to extract the time at half-maximum intensity (t_{50}). All reactions were measured on 20 μ M hIAPP in standard conditions with 50 μ M ThT and at 30°C. t_{50} values were measured with 1 M urea, 20 mM bicelles, 5 mM liposomes, or 2% TFE and reported relative to the t_{50} in standard buffer conditions. All values reported are the mean for triplicate reactions $\pm$ one stdev. (**Inset**) Representative IAPP kinetic data (squares) showing the fit (line) and t_{50} value (dashed line).

Figure 10.

(a) Model mechanism for solution and membrane-mediated IAPP amyloid formation. (i) IAPP is natively disordered in solution. (ii) IAPP samples transient helical structure in solution (blue) and fully helical states on membranes (red). (iii) IAPP forms parallel helical oligomers on the membrane surface that facilitate amyloid nucleation in the non-helical domain. We suggest this same process occurs to a lesser extent in solution. (iv) Subsequent nucleation and elongation events lead to formation of mature amyloid fibers. This model does not preclude other parallel assembly mechanisms. (b) Energy diagram of helical stability in relation to amyloid formation.