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Association of Highly Compact Type II Diabetes Related Islet Amyloid Polypeptide Intermediate Species at Physiological Temperature Revealed by Diffusion NMR Spectroscopy

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Abstract: Self-association of human islet amyloid polypeptide (hIAPP) is correlated with the development of type II diabetes by the disruption of cellular homeostasis in islet cells through the formation of membrane-active oligomers. The toxic species of hIAPP responsible for membrane damage has not been identified. In this study, we show by pulsed field gradient NMR spectroscopy that the monomeric form of the toxic, amyloidogenic human variant of IAPP (hIAPP) adopts a temperature dependent compact folded conformation that is absent in both the nontoxic and nonamyloidogenic rat variant of IAPP and absent in hIAPP at low temperatures, suggesting this compact form of monomeric hIAPP may be linked to its later aggregation and cytotoxicity. In addition to the monomeric form of hIAPP, a large oligomeric species greater than 100 nm in diameter is also present but does not trigger the nucleation-dependent aggregation of IAPP at 4 °C, indicating the large oligomeric species may be an off-pathway intermediate that has been predicted by kinetic models of IAPP fiber formation. Furthermore, analysis of the polydispersity of the calculated diffusion values indicates small oligomeric species of hIAPP are absent in agreement with a recent ultracentrifugation study. The absence of small oligomeric species in solution suggests the formation of small, well-defined ion channels by hIAPP may proceed by aggregation of monomeric IAPP on the membrane, rather than by the insertion of preformed structured oligomers from the solution state as has been proposed for other amyloidogenic proteins.

A common pathology shared among type II diabetic patients is the accumulation of islet amyloid polypeptide (IAPP, also known as amylin) in an insoluble fibrillar form in the pancreas.[1] Similar accumulations of misfolded amyloid proteins have also been found to be characteristic of other diseases that strike primarily late in life, including Alzheimer's, Parkinson's, and Huntington's.[2] A number of in vivo and in vitro studies have shown the spontaneous aggregation of IAPP has deleterious effects on the β-cell membrane with severe consequences for insulin production.[3−5] Despite the prevalence of amyloid fibers in a growing list of human pathologies, a causative role for amyloid fibers of IAPP in type II diabetes pathology has been difficult to establish. Mature IAPP amyloid fibers are essentially nontoxic to cells.[6−10] Amyloid fiber formation also does not correlate perfectly with the loss of β-cells in type II diabetes; IAPP amyloid deposits are both found in nondiabetic patients and are not found in all patients with type II diabetes.[11,12] Furthermore, there is frequently poor correlation both temporally and spatially between amyloid deposits of IAPP and active sites of β-cell apoptosis.[13−15]

In addition to amyloid fibers, amyloidogenic proteins aggregate to a large number of other oligomeric species, which can be either on or off the pathway to amyloid fiber formation and either transient or stable in nature.[16] Rather than mature amyloid fibers, the formation of smaller oligomeric species,

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(1) Lorenzo, A.; Razzaboni, B.; Weir, G. C.; Yankner, B. A. *Nature* **1994**, *368*, 756–760.
(2) Chiti, F.; Dobson, C. M. *Annu. Rev. Biochem.* **2006**, *75*, 333–366.
(3) Haataja, L.; Gurlo, T.; Huang, C. J.; Butler, P. C. *Endocr. Rev.* **2008**, *29*, 302–316.
(4) Hoppener, J. W. M.; Ahren, B.; Lips, C. J. M. *N. Engl. J. Med.* **2000**, *343*, 411–419.
(5) Meier, J. J. *Diabetologia* **2008**, *51*, 703–13.
(6) Demuro, A.; Mina, E.; Kayed, R.; Milton, S. C.; Parker, I.; Glabe, C. G. *J. Biol. Chem.* **2005**, *280*, 17294–17300.
(7) Janson, J.; Ashley, R. H.; Harrison, D.; McIntyre, S.; Butler, P. C. *Diabetes* **1999**, *48*, 491–498.
(8) Konarkowska, B.; Aitken, J. F.; Kistler, J.; Zhang, S. P.; Cooper, G. J. S. *FEBS J.* **2006**, *273*, 3614–3624.
(9) Meier, J. J.; Kayed, R.; Lin, C. Y.; Gurlo, T.; Haataja, L.; Jayasinghe, S.; Langen, R.; Glabe, C. G.; Butler, P. C. *Am. J. Physiol.* **2006**, *291*, E1317−E1324.
(10) Ritzel, R. A.; Butler, P. C. *Diabetes* **2003**, *52*, 1701–1708.
(11) Tasaka, Y.; Nakaya, F.; Matsumoto, H.; Iwamoto, Y.; Omori, Y. *Pancreas* **1995**, *11*, 303–308.
(12) Bell, E. T. *Am. J. Pathol.* **1959**, *35*, 801–805.
(13) Huang, C. J.; Haataja, L.; Gurlo, T.; Butler, A. E.; Wu, X.; Soeller, W. C.; Butler, P. C. *Am. J. Physiol.* **2007**, *293*, E1656−62.
(14) Janson, J.; Soeller, W. C.; Roche, P. C.; Nelson, R. T.; Torchia, A. J.; Kreutter, D. K.; Butler, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7283–7288.
(15) Butler, A. E.; Janson, J.; Soeller, W. C.; Butler, P. C. *Diabetes* **2003**, *52*, 2304–2314.

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Human IAPP: KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
Rat IAPP: KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY

Figure 1. Amino acid sequences of human and rat islet amyloid polypeptides with nonconserved residues shown in red color. Both peptides are amidated at the C-terminus and have a disulfide bridge from C2 to C7 like the physiologically expressed peptides.

either on or off the cell membrane, has been identified as a critical step in amyloid-induced cell death.[17−24,45] Although intensively studied, little is known about the equilibrium between intermediate states and the formation of early oligomers of IAPP. The initial trigger for IAPP aggregation is particularly mysterious, as pathological IAPP aggregation is not associated with any common mutations.[25,26] A cross-species comparison of IAPP sequences has been helpful in this regard, in particular comparisons of human IAPP (hIAPP) with its nonamyloidogenic and noncytotoxic rat variant (rIAPP); amino acid sequences of these peptides are given in Figure 1. Significantly, while rats do not ordinarily suffer from type II diabetes transgenic mice expressing hIAPP develop a diabetes-like condition when fed a high-fat diet.[27]

It is clear that knowledge of intermediates of amyloid formation is critical for strategies aimed at reducing the toxicity of amyloidogenic proteins. The targeting of compounds to the end-stage of aggregation, the largely inert amyloid fibers, has been shown in some cases to be ineffective at reducing the toxic effect of amyloidogenic proteins.[9,28] While the targeting of compounds specifically to toxic species may yield better results, characterization of earlier oligomeric species has been hampered by experimental limitations in characterizing unstable heterogeneous mixtures that contain a wide range of molecular sizes. Size exclusion chromatography has been an invaluable technique for studying the aggregation of proteins and other biopolymers. However, the dissociation of protein complexes during elution from the column can introduce some artifacts into the determination of oligomer size distributions.[29,30] Analytical ultracentrifugation is another technique which has also afforded valuable insight into the amyloid aggregation process but is hampered by long experimental times that limit the detection of transient species.[31,32] Similarly, light scattering and cross-linking experiments also suffer from experimental limitations on the quantitation of oligomeric species.[33,34] To identify possible early oligomers of IAPP aggregation and to analyze the differences between nontoxic and nonamyloidogenic rIAPP and the amyloidogenic and toxic hIAPP, we report the characterization of the species present in solutions of hIAPP and rIAPP by pulsed field gradient (PFG) NMR spectroscopy.

PFG NMR spectroscopy is commonly used in the determination of the translational diffusion of macromolecules, particularly proteins and peptides, in solution. The size of a water-soluble protein is often a function of its degree of folding and therefore the hydrodynamic radius determined by PFG can be used to estimate the degree of protein misfolding under various conditions.[35−38] In addition to providing information on protein folding, PFG NMR can also yield important insights into the association of peptides in solution.[39−42] Therefore, PFG NMR spectroscopy is a natural choice for the interrogation of IAPP aggregation in solution including amyloidogenic peptides such as Aβ and α-synuclein.[38−42] These studies have provided valuable insights into the size and dynamics of different oligomers in solution. Accordingly, we utilized ^{1}H PFG NMR experiments to characterize the species present in solutions of IAPP peptides.

Materials and Methods

Sample Preparation. hIAPP and rIAPP with amidated C-termini (>95% purity) were purchased from SynBioSci and Genscript, respectively. Preformed aggregates were broken down by initially dissolving the peptides in a mixture of trifluoroacetic acid and hexafluoroisopropanol (1:1 ratio by volume) at a concentration of 10 mg/mL. This solution was then subjected to three repeat cycles of lyophilization and resolubilization in the trifluoroacetic acid/hexafluoroisopropanol mixture. In the final cycle, the sample was resuspended in pure hexafluorisopropanol and lyophilized under high vacuum for 8 h. Immediately before the NMR experiments, the peptides were first resuspended in cold (~4 °C) D_2O and briefly vortexed before adding concentrated (10×) nondeuterated buffer.

(16) Caughey, B.; Lansbury, P. T. *Annu. Rev. Neurosci.* **2003**, *26*, 267–298.
(17) Lambert, M. P.; Barlow, A. K.; Chromy, B. A.; Edwards, C.; Freed, R.; Liosatos, M.; Morgan, T. E.; Rozovsky, I.; Trommer, B.; Viola, K. L.; Wals, P.; Zhang, C.; Finch, C. E.; Krafft, G. A.; Klein, W. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6448–6453.
(18) Shankar, G. M.; Li, S. M.; Mehta, T. H.; Garcia-Munoz, A.; Shepardson, N. E.; Smith, I.; Brett, F. M.; Farrell, M. A.; Rowan, M. J.; Lemere, C. A.; Regan, C. M.; Walsh, D. M.; Sabatini, B. L.; Selkoe, D. J. *Nat. Med.* **2008**, *14*, 837–842.
(19) Townsend, M.; Shankar, G. M.; Mehta, T.; Walsh, D. M.; Selkoe, D. J. *J. Physiol.-London* **2006**, *572*, 477–492.
(20) Cleary, J. P.; Walsh, D. M.; Hofmeister, J. J.; Shankar, G. M.; Kuskowski, M. A.; Selkoe, D. J.; Ashe, K. H. *Nat. Neurosci.* **2005**, *8*, 79–84.
(21) Bitan, G.; Lomakin, A.; Teplow, D. B. *J. Biol. Chem.* **2001**, *276*, 35176–35184.
(22) Gong, Y.; Chang, L.; Viola, K. L.; Lacor, P. N.; Lambert, M. P.; Finch, C. E.; Krafft, G. A.; Klein, W. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10417–22.
(23) Huang, T. H. J.; Yang, D. S.; Plaskos, N. P.; Go, S.; Yip, C. M.; Fraser, P. E.; Chakrabartty, A. *J. Mol. Biol.* **2000**, *297*, 73–87.
(24) Lesne, S.; Koh, M. T.; Kotilinek, L.; Kayed, R.; Glabe, C. G.; Yang, A.; Gallagher, M.; Ashe, K. H. *Nature* **2006**, *440*, 352–357.
(25) Esapa, C.; Moffitt, J. H.; Novials, A.; McNamara, C. M.; Levy, J. C.; Laakso, M.; Gomis, R.; Clark, A. *Biochim. Biophys. Acta* **2005**, *1740*, 74–8.
(26) Pildal, J.; Lajer, M.; Hansen, S. K.; Almind, K.; Ambye, L.; Borch-Johnsen, K.; Carstensen, B.; Hansen, T.; Pedersen, O. *Diabet. Med.* **2003**, *20*, 491–4.
(27) Matveyenko, A. V.; Butler, P. C. *ILAR J.* **2006**, *47*, 225–233.
(28) Shoval, H.; Weiner, L.; Gazit, E.; Levy, M.; Pinchuk, I.; Lichtenberg, D. *Biochim. Biophys. Acta* **2008**, *1784*, 1570–1577.
(29) Yu, C. M.; Mun, S.; Wang, N. H. L. *J. Chromatogr. A* **2008**, *1192*, 121–129.
(30) Yu, C. M.; Mun, S.; Wang, N. H. L. *J. Chromatogr. A* **2006**, *1132*, 99–108.
(31) Mok, Y. F.; Howlett, G. J. *Methods Enzymol.* **2006**, *413*, 199–217.
(32) Vaiana, S. M.; Ghirlando, R.; Yau, W. M.; Eaton, W. A.; Hofrichter, J. *Biophys. J.* **2008**, *94*, L45−L47.
(33) Bitan, G. *Methods Enzymol.* **2006**, *413*, 217–236.
(34) Lomakin, A.; Benedek, G. B.; Teplow, D. B. *Methods Enzymol.* **2006**, *413*, 429–459.
(35) Bertoncini, C. W.; Rasia, R. M.; Lamberto, G. R.; Binolfi, A.; Zweckstetter, M.; Griesinger, C.; Fernandez, C. O. *J. Mol. Biol.* **2007**, *372*, 708–722.
(36) Li, Y. J.; Kim, S.; Brodsky, B.; Baum, J. *J. Am. Chem. Soc.* **2005**, *127*, 10490–10491.
(37) Wilkins, D. K.; Grimshaw, S. B.; Receveur, V.; Dobson, C. M.; Jones, J. A.; Smith, L. J. *Biochemistry* **1999**, *38*, 16424–16431.
(38) McNulty, B. C.; Tripathy, A.; Young, G. B.; Charlton, L. M.; Orans, J.; Pielak, G. J. *Protein Sci.* **2006**, *15*, 602–608.
(39) Danielsson, J.; Jarvet, J.; Damberg, P.; Graslund, A. *Magn. Reson. Chem.* **2002**, *40*, S89−S97.
(40) Mansfield, S. L.; Jayawickrama, D. A.; Timmons, J. S.; Larive, C. K. *Biochim. Biophys. Acta* **1998**, *1382*, 257–265.
(41) Narayanan, S.; Reif, B. *Biochemistry* **2005**, *44*, 1444–1452.
(42) Rabanal, F.; Tusell, J. M.; Sastre, L.; Quintero, M. R.; Cruz, M.; Grillo, D.; Pons, M.; Albericio, F.; Serratosa, J.; Giralt, E. *J. Pept. Sci.* **2002**, *8*, 578–588.

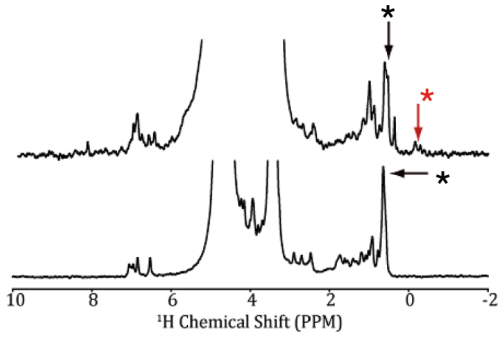

Figure 2. ^{1}H NMR of hIAPP (top) and rIAPP (bottom) at 4 °C. Arrows mark the resonances for which the echo attenuation was monitored from the PFG NMR experiments.

The final buffer used was 10 mM sodium phosphate with 100 mM NaCl, pH 7.3, and the final peptide concentration was 1 mg/mL (256 and 255 μM for hIAPP and rIAPP, respectively). Although rIAPP was readily soluble at concentrations as high as 2 mg/mL using this procedure, attempts to solubilize hIAPP at concentrations higher than 1 mg/mL at pH 7.3 were not successful.

NMR Spectroscopy. ^{1}H NMR diffusion measurements were carried out at 499.78 MHz using the stimulated echo (STE) pulsed field gradient (PFG) pulse sequence with squared gradient pulses of constant duration (5 ms) and variable gradient amplitude along the longitudinal axis.[43] Typical acquisition parameters used in NMR experiments were as follows. A 90° pulse width of 15 μs, a spin echo delay of 10 ms, a stimulated echo delay of 350 ms, a recycle delay of 5 s, a spectral width of 10 kHz, and 4048 data points. Radio frequency pulses were phase cycled to remove unwanted echoes. All spectra were processed with an exponential multiplication equivalent to a 5 Hz line broadening prior to Fourier transformation and were referenced relative to tetramethylsilane (TMS). The gradient strength was calibrated ($G = 3.28$ T m^{-1}) from the known diffusion coefficient of HDO in D_2O at 25 °C ($D_0 = 1.9 \times 10^{-9}$ m^2 s^{-1}).[44]

Circular Dichroism Spectroscopy. The change in secondary structure with temperature was measured for rIAPP and hIAPP using a Jasco J-715 CD spectrometer with a Peltier temperature-controlled cell holder. Samples were prepared identically to that of the samples used in NMR experiments except the concentration of the peptide was 25 μM and NaF was used instead of NaCl. Following a preincubation period of 10 min at 4 °C, $\Delta\varepsilon_{218}$ was measured as a function of temperature using a heating rate of 1 °C min^{-1}. Data were acquired every 1 °C for rIAPP and every 0.2 °C for hIAPP, which displayed a higher dependence of the secondary structure on temperature. The reversibility of the hIAPP transition was checked by cooling the same sample from 50 °C after a 10 min incubation.

Results

Figure 2 shows a comparison of 1D proton chemical shift spectra of solutions of hIAPP and rIAPP in the absence of a magnetic field gradient at 4 °C. The ^{1}H chemical shift spectra of both peptide solutions are similar, as can be expected due to the high degree of sequence homology between hIAPP and rIAPP, but are not identical (Figure 1). Notably, the signal intensity of hIAPP is significantly reduced as compared to that of the nonamyloidogenic rIAPP peptide. The decrease in signal intensity for the aggregating hIAPP sample relative to the nonaggregating rIAPP sample indicates that a significant population of hIAPP initially exists as oligomers similar to what has been observed for the Aβ_{1-40} peptide.[41] NMR signals from a large oligomeric hIAPP species, which tumbles slowly on the NMR time-scale and could behave like a solid, are very broad and therefore is insensitive to detection by solution NMR experiments. As discussed below, there is a broad peak near 0 ppm in Figure 2, which is present in the hIAPP sample but not observed in the rIAPP sample and can be assigned to large oligomeric species of hIAPP present in the initial spectrum completed within 2 h.

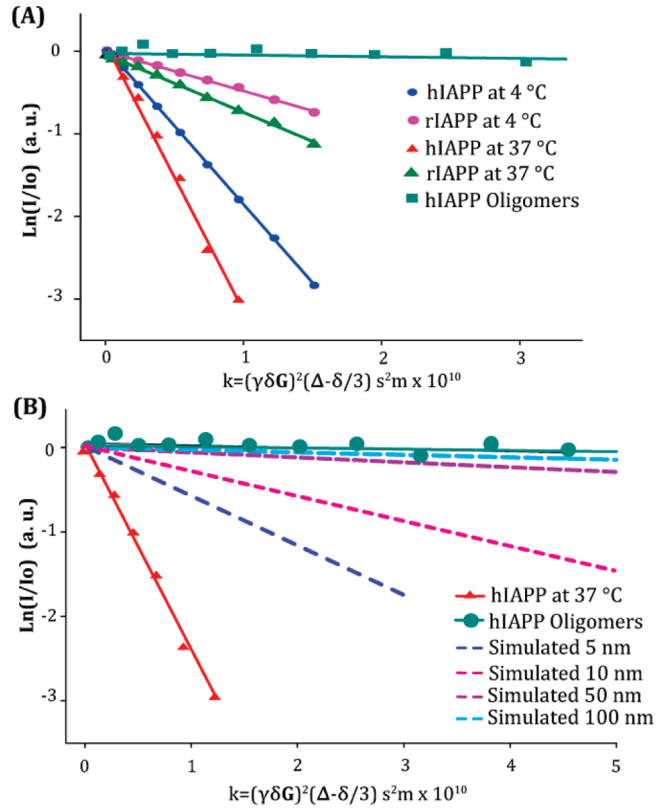

Figure 3. (A) Normalized stimulated echo intensity decays from STE (stimulated echo) PFG ^{1}H NMR spectra of hIAPP at 4 (blue circles) and 37 °C (red triangles) and rIAPP at 4 (magenta circles) and 37 °C (green triangles). The decay of the broad resonance at ~0 ppm (from Figure 1, top) is marked by blue rectangles. (B) Stimulated echo decays of hIAPP at 37 °C and the broad resonance at ~0 ppm, along with simulated curves illustrating the expected echoes from particles of 5, 10, 50, and 100 nm hydrodynamic radii.

Table 1. Hydrodynamic Parameters of hIAPP and rIAPP Measured from ^{1}H PFG NMR Experiments

	hIAPP			rIAPP		
T (°C)	D (m^2/s)	R_H (Å)	C_p	D (m^2/s)	R_H (Å)	C_p
4	7.03×10^{-11}	15.3	0.673	4.80×10^{-11}	22.4	0.265
37	3.60×10^{-10}	8.1	1.080	1.89×10^{-11}	14.1	0.745

To estimate the relative size and degree of folding of hIAPP and rIAPP, we determined the diffusion coefficients of both peptides at 4 and at 37 °C using PFG NMR (Figure 3). The diffusion coefficient is inversely related to the hydrodynamic radius of the particle, which in turn is determined by the compactness of the monomeric state and also the degree of self-association. A single form of rIAPP and two distinct species of hIAPP were detected using the diffusion coefficients of the narrow peak at ~0.5 ppm that is present in both peptide samples and the broad resonance at ~0 ppm found only in the amyloidogenic hIAPP peptide (Figure 3).

(43) Tanner, J. E. *J. Chem. Phys.* **1970**, *52*, 2523–2526.
(44) Mills, R. *J. Phys. Chem.* **1973**, *77*, 685–688.

The narrow peak at ~0.5 ppm for both peptides corresponds to a single, small, and rapidly diffusing species. Human IAPP is significantly more compact than rIAPP, suggesting a higher degree of folding as discussed below. The degree of compactness was quantified and compared to known values for natively folded and unfolded proteins by a compaction factor (C_p) (see Table 1) defined in the equation below.[37]

$$C_p = (R_H^D - R_H)/(R_H^D - R_H^N) \quad (1)$$

where R_H^D (17.3 Å) is the expected hydrodynamic radius of a completely denatured protein of IAPP's molecular weight, R_H is the measured hydrodynamic radius, and R_H^N is the expected hydrodynamic radius of a folded protein (13.5 Å).[37]

A C_p value of 1 corresponds to a protein with a similar amount of structure as a native protein, while a C_p value of 0 indicates a random coil conformation lacking both secondary structure and other intramolecular contacts. Although both hIAPP and rIAPP are commonly viewed as unstructured by spectroscopic techniques that are sensitive to secondary structure, the C_p values indicate a significant amount of intermolecular contacts in hIAPP but not in rIAPP. At 4 °C, the C_p values of hIAPP and rIAPP are 0.67 and 0.28, respectively, indicating a partially collapsed structure for hIAPP but an almost totally extended structure for rIAPP, in agreement with FRET data from rIAPP and hIAPP.[45] At 37 °C, the C_p of hIAPP increases to a value of 1, indicating the existence of a highly compact species with the same degree of folding as a natively folded protein. The compact factor of rIAPP at 37 °C is 0.75, indicating a partially collapsed structure with a similar degree of compaction as hIAPP at 4 °C. This result is in agreement with solution NMR studies that have shown that the monomeric forms of hIAPP and rIAPP are predominantly unstructured but transiently sample helical states.[46,47] While helical propensities were detected for both peptides at 5 °C, the C-terminus of hIAPP is more structured than that of rIAPP.[46,47] Our results suggest this difference between hIAPP and rIAPP is even more apparent at physiological temperatures.

hIAPP has been shown to have a sharp transition to a more helical intermediate at ~45 °C.[48] However, these studies were performed with 1% HFIP as a cosolvent, which has strong effects on IAPP aggregation and amyloid formation.[49,50] Furthermore, the conformational change with temperature was not completely reversible under these conditions. Therefore, we measured the CD signal as a function of temperature under conditions similar to the PFG experiments, albeit with a lower concentration of IAPP (25 μM) (Figure 4). hIAPP was found to undergo a reversible increase in secondary structure content as the temperature was raised from 4 to 50 °C. In contrast to the previous experiments performed with 1% HFIP, the change in secondary structure content is nearly linear with the temperature increase and lacks the sharp transition near 45 °C found in the HFIP containing solutions. This conformational transition is absent in rIAPP (see Figure 4). In summary, monomeric hIAPP is subject to a thermally induced change in both secondary structure and compaction, while rIAPP is only subject to a change in compaction such as the formation of hydrophobic clusters.

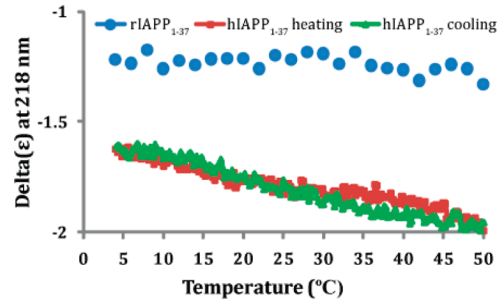

Figure 4. Plot of the CD signal at 218 nm as a function of temperature. Samples of 25 μM rIAPP (blue) and hIAPP (red) at pH 7.3 in 10 mM sodium phosphate with 100 mM NaF were heated from 4 to 50 °C at a rate of 1 °C min^{-1}. After 10 min at 50 °C the hIAPP sample was cooled from 50 to 4 °C and the reverse transition was followed (green).

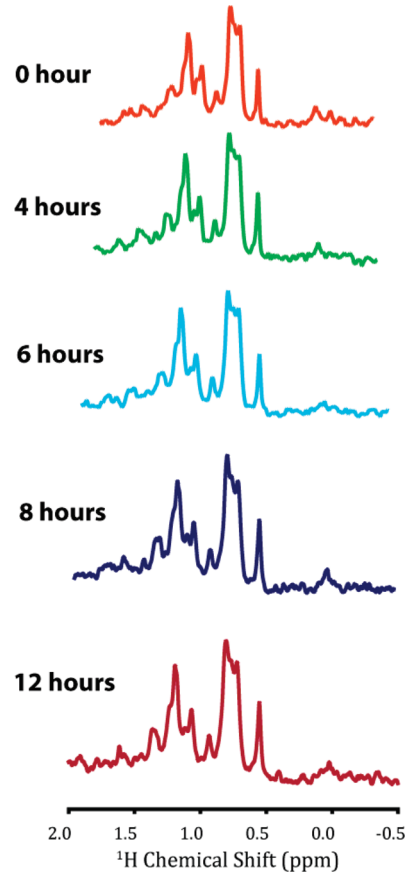

Figure 5. Time dependence of the ^{1}H chemical shift spectrum of hIAPP at 4 °C. The ^{1}H NMR spectra are invariant over time, indicating the aggregation process is very slow and the majority of the peptide is arrested in the monomeric state.

Importantly, hIAPP is much less prone to aggregation at 4 °C than at 37 °C.[32] PFG experiments for hIAPP were repeated over a period of 10 h without changes in the spectra (or calculated diffusion constant) that would be reflective of aggregation (Figure 5). In contrast, after the initial PFG experiment at 37 °C the signal for hIAPP disappeared and the sample had visibly aggregated. Similar results have been observed in a recent ultracentrifugation study of hIAPP where only monomers could be detected at 4 °C over the duration of

(45) Padrick, S. B.; Miranker, A. D. *J. Mol. Biol.* **2001**, *308*, 783–794.
(46) Williamson, J. A.; Miranker, A. D. *Protein Sci.* **2007**, *16*, 110–117.
(47) Yonemoto, I. T.; Kroon, G. J.; Dyson, H. J.; Balch, W. E.; Kelly, J. W. *Biochemistry* **2008**, *47*, 9900–10.
(48) Kayed, R.; Bernhagen, J.; Greenfield, N.; Sweimeh, K.; Brunner, H.; Voelter, W.; Kapurniotu, A. *J. Mol. Biol.* **1999**, *287*, 781–796.
(49) Capone, R.; Quiroz, F. G.; Prangkio, P.; Saluja, I.; Sauer, A. M.; Bautista, M. R.; Turner, R. S.; Mayer, M. *Neurotoxicity Res.* **2008**, *15*, 608–650.
(50) Padrick, S. B.; Miranker, A. D. *Biochemistry* **2002**, *41*, 4694–4703.

the experiment (96 h), but aggregation was immediately apparent at higher temperatures (≥20 °C).[32]

In contrast to the rapidly diffusing resonance at ~0.5 ppm, the broad resonance near 0 ppm is nearly stationary with an estimated diffusion constant of 2.12×10^{-12} m^2 s^{-1}, corresponding to a particle with a hydrodynamic radius of ~100 nm or 1 million monomers (Figure 3b). A similar peak was detected previously for the $A\beta_{1-40}$ peptide and was assigned to large, slowly tumbling oligomeric species.[41] Similarly, a combined ultracentrifugation and electron microscopy study has shown the presence of a small percentage of spherical oligomers in coexistence with hIAPP monomers.[32]

Analysis of the linearity of the stimulated echo plot can reveal if the two species exchange on the millisecond time scale, as well as the polydispersity associated with each diffusing peak. The very high degree of linearity of the plot of the attenuation of the signal versus the gradient strength indicates the rapidly diffusing resonance at 0.5 ppm corresponds to a single species, most likely a monomer.[32] On the other hand, a polydisperse sample would give rise to a nonlinear relationship if the exchange process is slow on the time-scale of the diffusion of the peptide, as has been seen for the $A\beta_{12-28}$ peptide.[40] While a linear relationship between the gradient strength and signal attenuation is possible if the system is under fast exchange, the calculated hydrodynamic radius for this resonance of hIAPP is considerably smaller than the corresponding rIAPP sample. Since rIAPP does not self-associate in solution, it is very likely that the rapidly diffusing resonance is associated only with the monomeric form of hIAPP and not oligomeric forms of hIAPP.[32]

Discussion

Our results show that a large, slow diffusing species of hIAPP can coexist in solution with the monomeric peptide (at 4 °C), without triggering aggregation. The existence of this off-pathway intermediate is in agreement with kinetic models of IAPP which evoke a reservoir population of IAPP that does not participate in the aggregation reaction to explain the independence of the aggregation kinetics of IAPP and IAPP fragments on IAPP concentration.[50,51] The morphology of the slowly diffusing aggregates cannot be determined from the experiments described here, although apparently nonfibrillar structures have been observed in coexistence with IAPP amyloid fibers both in vitro[32,52,53] and in situ.[7,54]

The increase in the rate of aggregation as the temperature is raised from 4 to 37 °C is in agreement with a previous study on the temperature dependence of the aggregation kinetics of hIAPP,[48] and corresponds to an increase in both secondary structure and compaction of the peptide. It has been noted that many amyloid proteins are neither completely unfolded nor structured like globular proteins but rather share many characteristics of premolten globule proteins.[55] Proteins in the premolten globule state possess an intermediate amount of secondary structure, are more compact than random coil proteins, and possess some hydrophobic clusters as revealed by ANS binding.[48,55] These structural features are characteristic of hIAPP at 37 °C but not at 4 °C. However, no such features were observed for rIAPP at any of the temperatures studied, indicating a partially folded conformation may be critical for aggregation for human IAPP.[56]

The absence of small oligomers of hIAPP in solution is highly significant for the cytotoxic mechanism of hIAPP. A current hypothesis holds that the most toxic species of amyloid proteins are small to intermediate size (~5−20 nm in diameter) oligomers.[3,57] However, the mechanism by which these oligomers assemble is not well understood.[58] An important and still controversial question in this area is whether the membrane-active oligomeric species are preassembled in solution before attaching to the cell membrane or are assembled on the membrane from IAPP monomers.[59,60] In contrast to other amyloidogenic peptides where preincubation of the peptide in solution is necessary for a toxic effect against membranes, membrane permeabilization by IAPP can be detected immediately after the addition of the peptide to membranes.[53,61] The relative insensitivity of IAPP toxicity and membrane binding to preaggregation is in marked contrast to other amyloid peptides. Oligomers of the $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides implicated in Alzheimer's disease are among the best studied examples.[57] Lambert et al. have shown that small spherical $A\beta_{1-42}$ oligomers of ~5−6 nm in size composed of ~4−10 monomer units (ADDLs, amyloid derived diffusible ligands) are toxic to mice at nanomolar doses.[17] Later research has implicated the formation of dimers, trimers, and other small oligomers as factors contributing to the toxicity of $A\beta_{1-40}$ and $A\beta_{1-42}$.[18−24,62] Many other amyloids show similar increases in membrane disruption and cytotoxicity with preaggregation of the peptide in solution.[63,64] Membrane binding of α-synuclein, for example, increases ~100 fold after 24 h of preaggregation in solution before addition of the peptide to the membrane.[64] Significantly, α-synuclein is not strictly monomeric in solution but includes dimers, trimers, tetramers, and pentamers as major species.[65] The immediate increase in membrane permeabilization after the addition of IAPP for some sample conditions suggests the formation of these soluble oligomers may not be as critical for IAPP as with other amyloidogenic peptides and other factors, largely unknown at present, control the relative toxicity of the peptide.

The mechanism by which hIAPP oligomers assemble is not well understood. Ultracentrifugation experiments have established that monomers are the dominant species present before fiber formation; however, the time-resolution of these experiments is limited (96 h until completion) and therefore the experiments could only be carried out at either low temperatures or at a low pH where hIAPP displays a greatly reduced rate of aggregation.[32] However, the ultracentrifugation experiments have a lower detection limit than NMR and are complementary to our studies. Combined results from both studies indicate that IAPP does not form stable low-molecular-weight oligomers in solution under aggregating conditions in vitro. Interestingly, while low-molecular-weight oligomers are not found in vitro, they have been isolated

(51) Rhoades, E.; Agarwal, J.; Gafni, A. *Biochim. Biophys. Acta* **2000**, *1476*, 230–238.
(52) Green, J. D.; Goldsbury, C.; Kistler, J.; Cooper, G. J. S.; Aebi, U. *J. Biol. Chem.* **2004**, *279*, 12206–12212.
(53) Porat, Y.; Kolusheva, S.; Jelinek, R.; Gazit, E. *Biochemistry* **2003**, *42*, 10971–10977.
(54) Zhao, H. L.; Sui, Y.; Guan, J.; He, L.; Gu, X. M.; Wong, H. K.; Baum, L.; Lai, F. M.; Tong, P. C.; Chan, J. C. *Transl. Res* **2009**, *153*, 24–32.
(55) Uversky, V. N.; Fink, A. L. *Biochim. Biophys. Acta* **2004**, *1698*, 131–53.
(56) Abedini, A.; Raleigh, D. P. *Physical Biol.* **2009**, *6*, 15005.
(57) Ferreira, S. T.; Vieira, M. N. N.; De Felice, F. G. *IUBMB Life* **2007**, *59*, 332–345.

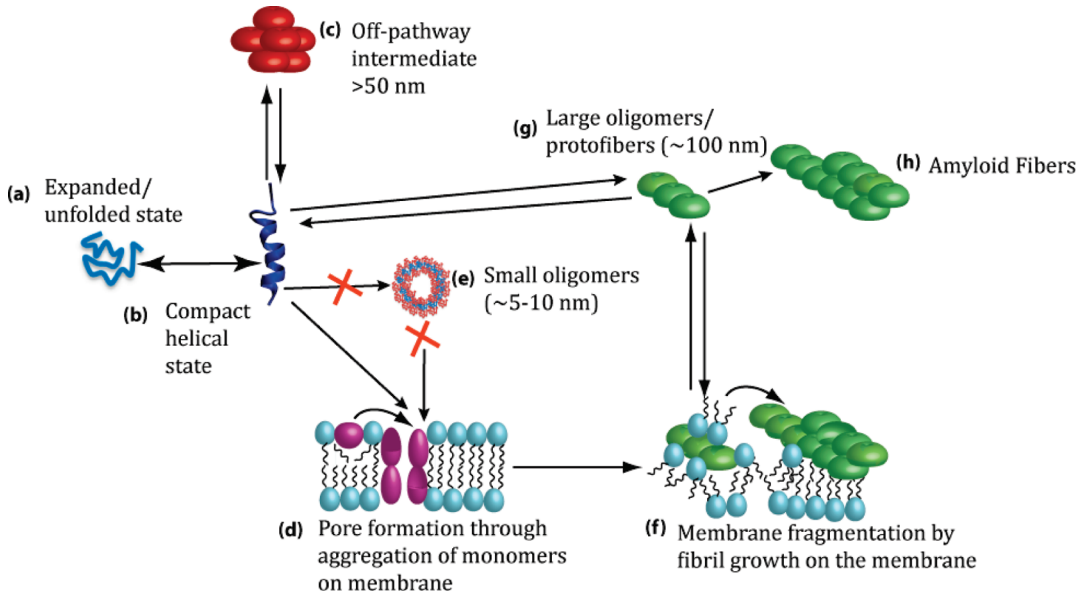

Figure 6. Hypothetical mechanism for membrane disruption by IAPP based on the PFG experiments reported here and other previous studies. Explanations for the individual steps are given in the main text. Drawing is not to scale.

from the pancreases of diabetic patients, suggesting an additional unknown factor present in vivo may catalyze or stabilize the formation of these intermediates.[54] However, it is worth noting that a conformation-specific antibody that recognizes ADDLs of many amyloidogenic proteins did not protect mice against hIAPP induced β-cell apoptosis, in contrast to the success with other amyloidogenic proteins.[66,67]

A possible mechanism for membrane disruption by hIAPP constructed on the basis of our results in combination with ultracentrifugation,[32] atomic force microscopy,[68,69] and lipid monolayer and spectroscopy studies, is presented in Figure 6.[58−60,70−72] Before binding to the membrane, hIAPP exists primarily as a monomer with larger oligomers as a minor species (Figure 6a and b). The larger oligomers appear to be off-pathway intermediates, based on the apparent stability of the sample at 4 °C and kinetic models of IAPP aggregation (Figure 6c).[50] While the monomeric species largely lacks secondary structure, it is compact and transiently samples helical states, with the degree of helicity dependent on the temperature of the sample.[46,47] The monomeric peptide can bind to the membrane in a cooperative manner where it can then aggregate to form stable, possibly toroidal, pores (Figure 6d).[59−61,68,73−77] Pores are apparently formed from the aggregation of the monomeric peptide on the membrane, as small oligomeric species indicative of preformed pores have not been detected in this study or in ultracentrifugation studies (Figure 6e).[32] However, the possibility of transient formation of small oligomeric species cannot be eliminated due to the limited time resolution of current studies. Furthermore, small oligomeric species may form in alternative experimental conditions. The continued aggregation and growth of amyloid fibers of hIAPP on the membrane leads to complete fragmentation of the membrane (Figure 6f).[6,69−71,78,79] Alter-

(58) Khemtemourian, L.; Killian, J. A.; Hoppener, J. W.; Engel, M. F. *Exp. Diab. Res.* **2008**, *2008*, 421287.
(59) Knight, J. D.; Hebda, J. A.; Miranker, A. D. *Biochemistry* **2006**, *45*, 9496–9508.
(60) Engel, M. F. M.; Yigittop, H.; Elgersma, R. C.; Rijkers, D. T. S.; Liskamp, R. M. J.; de Kruijff, B.; Hoppener, J. W. M.; Killian, J. A. *J. Mol. Biol.* **2006**, *356*, 783–789.
(61) Anguiano, M.; Nowak, R. J.; Lansbury, P. T. *Biochemistry* **2002**, *41*, 11338–11343.
(62) Hepler, R. W.; Grimm, K. M.; Nahas, D. D.; Breese, R.; Dodson, E. C.; Acton, P.; Keller, P. M.; Yeager, M.; Wang, H.; Shughrue, P.; Kinney, G.; Joyce, J. G. *Biochemistry* **2006**, *45*, 15157–15167.
(63) El-Agnaf, O. M. A.; Nagala, S.; Patel, B. P.; Austen, B. M. *J. Mol. Biol.* **2001**, *310*, 157–168.
(64) Smith, D. P.; Tew, D. J.; Hill, A. F.; Bottomley, S. P.; Masters, C. L.; Barnham, K. J.; Cappai, R. *Biochemistry* **2008**, *47*, 1425–1434.
(65) Giannakis, E.; Pacifico, J.; Smith, D. P.; Hung, L. W.; Masters, C. L.; Cappai, R.; Wade, J. D.; Barnhain, K. J. *Biochem. Biophys. Acta* **2008**, *1778*, 1112–1119.
(66) Lin, C. Y.; Gurlo, T.; Kayed, R.; Butler, A. E.; Haataja, L.; Glabe, C. G.; Butler, P. C. *Diabetes* **2007**, *56*, 1324–1332.
(67) Bard, F.; Cannon, C.; Barbour, R.; Burke, R. L.; Games, D.; Grajeda, H.; Guido, T.; Hu, K.; Huang, J. P.; Johnson-Wood, K.; Khan, K.; Kholodenko, D.; Lee, M.; Lieberburg, I.; Motter, R.; Nguyen, M.; Soriano, F.; Vasquez, N.; Weiss, K.; Welch, B.; Seubert, P.; Schenk, D.; Yednock, T. *Nat. Med.* **2000**, *6*, 916–919.
(68) Quist, A.; Doudevski, L.; Lin, H.; Azimova, R.; Ng, D.; Frangione, B.; Kagan, B.; Ghiso, J.; Lal, R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10427–10432.
(69) Green, J. D.; Kreplak, L.; Goldsbury, C.; Blatter, X. L.; Stolz, M.; Cooper, G. S.; Seelig, A.; Kist-Ler, J.; Aebi, U. *J. Mol. Biol.* **2004**, *342*, 877–887.
(70) Sparr, E.; Engel, M. F. M.; Sakharov, D. V.; Sprong, M.; Jacobs, J.; de Kruijff, B.; Hoppener, J. W. M.; Killian, J. A. *FEBS Lett.* **2004**, *577*, 117–120.
(71) Engel, M. F.; Khemtemourian, L.; Kleijer, C. C.; Meeldijk, H. J.; Jacobs, J.; Verkleij, A. J.; de Kruijff, B.; Killian, J. A.; Hoppener, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 6033–8.
(72) Harroun, T. A.; Bradshaw, J. P.; Ashley, R. H. *FEBS Lett.* **2001**, *507*, 200–204.
(73) Brender, J. R.; Lee, E. L.; Cavitt, M. A.; Gafni, A.; Steel, D. G.; Ramamoorthy, A. *J. Am. Chem. Soc.* **2008**, *130*, 6424–6429.
(74) Brender, J. R.; Hartman, K.; Reid, K. R.; Kennedy, R. T.; Ramamoorthy, A. *Biochemistry* **2008**, *47*, 12680–12689.
(75) Jang, H.; Ma, B.; Lal, R.; Nussinov, R. *Biophys. J.* **2008**, *95*, 4631–42.
(76) Nanga, R. P. R.; Brender, J. R.; Xu, J.; Veglia, G.; Ramamoorthy, A. *Biochemistry* **2008**, *47*, 12689–12697.
(77) Mirzabekov, T. A.; Lin, M. C.; Kagan, B. L. *J. Biol. Chem.* **1996**, *271*, 1988–1992.
(78) Domanov, Y. A.; Kinnunen, P. K. J. *J. Mol. Biol.* **2008**, *376*, 42–54.
(79) Brender, J. R.; Dürr, U. H. N.; Heyl, D.; Budarapu, M. B.; Ramamoorthy, A. *Biochim. Biophys. Acta* **2007**, *1768*, 2026–2029.

natively, hIAPP forms amyloid fibers in solution after the formation of transient nuclei (Figure 6g and h).[80] The mechanism of IAPP-induced membrane disruption is complex and awaits further elucidation, particularly in the cellular environment.

(80) Goldsbury, C.; Goldie, K.; Pellaud, J.; Seelig, J.; Frey, P.; Muller, S. A.; Kistler, J.; Cooper, G. J. S.; Aebi, U. *J. Struct. Biol.* **2000**, *130*, 352–362.

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