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NMR Spectroscopic Investigation of Early Events in IAPP Amyloid Fibril Formation

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The islet amyloid polypeptide (IAPP) is a hormone, secreted by pancreatic β-cells along with glucagon and insulin. A balanced ratio of insulin and IAPP keeps the glucose metabolism of humans under control.[1] Disturbance of this balance results in type II diabetes mellitus. Most patients diagnosed with type II diabetes have a deposition of extracellular amyloid in the pancreas, and biochemical analysis has revealed that the main component of the amyloid deposit is IAPP. IAPP comprises 37 amino acids and is C-terminally amidated. In addition, cysteines 2 and 7 form a disulfide bond.[2,3] Under physiological conditions, IAPP remains soluble and a random coil structure has been reported by circular dichroism (CD) spectroscopy.[4] However, in the pathological condition of type II diabetes, IAPP adopts a fibrillar structure rich in β-sheets.[5]

It is still unclear what triggers the conversion of soluble monomeric IAPP into insoluble amyloid fibrils. Mostly biophysical and mutagenesis approaches have been used to understand IAPP fibril formation in vitro.[6,7] A wealth of information about the role of the amino acid sequence in IAPP fibril formation has been obtained from rat IAPP (rIAPP), which differs in only six amino acid residues but does not form amyloid fibrils.[8] The non-aggregating nature of rIAPP was attributed to three proline residues in region 20–29 in the peptide. One other important difference in IAPP residues in the two species is the presence of arginine at position 18 in rat and histidine in humans. Although the preventive properties of the proline residues in IAPP fibrillation have been well studied,[8] biophysical studies that pinpoint the roles of specific residues in the initiation of IAPP amyloid formation are missing. Various real-time spectroscopic methods have been used to monitor amyloid fibril formation in bulk solution,[9,10] as well as in the presence of membranes,[11,12] but residue-specific information on the native peptide, which can best be obtained by NMR spectroscopy, is so far lacking. In a recent NMR spectroscopic study it was shown that rIAPP forms transient stretches of helical structure in its monomeric form.[13] In another study on human IAPP free acid, it has been indicated that the N terminus has a propensity for α-helical structure formation, but owing to the fast aggregation rate these studies were carried out only on the charged IAPP free acid.[14] In an attempt to study the early stages of glucagon amyloid fibril formation, one-dimensional, correlation and diffusion experiments were carried out by NMR spectroscopy.[15] Other approaches such as the application of high pressure to dissociate the fibril or to stabilize oligomeric structures during amyloid formation have also been shown to provide insight into the fibrillation reaction.[16,17]

In this study we elucidated the monomeric structure of full-length human IAPP (hIAPP, Figure 1) and the role of specific amino acid residues in the initiation of IAPP fibril formation by

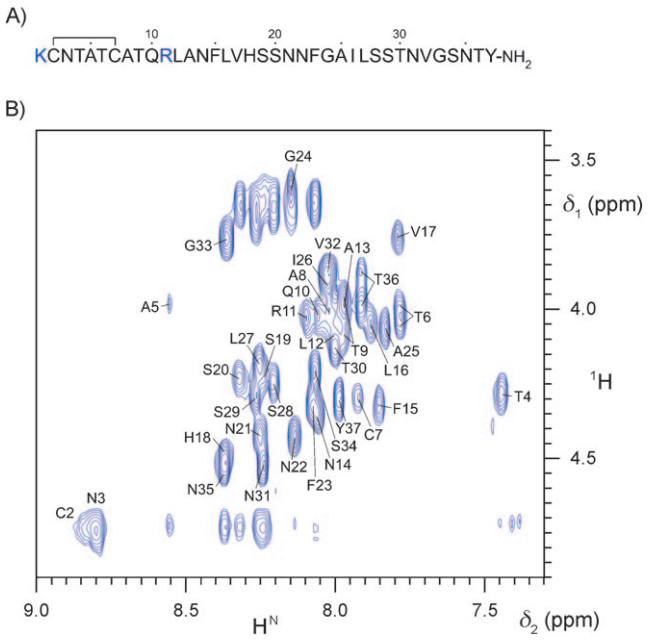

Figure 1. NMR fingerprint region of human IAPP. A) Amino acid sequence of native human IAPP. B) 2D $^{1}H,^{1}H$ TOCSY spectrum of human IAPP at pH 5.5, 10 °C. NH,Hα assignments of the amino acid residues are labelled.

NMR spectroscopy. IAPP is known to be one of the most amyloidogenic, and hence most rapidly aggregating, proteins, which is one of the reasons why it is difficult to find spectroscopic methods capable of providing critical information relating to changes in secondary structure and the role of specific residues in conformational changes. Through optimization of the NMR conditions we were able to identify the initial events of IAPP fibril formation, and it proved possible to monitor the transition from soluble IAPP to insoluble fibrillated IAPP by NMR spectroscopy.

It had previously been difficult to characterize the monomeric state of native human IAPP by NMR spectroscopy, due to the highly aggregation prone nature of IAPP and the rather high peptide concentrations required for NMR studies. Previ-

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ous studies by CD spectroscopy reported a random coil-like structure for native IAPP in aqueous solution.[4] Structure determination of soluble monomeric IAPP under physiological conditions at pH 7.4 and 37 °C appears almost impossible, due to its high rate of aggregation under the concentration conditions needed for NMR. To overcome this problem, the pH can be reduced, which is in fact reasonable, because IAPP is stored in secretory granules in which the pH is moderately acidic (around pH 5.5).[18] A moderately acidic pH might result in protonation of the histidine, which would in turn be analogous to the situation in rat IAPP, in which histidine is replaced by arginine. This should reduce the rate of aggregation of human IAPP. The low pH and the environment in secretory granules prevent IAPP from forming amyloid. In addition, to reduce the rate of aggregation further, the temperature in the NMR measurements was set to 10 °C. Moreover, we also optimized the NMR conditions so that we could work with IAPP concentrations as low as 40 μM. The combination of low pH, low temperature and low concentration finally allowed us to carry out kinetic measurements on IAPP fibril formation by real-time NMR spectroscopy.

2D NMR spectra obtained at pH 5.5 revealed a good dispersion of the chemical shifts, as shown in the fingerprint region of the TOCSY spectrum (Figure 1 B). Residue assignments were carried out with the help of intraresidual and sequential NH,Hα and NH,Hβ resonances by standard correlation and NOE connectivities.[19,20] Except for the amino-terminal lysine and cysteine 2, all residues could be unambiguously identified as shown in Figure 1 and in Table S1 in the Supporting Information. The chemical dispersion indicates a largely random-coil-like structure of monomeric IAPP in solution. No long-range NOEs were observed, indicating the absence of secondary and tertiary structure under these conditions.

The amyloid fibril formation is a multistep event in which the random-coil-like conformation of the monomer changes into a cross-β-sheet fibrillar structure.[21] During the early stages of fibril formation nucleation takes place, and further addition of monomers or oligomers leads to the formation of mature fibrils. Whereas solution NMR spectroscopy is the method of choice for study of the soluble structure, solid-state NMR on powder samples is appropriate for analysis of the fibrillized form. Because the rate of aggregation is very fast after nucleation, further events cannot be captured by solution NMR spectroscopy, due to the peak broadening and finally the complete loss of NMR signal intensity. In addition, the polydispersity of the evolving aggregate structures makes retrieval of structural information almost impossible. However, NMR spectroscopy is at the same time the most powerful technique for observation of changes in monomer structure at a residue-specific level and for capturing of the transient species at the onset of the nucleation and aggregation process. So far, there are no reports in which early fibrillation events during the lag phase of IAPP have been captured by NMR.

For the observation of the IAPP aggregation kinetics under NMR conditions, a pH titration series was performed, in order to allow determination of the best conditions for recording of well-resolved TOCSY spectra during the lag time of the fibrillation process. For a 40 μM hIAPP sample resolved in aqueous buffer, a retarded fibrillation process over 140 h was achieved at pH 5.65 and 10 °C. Collection of each TOCSY spectrum was adjusted to 18 h. Thus, to observe the fibrillation process in the NMR tube, a total of eight TOCSY spectra were recorded

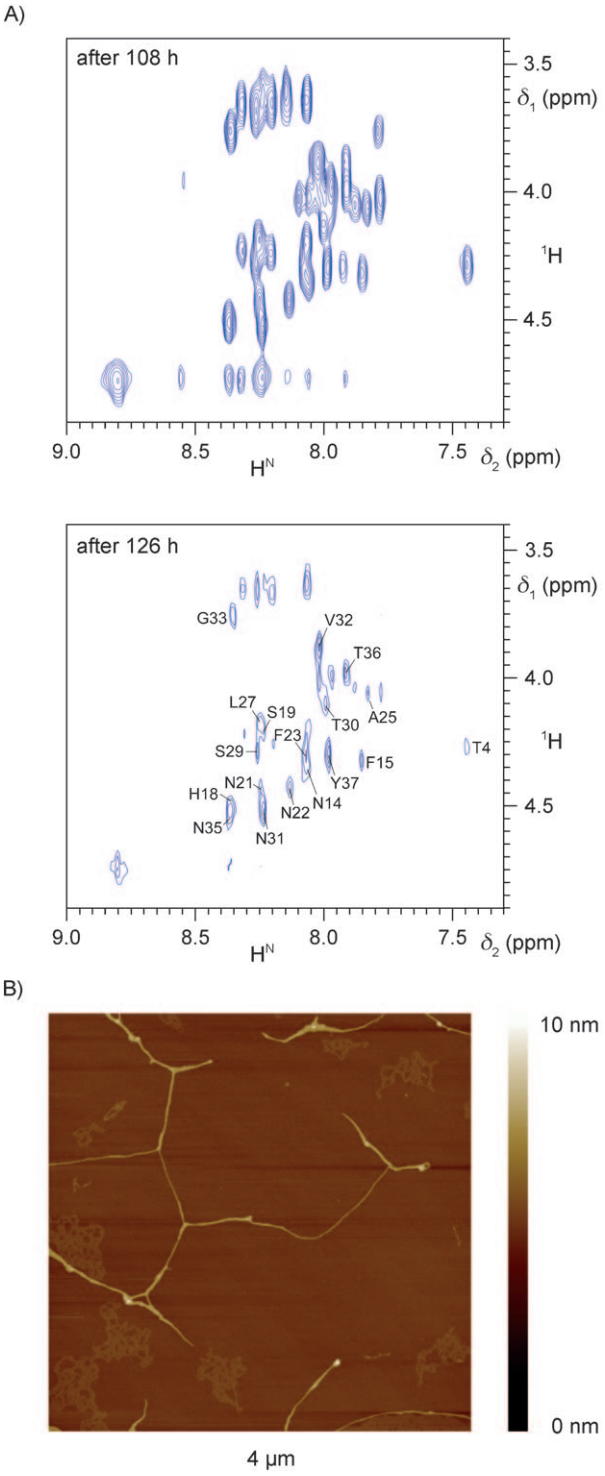

Figure 2. Kinetics of IAPP fibril formation observed by NMR spectroscopy. A) TOCSY spectra of IAPP (40 μM, pH 5.65, 10 °C): top: after 108 h, and bottom: after 126 h. B) AFM image of the fibrils formed after 144 h under the same conditions.

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with a mixing time of 100 ms. In between each TOCSY spectrum, 1D spectra were recorded to ensure the homogeneity of the solution. No significant changes were seen in the first six TOCSY spectra recorded. In the seventh TOCSY spectrum, recorded after 108 to 126 h lag time, however, some of the peaks had completely disappeared whereas other peak intensities still remained resolved, indicating the onset of the aggregation reaction in this time regime. During recording of the eighth TOCSY spectrum (at 126 to 144 h) all peaks finally vanished. The transition occurring between the sixth and the seventh TOCSY spectra is shown in Figure 2A, and the subsequent fibril formation was confirmed by atomic force microscopy (AFM), which displays the typical fibril height of 3 nm (Figure 2B). We also carried out kinetic AFM experiments under NMR conditions at concentrations of up to 100 μM, which showed the appearance of the first amyloid fibrils in the same time range of 100–120 h. Exact comparison, particularly for the transition stage as observed in the NMR spectra, is difficult to achieve, however, because different sizes of species are probed by the two techniques.

As depicted in Figure 3 (for better visualization, an enlarged version of Part A is also included in the Supporting Information), a detailed analysis by integration of the cross-peak volumes showed that overall the N-terminal residues 1–17 disappear first, whereas the peak intensities of residues 18–37 vanish at a later stage. The disappearance of the NH,Cα cross-peaks suggests that the N-terminal residues are involved in the initiation of amyloid fibril formation solely through intermolecular interaction, and it is only at a later stage that reorientation of the C-terminal residues to form fibrillar structures takes place. Only residues T4 and F15 do not seem to be involved in this initiation process.

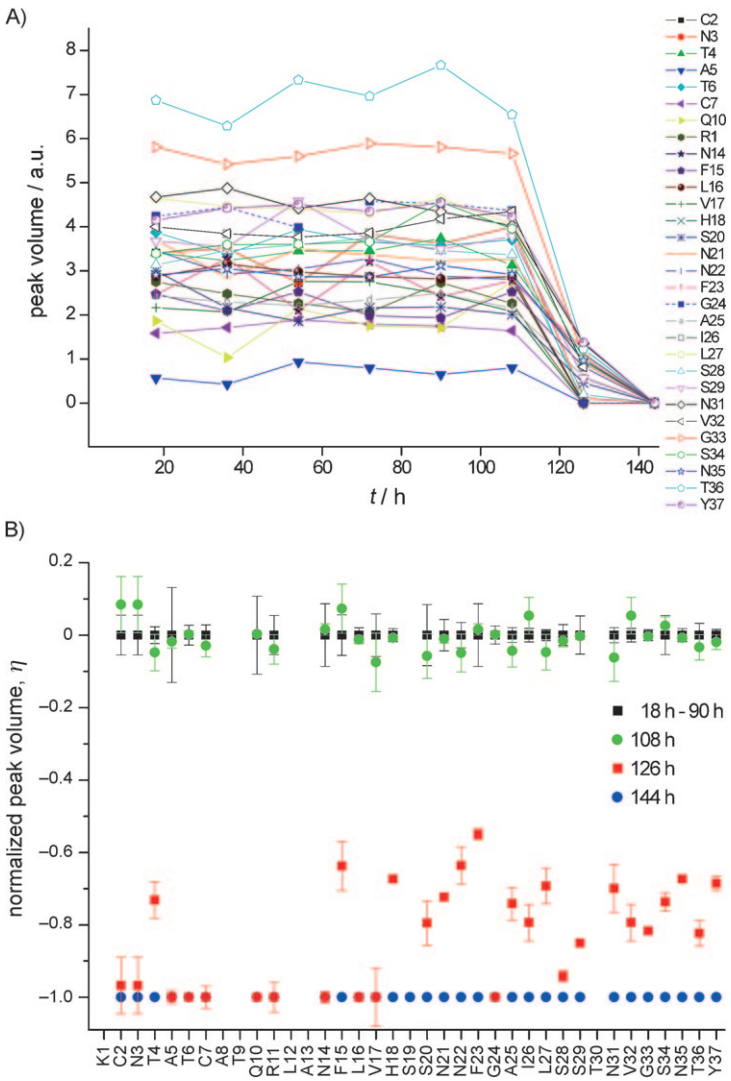

Figure 3. Kinetics of IAPP fibril formation as observed by 2D NMR spectroscopy. Successive TOCSY spectra were collected to follow the disappearance of the amide protons upon onset of the nucleation and fibrillation reaction. The figure shows A) the time evolution of the changes in peak volumes, and B) for better visualization the corresponding peak volume changes normalized to the initial peak volume—$\eta = [I(t) - I(0)]/I(0)$ ($\eta = -1$ if the peak volume approaches zero)—between 18–90, 108, 126 and 144 h of incubation at $T = 10\,^{\circ}C$.

The lack of involvement of the C-terminal residues in the initiation of the fibrillation reaction is surprising. In the current view of hIAPP fibril formation, the core amyloidogenic region of the hIAPP amyloid is believed to be region 20–29.[8] Moreover, EPR[22] and solid-state NMR studies[23] on full-length IAPP amyloid fibrils indicate that the N-terminal region is less ordered in the fibril.

In another set of experiments, the kinetics of IAPP fibril formation were also studied at slightly higher pH (pH 5.8) with reduced lag phase. The data show the same trend: that is, the N-terminal residues disappear first and the C-terminal residues at a later stage (Figure S1).

In conclusion, these NMR data reveal new atomic-level structural details of the prefibrillar state of the hIAPP aggregation reaction. The chemical-shift dispersion of native hIAPP is typical of a largely disordered peptide, in agreement with literature data.[14] Our data strongly suggest that the N-terminal region of hIAPP (residues 1–17), and not the main amyloidogenic region (residues 20–29) of hIAPP, is involved in the initial self-association of the peptide in bulk solution. Interestingly, it has been shown recently that hIAPP aggregation and fibrillation are drastically heightened in the presence of anionic lipid membranes and also occur through insertion of the N-terminal region of hIAPP, which is followed by formation of α-helical intermediate structures.[11,12] Hence, we can conclude that fibrillation in the bulk and in the presence of anionic membranes follows the same route and that the transient α-helical sampling of IAPP in solution could be a structural reflection of similar states stabilized by anionic lipid membranes.[13] Our findings might also be of significance for optimization of small-molecule inhibitors for IAPP fibril formation,[24] which has so far been hampered by the lack of structural information on the early events of fibril formation.

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Experimental Section

Synthetic human IAPP was obtained from Calbiochem (Germany), and hexafluoroisopropanol (HFIP) from Riedel–de Haen (Germany). Fibril formation was carried out essentially as described earlier.[24] For the NMR measurements, the peptide was dissolved in HFIP to disaggregate the sample, followed by removal of the solvent by lyophilization. The lyophilized peptide was dissolved in sodium acetate buffer (10 mM, pH 5.5) to a final concentration of 40 μM. NMR experiments were performed on a 600 MHz Varian INOVA spectrometer at 10 °C. The instrument was equipped with a single gradient axis and triple resonance probe. TOCSY and NOESY spectra were acquired in the phase-sensitive mode and watergate suppression was used to reduce the water peak. For the monomeric structure of IAPP, TOCSY spectra were recorded with 60 to 100 ms mixing times by use of DIPSI spinlock and an 8 kHz RF field. NOESY spectra were recorded with 200, 300, 400 and 600 ms mixing times. The fibril formation was started by placing the IAPP solution in the NMR tube. In between each TOCSY, 1-D spectra were recorded. During the kinetics of IAPP fibril formation, each TOCSY was recorded with use of a 100 ms mixing time with 160 scans, 152 increments and 3276 data points. The data were acquired by use of VNMR software. Proton assignments of backbone and side chain resonances were carried out from homonuclear 2D TOCSY and NOESY spectra, and peak volumes were determined to the noise level with the aid of the Aurelia software by an integration routine optimized for the resolution of overlapping peaks.[25] Figure 3B has been plotted with initial volumes taken from the mean of measurements 1–5 (18–90 h, which exhibit no changes in the spectral patterns) for each residue and the error bars are given. The error bars for the sixth (108 h) and seventh (126 h) measurement have been calculated with the statistical accuracy of all measurements taken into account. AFM was performed as described earlier.[24]

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