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Stepwise oligomerization of murine amylin and assembly of amyloid fibrils

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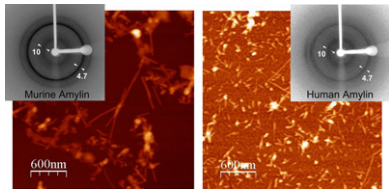
HIGHLIGHTS

- Murine amylin spontaneously associate in low and high order oligomers.
- Murine amylin forms aggregates with amyloid fibril morphology.
- Proline-rich amylin is also capable to form amyloid structures *in vitro*.

GRAPHICAL ABSTRACT



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ABSTRACT

Amylin is a pancreatic hormone co-secreted with insulin. Human amylin has been shown to form dimers and exhibit high propensity for amyloid fibril formation. We observed the ability of the water-soluble murine amylin to aggregate in water resulting in an insoluble material with Thioflavin T binding properties. Infrared spectroscopy analysis revealed beta-sheet components in the aggregated murine amylin. Morphological analysis by transmission electron microscopy and atomic force microscopy provided access to the fibril nature of the murine amylin aggregate which is similar to amyloid fibrils from human amylin. X-ray diffraction of the murine amylin fibrils showed peaks at 4.7 Å and 10 Å, a fingerprint for amyloid fibrils. Electron spray ionization–ion mobility spectroscopy–mass spectrometry (ESI-IMS-MS) analysis and crosslinking assays revealed self-association intermediates of murine amylin into high order oligomeric assemblies. These data demonstrate the stepwise association mechanism of murine amylin into stable oligomers, which ultimately converges to its organization into amyloid fibrils.

1. Introduction

Amylin (also known as islet amyloid polypeptide, IAPP) was discovered from amyloid deposit in pancreas by two independent groups [1,2]. Amyloid deposits of amylin can be found in pancreas of both diabetic and normal individuals, as well as in individuals with insulinoma [3]. The 37 amino acid peptide, amidated and with a disulfide bond between Cys2 and Cys7, is cosecreted with insulin [4] and displays several physiological functions including regulation

Abbreviations: IAPP, islet amyloid polypeptide; ESI-IMS-MS, Electrospray Ionization–Ion Mobility Spectrometry–Mass Spectrometry.

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of food intake, satiety, gastric emptying, regulation of glucose and lactate homeostasis, among others [5–8].

Human amylin displays restricted solubility in aqueous milieu, in the nanomolar concentration range [9]. Human and murine amylin are very similar, differing only in four amino acids, His^{18} and $Pro^{25,28,29}$. Proline-rich amylin variants display enhanced aqueous solubility [10]. It has been suggested that proline-rich variants of amylin in the segment comprising residues 20–29 are not prone to aggregation, and this feature would correlate with the propensity for amyloid deposition *in vivo* in mammalians [10,11]. However, metabolic disturbance in beta cells could also exert important roles in the formation of amyloid deposits containing non-proline-rich amylin [12].

These features have inspired the strategy behind the development of amylin derivatives [13,14] and amylinomimetic compounds, such as the triple mutant $Pro^{25,28,29}$ amylin analog named pramlintide, available since 2005 in the therapeutic practice for mimicking the post-prandial amylin levels in diabetic individuals [15,16]. Despite the enhanced solubility of the proline-rich variants, evidences have been found elsewhere that such human amylin analog peptides are prone to assembly into amyloid fibrils. Segments 8–20 and 14–20 of human amylin are able to form amyloid fibrils [17,18]. Also, it has been found that the equivalent segment of murine amylin spanning amino acids 8–20 (differing from human amylin solely by the His^{18}) also forms amyloid fibrils [18]. A peptide comprising the region 30–37, which share the same sequence in both human and murine amylin, also can be structured into amyloid fibrils [19]. Single-point mutation in the murine amylin sequence enhances the propensity for amyloid formation in murine amylin [20]. In fact, murine amylin has been found to form amyloid fibrils under very particular circumstances, by dissolving lyophilized amylin directly with Tris–HCl buffer pH 7.4, while attempts to dissolve with water or phosphate buffered saline did not result into amyloid fibrils [21]. Moreover, formation of murine amylin amyloid assemblies has been reported with discretion in the literature [20,22], and so far these data have not received much attention.

The collected evidences concerning amyloid fibrils formation from murine amylin fragments have been indicative that murine amylin can in fact perform homoassociation, resulting in the assembly of high order oligomers that ultimately could result in the formation of amyloid fibrils. However, the lack of experimental evidences for such intermediates and the lack of detection of amyloid deposits in rodent has been used as an excluding criteria in the assignment of murine amylin as an amyloidogenic peptide.

Amyloid assembly requires extensive conformational conversion and association of protein subunits into oligomeric assemblies which ultimately will acquire a final amyloid fibril conformation [23–28]. Indeed, evidence from distinct groups using varying methods have demonstrated the association of amylin into dimers, both murine and human amylin variants [23,29–36]. However, higher order association species have not been described, which has limited the description of a pathway for amyloid formation.

In this work we report the characterization of murine amylin self-association and amyloid fibril formation in the absence of molecular cofactors. In another aspect, our data elicits an equilibrium transition between a large conformational variability of amylin monomers and an ensemble of higher order oligomers. We discuss the self-association and further amyloid fibril formation as a general propensity of amylin, which might be under tight control of homeostasis *in vivo* which is likely to rule over the prevention of amylin amyloid aggregation.

2. Material and methods

2.1. Reagents

Murine (molecular weight 3920.045 Da; lot 129962001070611XB) and human (molecular weight 3903.33 Da; lot 81709XB) amylin (CAS 122384-88-7), carboxy-amidated and with a disulfide bond between C2 and C7 were obtained from GenScript and Genemed (murine amylin, lot 87506). The purity and identity were confirmed by electron-spray-ionization–mass-spectrometry (ESI-MS), Matrix-Assisted Laser Desorption–Time of Flight–Mass Spectrometry (MALDI-ToF-MS) analysis, SDS-PAGE and C18-reversed-phase HPLC, both by the manufacturer and the present authors (data not shown). Dry peptides were stored at −20 °C until use, and once in solution they were kept at 4 °C unless otherwise stated in the text. Type I water was obtained from distilled water by deionizing to less than 1.0 μS and filtered through a 0.22 μm pore-size membrane in a water purification system (milliQ Academic; Merck-Millipore, Brazil) immediately prior to use. All other reagents were of analytical grade. All buffers and solutions were prepared immediately prior use.

2.2. Crosslinking analysis

Crosslinking was performed by incubation of 40 μg murine amylin with 0.5% v/v glutaraldehyde for 30 min in 15 μL total volume and followed by 22.5% polyacrylamide gel electrophoresis (PAGE) containing 0.01% sodium dodecylsulfate (SDS) [37]. The gels were stained with Coomassie brilliant blue R-250 and then digitalized for further data analysis through integration using ImageJ [38]. Peak analysis was performed through a curve-fitting and integration routine using Fityk [39], assuming Gaussian functions for each peak.

2.3. Electrospray ionization–ion mobility spectrometry–mass spectrometry (ESI-IMS-MS)

ESI-IMS-MS measurements were performed in a MALDI-Synapt G1 (Waters Brazil) high definition mass spectrometer (HDMS) quadrupole-traveling wave mass spectrometer. Amylin samples were diluted to 0.5 mg/mL final concentration in 100 mM ammonium acetate buffered to pH 7.4 from 10 mg/mL stock solution, and injected at a rate of 100 nL/min, using a positive ESI with a capillary voltage of 2.8 kV and N_2g at 0.4 bar. Data were acquired over the range of *m/z* 500 to 3000 for 20 min per acquisition with repeated 3 s acquisition time per point. Mass calibration was performed on a dynamic mode with phosphoric acid. Other typical instrumental settings are as described previously [40]. Data were analyzed using DriftScope 2.1 [41] (Waters Corporation, Brazil) and MassLynx 4.1 (Waters Corporation, Brazil). Human amylin shows thus a very weak signal in the ESI-MS at aqueous buffer pH 7.4 due to a fast amyloid aggregation, as also previously reported [42], and thus a detailed ESI-IMS-MS characterization of the human amylin in similar conditions of measurements performed for murine amylin was not possible.

2.4. Atomic force microscopy (AFM)

Murine and human amylin (10 mg/mL in water) were left stand to aggregate for 1 day at 25 °C and followed by dilution to 8 μg/mL with water. From each sample 20 μL was separately applied onto freshly cleaved muscovite mica and allowed to air dry at 40% relative humidity. Samples were imaged by AFM (JPK Nanowizard III, JPK Instruments AG) operating in intermittent contact mode, using conventional silicon cantilevers (OLTESPA — Bruker Probes) at room temperature. Several images were acquired at different regions to assess the homogeneity along the surface of the samples. Images were acquired at resolution 512 × 512 pixels. AFM data analysis was performed using the software WSxM [43].

2.5. Transmission electron microscopy (TEM)

Murine and human amylin dispersed in water at 1 mg/mL were diluted to 100 μg/mL with water and placed on a carbon-coated grid for 5 min, blotted to remove excess material, and stained for 2 min with a 2% solution of uranyl acetate prepared in water. Images were digitally