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Amyloid Formation in Heterogeneous Environments: Islet Amyloid Polypeptide Glycosaminoglycan Interactions

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Abstract

Amyloid formation plays an important role in a broad range of diseases and the search for amyloid inhibitors is an active area of research. Amyloid formation takes places in a heterogeneous environment *in vivo* with the potential for interactions with membranes and with components of the extracellular matrix. Naturally occurring amyloid deposits are associated with sulfated proteoglycans and other factors. However, the vast majority of *in vitro* assays of amyloid formation and amyloid inhibition are conducted in homogeneous solution where the potential for interactions with membranes or sulfated proteoglycans is lacking and it is possible that different results may be obtained in heterogeneous environments. We show that variants of islet amyloid polypeptide, which are non-amyloidgenic in homogeneous solution, can be readily induced to form amyloid in the presence of glycosaminoglycans. Glycosaminoglycans are found to be more effective than anionic lipid vesicles at inducing amyloid formation on a per charge basis. Several known inhibitors of IAPP amyloid formation are shown to be less effective in the presence of glycosaminoglycans.

Keywords

IAPP; amylin; glycosaminoglycan; extracellular matrix; amyloid; inhibitor

Introduction

Amyloid fiber formation, the aggregation of normally soluble proteins into partially ordered β -rich structures, plays a role in a broad range of diseases including type 2 diabetes and Alzheimer's disease.^{1,2} Amyloid, or the process of its formation contributes to the pathology of many diseases^{3–5} and the search for inhibitors of amyloid formation is an active area of research.^{6–13}

Amyloid formation takes places in a heterogeneous environment *in vivo* with the potential for interactions with membranes and with components of the extracellular matrix. All naturally occurring amyloid deposits are associated with sulfated proteoglycans and other factors.^{14–19} However, the vast majority of *in vitro* assays of amyloid formation and amyloid inhibition are conducted in homogeneous solution where the potential for interactions with membranes or sulfated proteoglycans is lacking and it is possible that different results may be obtained in heterogeneous environments. In addition, studies of proteins in buffer have been widely used to develop propensity scales for amyloid formation,^{20–22} but it is not

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certain that these scales will have the same quantitative predictive power in heterogeneous environments.

Islet amyloid polypeptide (IAPP, also known as amylin) forms pancreatic islet amyloid deposits in type-2 diabetes. IAPP amyloid formation is toxic to cultured cells, suggesting that it contributes to the disease by leading to β -cell dysfunction and death.^{23–27} Recent work also implicates amyloid formation as a cause of graft failure in islet cell transplantation.^{28,29} A wide range of studies have shown that model membranes which contain anionic lipids accelerate amyloid formation by IAPP,^{30–32} but less is known about the effects of glycosaminoglycans (GAGs) on IAPP amyloid formation.

Immunohistochemical studies have shown that heparan sulfate proteoglycans are associated with islet amyloid isolated from patients with type 2 diabetes,¹⁹ and GAGs can enhance amyloid formation by human proIAPP processing intermediates and by human IAPP.^{33,34} IAPP is one of the most amyloidogenic naturally occurring polypeptides, but it is not known if GAGs can induce amyloid formation in apparently non-amyloidogenic variants of IAPP, nor, with a few exceptions, have the effects of GAGs on IAPP inhibitors been generally considered. Here we examine the effects of GAGs on two apparently non-amyloidogenic variants of IAPP and study the impact of GAGs on IAPP inhibitors. The I26P point mutant of human IAPP (I26P-IAPP), renders the protein non-amyloidogenic and converts it into an inhibitor of amyloid formation by wild type IAPP.¹² The doubly N-methylated variant of human IAPP, G24-N-Methyl, I26-N-Methyl-IAPP (NMe-G24, NMe-I26-IAPP), is a potent inhibitor of IAPP amyloid formation in homogeneous solution and is itself not amyloidogenic.¹¹ The mode of action of these inhibitors is not known, but the substitutions are located in a region of the chain which has been highlighted as important for amyloid formation.^{35,36} We show that these two variants of IAPP can be readily induced to form amyloid by GAGs and that GAGs are more effective than model lipid micelle systems in inducing them to form amyloid. We also demonstrate that some potent inhibitors of IAPP amyloid formation are less effective in the presence of GAGs. Polypeptide-GAG interactions have been postulated to play an important role in islet amyloid formation *in vivo*,^{15–19,33,37–40} and our results highlight the importance of considering these effects *in vitro* and in inhibitor design.

Results and Discussions

Heparan sulfate induces amyloid formation by non-amyloidogenic variants of IAPP

The sequences of wild type IAPP, the I26P-IAPP and NMe-G24, NMe-I26-IAPP variants are shown in Figure 1. No data are available on the effects of GAGs on the ability of either of these variants to form amyloid. We first studied amyloid formation by I26P-IAPP in the presence of the model GAG heparan sulfate (HS). High molecular weight (10 to 14kd) HS was used for these studies. Alexandrescu and coworkers examined the length dependence of heparin upon amyloid formation by wild type IAPP and showed that length dependent effects leveled off above 12 monosaccharide units.³⁹ The high molecular weight HS used here was above the threshold observed for heparin. Figure 2a compares the ability of the I26P point mutant to form amyloid in the absence and presence of HS. The peptide was not amyloidogenic in homogeneous solution over the 10 hr time course of the experiment as judged by thioflavin-T binding assays, transmission electron microscopy (TEM) and circular dichroism (CD). In contrast, wild type IAPP formed amyloid with a T_{50} , the time required for the reaction to reach 50% of the final fluorescence intensity, of 21 mins under these conditions in the absence of GAG. The mutant formed amyloid in the presence of HS. A rapid increase in thioflavin-T fluorescence was observed for I26P-IAPP in the presence of HS with a T_{50} of 20 mins. TEM confirmed the results of the thioflavin-T studies, and dense mats of amyloid fibers were observed in the presence of HS, but not in its absence (Figure

2b, 2c). CD (Supplementary Material) revealed the presence of significant β -sheet structure in the presence of GAGs.

We analyzed a second non-amyloidogenic variant of IAPP, NMe-G24, NMe-I26-IAPP. This peptide is non-amyloidogenic and is one of the most effective inhibitors of amyloid formation by IAPP and protects against IAPP induced toxicity in cell culture.¹¹ Figure 3a compares kinetic curves collected in the presence and absence of heparan sulfate. No amyloid formation was observed in the absence of heparan sulfate, even for samples which were incubated for more than 20 times longer than the time required for wild type IAPP to form amyloid (Figure 3a). In contrast, rapid amyloid formation was observed when heparan sulfate was present as judged by thioflavin-T assays and TEM (Figure 3b) with a T_{50} of 30 mins. CD (Supplementary Material) confirmed the presence of β -sheet structure.

The experiments outlined above were carried out using standard protocols for biophysical studies of IAPP. These involved solubilizing the peptide in hexafluoroisopropanol (HFIP) and diluting the stock solution into buffer. This results in 2% residual HFIP by volume. Even this low percentage of organic co-solvent enhances significantly the kinetics of amyloid formation by IAPP.^{41,42} Thus we wanted to test if our results might be a consequence of the conditions used. We repeated the experiments using a different protocol which avoids the use of HFIP. Buffer was added directly to dried peptide and the time course of amyloid formation was followed. We obtained similar results; both I26P-IAPP and NMe-G24, NMe-I26-IAPP formed amyloid in the presence of heparan sulfate, but not in its absence (Supplementary Material). The T_{50} of I26P-IAPP in the presence of heparan sulfate under this condition was 465 mins and was 2010 mins for the NMe-G24, NMe-I26-IAPP, wild type IAPP formed amyloid with a T_{50} of 60 mins in the presence of heparan sulfate under these conditions.

FRET experiments reveal the co-localization of amyloid fibrils and GAGs

To further investigate the mechanism of the amyloid inducing effects of GAGs on I26P-IAPP and NMe-G24, NMe-I26-IAPP, we tested if GAGs are associated with amyloid fibrils. This is important because GAGs might exert their effects by binding to the IAPP variants or through non-specific polyanion effects. We used a recently developed assay based on FRET from thioflavin-T to fluorescein labeled heparin (FLH).³⁹ Labeled heparin with one fluorescein conjugated per heparin was used as a model GAG. The excitation maximum of thioflavin-T when bound to amyloid fibrils is near 450 nm and its emission maximum is near 485 nm. The excitation maximum of fluorescein is 488 nm and its emission maximum is 515 nm. Thus, the two dyes form a convenient FRET pair to monitor the proximity of GAG and amyloid fibrils. Figure 4a shows the kinetic profile of I26P-IAPP amyloid formation induced by FLH, monitored at an excitation wavelength of 440 nm and an emission wavelength of 510 nm. This pair of wavelengths was chosen to detect just the fluorescent signal due to FRET between fluorescein and thioflavin-T bound to fibrils by avoiding signal from direct excitation of bound thioflavin-T. FRET to fluorescein from fibril bound thioflavin-T was detected as amyloid formation by I26P-IAPP proceeds (Figure 4a, red curve). CD (Supplementary Material) and TEM images (Figure 4b) confirmed the existence of fibrils. In a control experiment containing I26P-IAPP and FLH without thioflavin-T, no significant increase in fluorescence intensity was detected (Figure 4a, black curve), although CD (Supplementary Material) and TEM (Figure 4) confirmed the presence of fibrils. We also studied the co-localization of FLH and NMe-G24, NMe-I26-IAPP using FRET. The results were very similar to those observed with I26P-IAPP. (Supplementary Material) The observation of FRET between FLH and thioflavin-T in both cases demonstrates an association of GAG with the peptides. The association may occur during the process of amyloid formation or might result from the IAPP peptides forming amyloid fibers in solution and then binding to GAG. We conducted additional experiments to test

these possibilities. We simultaneously monitored direct thioflavin-T fluorescence and thioflavin-T to FLH FRET for the same sample. (Supplementary Material) The two curves showed identical time courses; the simplest explanation is that the peptides bind GAGs before amyloid formation is complete.

The effect of heparan sulfate on I26P-IAPP amyloid formation can be screened by high ionic strength

IAPP and the two variants studied here all have a net charge between +2 and +4 at physiologically relevant pH, depending upon the exact pKa of the N-terminus and His-18, thus all are capable of making electrostatic interactions with heparan sulfate. In order to study the potential role of electrostatic interactions between I26P-IAPP and heparan sulfate, we examined amyloid formation kinetics in buffers with high concentrations of salt. Amyloid formation by wild type IAPP is very sensitive to salt.⁴³ Thioflavin-T fluorescence assays without HFIP were used to follow amyloid formation as a function of added salt. Figure 5a compares the ability of I26P-IAPP to form amyloid in the presence and absence of heparan sulfate as a function of NaCl in the assay. I26P-IAPP was still non-amyloidogenic in homogeneous solution when 150 mM NaCl was added to the buffer (Figure 5a, black curve). Heparan sulfate still induced amyloid formation under these conditions (Figure 5a, blue curve), but with a lower efficiency as indicated by a 3.3 fold longer lag phase (990 mins), compared to the kinetic profile without NaCl. (Supplementary Material). At higher NaCl, 500 mM, I26P-IAPP did form amyloid in the presence and in the absence of heparan sulfate (Figure 5a). Similar kinetic profiles were obtained with and without heparan sulfate, although slightly higher final fluorescence intensity was observed in the presence of heparan sulfate. TEM images collected at the end of each experiment (Figure 5) are consistent with the thioflavin-T fluorescence measurements. The observation that heparan sulfate no longer accelerates IAPP amyloid formation by I26P-IAPP at high salt relative to control suggests that electrostatic interactions make a significant contribution to GAG-peptide interactions.

GAGs are more effective than anionic vesicles at inducing amyloid formation by I26P-IAPP

A wide range of studies have shown that lipid membranes containing negatively charged lipids promote the formation of IAPP amyloid.^{30,32,44,45} Although the mechanism of the effect is not completely clear, it is believed that electrostatic interactions between positively charged peptides and negatively charged lipids play an important role. If the effects of GAGs and lipid vesicles depended only on the concentration of negative charges rather than on their spatial distribution, then we would expect similar effects when the total number of negatively charged sites were matched. To test if this is the case, we compared the effects of lipid vesicles and heparan sulfate on amyloid formation by I26P-IAPP. Anionic dioleoylphosphatidylglycerol (DOPG) which contains a single negative charge was chosen as a model lipid. DOPG has been widely used in model studies of IAPP membrane interactions.^{31,46,47}

DOPG induces amyloid formation by I26P-IAPP, but much less efficiently on a per negative charge basis than heparan sulfate. The concentration of heparan sulfate (0.97 μ M) and DOPG (48 μ M) used here correspond to a concentration of 48 μ M negative charges. The lag phase of I26P-IAPP amyloid formation in the presence of DOPG (675 mins) was almost 10 fold longer than observed with heparan sulfate (68 mins). (Figure 6) Peptide-lipid binding and lipid induced fiber formation are highly dependent on the concentration of lipids and on the lipid to peptide ratio. A 5 fold increase in the concentration of DOPG increased T_{50} two fold. (Supplementary Material) This dependence was weaker for HS. A 10 fold increase in HS concentration led to only a 1.5 fold increase in rate. (Supplementary Material) These results demonstrate that heparan sulfate is more efficient than DOPG in inducing amyloid

formation by I26P-IAPP at the same net total charge under the conditions used. Moreover, the effects of HS are less dependent on its concentration.

I26P-IAPP and NMe-G24, I26-IAPP can be induced to form amyloid by other GAGs

It is natural to inquire if the effect of heparan sulfate is specific to the structure of the GAG used. We studied amyloid formation by the IAPP variants in the presence of two other GAGs, chondroitin sulfate and dermatan sulfate. All three of these GAGs are composed of repeating disaccharide units. The most common unit of heparan sulfate is glucuronic acid (GlcA) linked to N-acetylglucosamine (GlcNAc). Chondroitin sulfate is composed mainly of alternating D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). When the GlcA is epimerized into L-iduronic acid (IdoA), the resulting GAG is denoted dermatan sulfate.

Figure 7a compares I26P-IAPP amyloid formation induced by the three GAGs. Chondroitin sulfate and dermatan sulfate also promoted amyloid formation by I26P-IAPP. A slightly shorter lag phase and lower final fluorescence intensity was observed in the presence of chondroitin sulfate (Figure 7a, red curve) than with heparan sulfate (Figure 7a, black curve), while the lag phase for dermatan sulfate induced amyloid formation was between that observed for chondroitin sulfate and heparan sulfate. TEM (Figure 7) and CD (Supplementary Material) confirmed that fibrils had formed at the end of each experiment. Similar results were observed with NMe-G24, NMe-I26-IAPP. The peptide was amyloidogenic in the presence of each of the three GAGs and the trend in the efficiency in inducing amyloid formation was the same as observed for I26P-IAPP as judged by the lag times (Supplementary Material). The results show that the amyloid inducing effect of GAGs on non-amyloidogenic IAPP variants is not specific to heparan sulfate. The difference in GAGs' structures and the arrangement of the charges are likely responsible for the slight variations in the kinetic parameters.

IAPP amyloid inhibitors are less effective in the presence of GAGs

I26P-IAPP and NMe-G24, NMe-I26-IAPP are both inhibitors of IAPP amyloid formation in homogeneous solution. We tested their efficiency in the presence of heparan sulfate. Figure 8a displays the results of an inhibition experiment using I26P-IAPP. In the absence of GAG, the lag phase of amyloid formation by IAPP was a factor of 3 times longer when the inhibitor was present, consistent with previous reports.⁸ Quite different results were obtained when GAG was present in the mixture; an initial, rapid increase in the thioflavin fluorescence was observed followed by an intermediate plateau. Similar biphasic behavior has been observed during GAG catalyzed amyloid formation by proIAPP processing intermediates and is thought to be due to the rapid formation of a GAG bound intermediate.³³ This was followed by a second growth phase leading to a final plateau. TEM analysis of aliquots removed during the first plateau revealed the presence of thin fibers as well as shorter fibril like objects (Figure 8). Numerous fibers were observed in the final plateau (Figure 8). The results of the thioflavin-T assays are difficult to unambiguously interpret since both polypeptides can form amyloid in the presence of GAGs. Thus the final thioflavin-T signal could arise from amyloid formation by one or both of the polypeptides. None the less, it is clear that mixtures of wild type IAPP with I26P-IAPP or with NMe-G24, NMe-I26-IAPP behave very differently in the presence of GAGs than in their absence.

This experiment was conducted by mixing IAPP, inhibitor and HS at the beginning of the experiment. The effect of the GAG could arise from sequestration of the inhibitor by HS. We conducted an additional experiment to test whether GAGs accelerated amyloid formation if they were added in the lag phase of the I26P-IAPP: IAPP mixture. In this experiment, IAPP and inhibitor were allowed to form a complex before addition of HS. HS

still accelerated amyloid formation (Figure 9), suggesting that simple sequestration of the inhibitor is not the only cause of the HS induced effects. This is reasonable since both IAPP and I26P-IAPP can bind HS and there is no reason that HS should selectively remove I26P-IAPP from solution.

We next examined the ability of NMe-G24, NMe-I26-IAPP to inhibit amyloid formation. Figure 10a compares thioflavin-T kinetic curves for wild type IAPP in the absence of heparan sulfate, a 1:1 mixture of wild type IAPP and inhibitor without heparan sulfate and a 1:1 mixture of wild type IAPP and inhibitor in the presence of heparan sulfate. NMe-G24, NMe-I26-IAPP is, as expected, an effective inhibitor in the absence of heparan sulfate. Under our conditions wild type IAPP forms amyloid with a lag time of 17 mins, in contrast, no significant increase in thioflavin-T fluorescence is observed for 150 mins in the presence of inhibitor although eventually a significant increase is detected around 400 mins. The inhibitor is less effective when heparan sulfate is present. A rapid increase in thioflavin-T fluorescence is observed, which is followed, quickly, by a second transition to a final plateau. TEM reveals some fibril like aggregates in the first plateau (Figure 10d). Dense mats of fibers were observed at the end of the reaction (Figure 10e).

We also analyzed the ability of the small molecule (–)-epigallocatechin 3-gallate (EGCG) to inhibit amyloid formation in the presence of heparan sulfate in order to test if GAGs can affect small molecule inhibitors as well as peptide-based inhibitors. EGCG is a green tea-derived flavanol that has been reported to inhibit amyloid formation by a wide range of natively unfolded polypeptides including IAPP.^{9,48–51} Previous work from our laboratory has shown that EGCG effectively inhibits *in vitro* IAPP amyloid formation and dissociates pre-formed fibrils into small aggregates.^{9,51} In the presence of heparan sulfate without EGCG, IAPP amyloid formation is greatly accelerated compared to the same reaction in homogeneous solution (Figure 11). No increase of thioflavin-T fluorescence is observed for the 1:1 mixture of IAPP with EGCG without heparan sulfate (Figure 11). However, when heparan sulfate and EGCG are present, an initial rapid increase of thioflavin-T fluorescence was observed followed by a slow decrease, which may indicate that aggregates formed immediately and then gradually dissociated into small aggregates or were restructured (Figure 11, red curve). The initial increase of fluorescence was lower for higher concentrations of added EGCG (Supplementary Material), but the effect was still observable even when EGCG was in 20 fold excess. TEM images confirmed the existence of small aggregates at the end of each experiment. (Figure 11, Supplementary Material) The aggregates did not show any obvious change in morphology as the concentration of EGCG is increased (Supplementary Material).

EGCG was still able to dissociate amyloid fibrils formed by IAPP in the presence of heparan sulfate (Figure 12a). The structures of the aggregates formed were similar to those formed when EGCG is added at the beginning of the reaction as judged by TEM images (Figure 12, Supplementary Material). CD confirmed that all of the aggregates contain β -sheet structure (Supplementary Material). These results indicate that EGCG is still able to inhibit IAPP amyloid formation and dissociate mature amyloid fibrils into small aggregates in the presence of heparan sulfate, but is less effective than in a homogeneous environment.

Conclusions

The analysis presented here reveals that IAPP amyloid formation in heterogeneous environment is very different than in homogeneous solutions: Apparently non-amyloidogenic variants can be readily induced to form amyloid in the presence of GAGs and some peptide based and small molecule amyloid inhibitors are less effective. The GAGs tested here are more effective at inducing amyloid formation than are anionic lipid vesicles on a per net

charge basis even when the total number of anionic sites is the same, suggesting that the spatial arrangement of negatively charged sites is important. This conjecture is supported by a recent study of the effects of varying the number of sulfated saccharide monomers in heparin on amyloid formation by wild type human IAPP.³⁹ Small fragments were less effective than large ones, but the length dependence leveled off beyond 12 sulfated saccharide units. Control experiments showed that the dependence was due to a length effect rather than an increase in the number of monomer units. The length dependent effects are believed to arise from the ability of the longer heparin fragments to adopt specific structures. Solution NMR studies have shown that heparin forms a left-handed helix structure with four saccharides per turn⁵² and the length dependence studies imply that heparin exerts its most potent effects on IAPP amyloid formation when it can form helix structures.³⁹ IAPP contains a positively charged N-terminus, a Lys at position 1, an Arg at position-11 and a His at position 18. All of these groups have been shown to be important for IAPP GAG interactions and the charge arrangement in helical heparin fragments is complimentary to the arrangement of positive charges in models of IAPP amyloid fibers.^{33,39,53} Modeling of A β GAG interactions also suggests a role for complimentary charged interfaces in the catalytic effects of GAGs on A β amyloid formation.⁵⁴

HSPGs are associated with *in vivo* islet amyloid and the interaction of proIAPP processing intermediates with HSPGs of the extracellular has been postulated to play a role in inducing amyloid formation.^{34,38} Studies with inhibitors of GAG synthesis show that decreasing GAG synthesis and protein glycosylation in islets results in reduced islet amyloid formation *in vitro*.⁵⁵ This result is consistent with earlier *in vivo* findings that these inhibitors reduce amyloid formation in mouse models of Amyloid A amyloidosis.⁵⁶ These *in vitro* and *in vivo* studies suggest that limiting IAPP GAG interaction might be a therapeutic method for amyloid related diseases.

Relatively little attention has been paid to the role of IAPP GAG interactions. The results presented here show they can strongly impact amyloid formation and the efficiency of amyloid inhibitors. Thus potential interactions with GAGs should be considered in inhibitor design and in the analysis of amyloidogenicity. GAGs have also been shown to induce amyloid formation by other polypeptides which appear to be non-amyloidogenic in homogeneous solution.⁵⁷ Thus the effects observed with IAPP may be one example of a broader role for GAGs in amyloid formation.

Materials and Methods

Peptide Synthesis and Purification

Peptides were synthesized on a 0.25 mmol scale using a CEM Liberty Microwave Peptide Synthesizer utilizing 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. 5-(4'-fmoc-aminomethyl-3',5-dimethoxyphenol) valeric acid (PAL-PEG) resin was used to form an amidated C-terminus. Fmoc protected pseudoproline (oxazolidine) dipeptide derivatives were incorporated to facilitate the synthesis as previously described.^{58,59} Standard Fmoc reaction cycles were used. The first residue attached to the resin, all β -branched residues and all pseudoproline dipeptide derivatives were double-coupled. N-methylated isoleucine was triple-coupled. The alanine that directly followed the N-methylated isoleucine was coupled 5 times. N-methylated glycine, the phenylalanine and the asparagines directly following the N-methylated glycine were double coupled. The peptides were cleaved from the resin through the use of standard trifluoroacetic acid (TFA) methods. Crude peptides were oxidized by dimethyl sulfoxide (DMSO) at room temperature. The peptides were purified via reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C18 preparative column.⁶⁰ Analytical HPLC were used to check the purity of the peptides before each experiment. The identity of the pure peptides was confirmed by MALDI-TOF MS.

Wild type IAPP expected 3902.9, observed 3902.7; I26P-IAPP expected 3888.3, observed 3888.2; NMe-G24, NMe-I26-IAPP expected, 3930.9, observed, 3930.8.

Sample preparation

Peptide stock solutions were prepared in 100% hexafluoroisopropanol (HFIP) at 1.6 mM. Low molecular weight heparan sulfate (10,000–14,000 molecular weight), chondroitin sulfate and dermatan sulfate were purchased from Sigma. Fluorescein-labeled heparin (FLH) with an average molecular weight of 18,000 was purchased from Invitrogen. All GAG stock solutions were prepared by dissolving GAG in 20 mM Tris-HCl (pH 7.4) at 2.2 mg/ml. EGCG was purchased from Sigma and dissolved in 20 mM Tris-HCl (pH 7.4) at 1.6 mM immediately before use.

Preparation of large unilamellar vesicles (LUVs)

The LUVs were composed of 100% 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), from Avanti Polar Lipids. Stock solutions of DOPG in chloroform at a concentration of 10 mg/mL were first evaporated with a stream of nitrogen gas and then dried under a vacuum overnight to completely remove the residual organic solvent. The resulting lipid film was hydrated in 20 mM Tris-HCl, pH 7.4 buffer for 1 hour, at a lipid concentration of 8 mM. The multilamellar vesicles were subjected to 10 freeze-thaw cycles and extruded 11 times through 100 nm pore size filters (Whatman, GE). The phospholipid concentration was determined by the method of Stewart.⁶¹ A fresh vesicle solution was used for each experiment.

Fluorescence Assays

Two types of ThioflavinT binding assays were utilized, one for samples with hexafluoroisopropanol (HFIP) and the other for samples which did not contain HFIP. Amyloid formations by I26P-IAPP and NMe-G24, NMe-I26-IAPP and their ability to inhibit amyloid formation by wild type human IAPP in the presence and absence of GAGs were first monitored by thioflavin-T binding assays conducted in the presence of 2% hexafluoroisopropanol (HFIP) and with continuous stirring at 25 °C. Fluorescence measurements were performed using an Applied Photon Technology fluorescence spectrophotometer with 450 nm excitation and 485 nm emission. The slit widths for excitation and emission were set at 4 nm and a 1.0 cm cuvette was used. Each point was averaged for 1 minute. Solutions were prepared by diluting filtered stock solution (0.45 μ M Acrodisc syringe filter with GHP membrane) into Tris-HCl buffer and thioflavin-T solution immediately before the measurement. The final concentration was 16 μ M peptide and 32 μ M thioflavinT in 20 mM Tris-HCl (pH 7.4). In the I26P-IAPP and NMe-G24, NMe-I26-IAPP amyloid formation experiments, GAGs (heparan sulfate, chondroitin sulfate and dermatan sulfate), when present, were at 1.3 μ M. In the inhibition experiments, heparan sulfate, when present, was at 2.6 μ M. FRET experiments involving fluorescein-labeled heparin (FLH) were also monitored using this assay in solutions which contained 2% HFIP. Fluorescence measurements were performed on the same instrument using 440 nm excitation and 510 nm emission. The final solution composition was 16 μ M peptide, 1.3 μ M FLH and 32 μ M thioflavinT, when present.

Amyloid formation by I26P-IAPP and NMe-G24, NMe-I26-IAPP in the presence and absence of heparan sulfate were also monitored by thioflavin-T binding assays in the absence of HFIP. These assays were conducted without stirring using a Beckman Coulter DTX 880 plate reader with multimode detector using 430 nm excitation and 485 nm emission at 25°C. Fluorescence solutions were prepared by lyophilizing filtered stock solution (0.45 μ M Acrodisc syringe filter with GHP membrane) for 22 hrs and then dissolving dry peptides into Tris-HCl buffer and thioflavin-T solution immediately before

the measurement. The final concentration was 16 μ M peptide and 32 μ M thioflavin-T in 20 mM Tris-HCl (pH 7.4). Heparan sulfate, when present, was at 1.3 μ M. Experiments involving high ionic strength, lipid micelle or EGCG were also monitored using this assay.

Circular Dichroism (CD)

Far-UV CD experiments were performed at 25°C on an Applied Photophysics Chirascan CD spectrophotometer. Aliquots from the kinetic experiments were removed at relevant time points during the experiments and the spectra were recorded. Spectra were the average of three repeats recorded over a range of 190–260 nm, at 1 nm intervals. A 0.1 cm quartz cuvette was used and a background spectrum was subtracted from the collected data.

Transmission Electron Microscopy (TEM)

TEM images were collected at the Life Science Microscopy Center at the State University of New York at Stony Brook. 15 μ L aliquots of the samples used for the kinetic studies were removed at relevant time points, placed on a carbon-coated 300-mesh copper grid for 1 min and then negatively stained with saturated uranyl acetate for 1 min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IAPP	islet amyloid polypeptide
I26P-IAPP	I26P point mutant of human IAPP
NMe-G24	NMe-I26-IAPP, G24-N-Methyl, I26-N-Methyl-IAPP
GAG	glycosaminoglycan
HS	heparan sulfate
FLH	fluorescein labeled heparin
HFIP	hexafluoroisopropanol
DMSO	dimethyl sulfoxide
LUV	large unilamellar vesicles
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
GalNAc	N-acetyl--galactosamine
IdoA	iduronic acid
CD	circular dichroism
TEM	transmission electron microscopy
EGCG	(-)-epigallocatechin 3-gallate

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Wild type IAPP: **I26P-IAPP:** **NMe-G24, NMe-I26-IAPP:** **Figure 1.**

Sequence of wild type IAPP, I26P-IAPP and G24-N-methyl, I26-N-methyl-IAPP (NMe denotes N-methylation). Each peptide has an amidated C-terminus and a disulfide bond.

Figure 2.

Amyloid formation by I26P-IAPP in the absence and presence of heparan sulfate. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. Black, I26P-IAPP in the absence of heparan sulfate; red, I26P-IAPP in the presence of heparan sulfate. The insert shows an expanded plot of the first 50 mins. (b) TEM image of I26P-IAPP in the presence of heparan sulfate. (c) TEM image of I26P-IAPP in the absence of heparan sulfate. Aliquots were removed at the end of each kinetic experiment for TEM analysis. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of I26P-IAPP was 16 μ M. Heparan sulfate, when present, was at 1.3 μ M.

Figure 3.

Amyloid formation by NMe-G24, NMe-I26-IAPP in the absence and presence of heparan sulfate. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. Black, NMe-G24, NMe-I26-IAPP in the absence of heparan sulfate; red, NMe-G24, NMe-I26-IAPP in the presence of heparan sulfate. The insert shows an expanded plot of the first 100 mins. (b) TEM image of NMe-G24, NMe-I26-IAPP in the presence of heparan sulfate. (c) TEM image of NMe-G24, NMe-I26-IAPP in the absence of heparan sulfate. Aliquots were removed at the end of each kinetic experiment for TEM analysis. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of NMe-G24, NMe-I26-IAPP was 16 μ M. Heparan sulfate, when present, was at 1.3 μ M.

Figure 4.

Heparan sulfate is associated with amyloid fibrils. FRET between fluorescein labeled heparin (FLH) and thioflavin-T bound to amyloid fibrils formed by I26P-IAPP. (a) Kinetic profile of I26P-IAPP in the presence of FLH monitored by FRET between FLH and thioflavin-T bound to amyloid fibrils. The fluorescence was measured using an excitation wavelength of 440 nm and an emission wavelegnth of 510 nm. Black, control experiment, I26P-IAPP in the presence of FLH, no thioflavin-T; red, I26P-IAPP in the presence of FLH and thioflavin-T. (b) TEM image of I26P-IAPP in the presence of FLH and thioflavin-T. An aliquot was removed at the end of the reaction for TEM analysis. (c) TEM image of I26P-IAPP in the presence of FLH, no thioflavin-T. An aliquot was removed at the end of the reaction for TEM analysis. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of I26P-IAPP was 16 μ M. FLH was at 1.3 μ M.

Figure 5.

I26P-IAPP amyloid formation in the presence and absence of heparan sulfate at different NaCl concentrations. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. The kinetic profiles were collected with 20 mM Tris-HCl and either 150 mM or 500 mM NaCl at pH 7.4, no HFIP, without stirring at 25 °C. Black and blue curves were collected in a buffer with 170 mM total salt concentration (20 mM Tris+150 mM NaCl). Black, I26P-IAPP in the absence of heparan sulfate; blue, I26P-IAPP in the presence of heparan sulfate. Green and pink curves were collected in a buffer with 520 mM salt concentration (20 mM Tris+500 mM NaCl). Green, I26P-IAPP in the absence of heparan sulfate; pink, I26P-IAPP in the presence of heparan sulfate. (b) TEM image of I26P-IAPP in the absence of heparan sulfate with 150 mM NaCl in the buffer. (c) TEM image of I26P-IAPP in the presence of heparan sulfate with 150 mM NaCl in the buffer. (d) TEM image of I26P-IAPP in the absence of heparan sulfate with 500 mM NaCl in the buffer. (e) TEM image of I26P-IAPP in the presence of heparan sulfate with 500 mM NaCl in the buffer. Aliquots were removed at the end of each experiment for TEM analysis. Scale bars represent 100 nm. The concentration of I26P-IAPP was 16 μ M. Heparan sulfate, when present, was at 1.3 μ M.

Figure 6.

Comparison of amyloid formation by I26P-IAPP in the presence of GAG and lipids. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. Black, I26P-IAPP in the presence of heparan sulfate; red, I26P-IAPP in the presence of 100 nm DOPG vesicles. (b) TEM image of I26P-IAPP in the presence of heparan sulfate. (c) TEM image of I26P-IAPP in the presence of 100 nm DOPG vesicles. Aliquots were removed at the end of each reaction for TEM analysis. Scale bars represent 100 nm. The kinetic profiles were collected with 20 mM Tris at pH 7.4, no HFIP, no stirring at 25°C. The concentration of IAPP is 16 μ M. Heparan sulfate, when present was at 0.97 μ M. DOPG, when present, was at 48 μ M. The concentration of heparan sulfate and DOPG were chosen so that the samples would contain the same number of negatively charged sites.

Figure 7.

Comparison of I26P-IAPP amyloid formation in the presence of different GAGs. (a) Kinetic profiles. Black, I26P-IAPP in the presence of heparan sulfate; red, I26P-IAPP in the presence of chondroitin sulfate; green, I26P-IAPP in the presence of dermatan sulfate. (b) TEM image of I26P-IAPP in the presence of chondroitin sulfate. (c) TEM image of I26P-IAPP in the presence of dermatan sulfate. Aliquots were removed at the end of each reaction for TEM analysis. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of I26P-IAPP was 16 μ M, and the concentration of GAG was 1.3 μ M.

Figure 8.

Inhibition of IAPP amyloid formation by I26P-IAPP in the absence and presence of heparan sulfate. (a) The results of thioflavin-T binding assays are plotted. Black, wild type IAPP in the absence of heparan sulfate; red, a 1:1 mixture of wild type IAPP and I26P-IAPP in the absence of heparan sulfate; green, a 1:1 mixture of wild type IAPP and I26P-IAPP in the presence of heparan sulfate. (b) TEM image of wild type IAPP in the absence of heparan sulfate. An aliquot was removed at the end of the reaction for TEM analysis as indicated by the black rectangle in panel A. (c) TEM image of a 1:1 mixture of wild type IAPP and I26P-IAPP in the absence of heparan sulfate. An aliquot was removed at the end of the reaction for TEM analysis as indicated by the red triangle in panel A. (d) TEM image of a 1:1 mixture of wild type IAPP and I26P-IAPP in the presence of heparan sulfate in the first plateau. An aliquot was removed in the middle of the first plateau (10 mins) for TEM analysis as indicated by the green circle in panel A. (e) TEM image of a 1:1 mixture of wild type IAPP and I26P-IAPP in the presence of heparan sulfate. An aliquot was removed at the end of the reaction for TEM analysis as indicated by the green star in panel A. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of wild type IAPP was 16 μ M. Heparan sulfate, when present, was at 2.6 μ M.

Figure 9.

Heparan sulfate can induce amyloid formation by a mixture of IAPP and I26P-IAPP. The results of thioflavin-T binding assays are plotted. Red, a 1:1 mixture of wild type IAPP and I26P-IAPP in the absence of heparan sulfate; blue, a 1:1 mixture of wild type IAPP and I26P-IAPP in the presence of heparan sulfate, heparan sulfate was added at the time point as indicated by the black arrow.

Figure 10.

Inhibition of IAPP amyloid formation by NMe-G24, NMe-I26-IAPP in the absence and presence of heparan sulfate. (a) The results of thioflavin-T binding assays are plotted. Black, wild type IAPP; red, a 1:1 mixture of wild type IAPP and NMe-G24, NMe-I26-IAPP in the absence of heparan sulfate; green, a 1:1 mixture of wild type IAPP and NMe-G24, NMe-I26-IAPP in the presence of heparan sulfate. (b) An expansion of the first 200 mins of panel A. The same color coding is used. (c) TEM image of a 1:1 mixture of wild type IAPP and NMe-G24, NMe-I26-IAPP in the absence of heparan sulfate. An aliquot was removed at the end of the reaction (563 min) for TEM analysis. (d) TEM image of a 1:1 mixture of wild type IAPP and NMe-G24, NMe-I26-IAPP in the presence of heparan sulfate in the first plateau. An aliquot was removed in the middle of the first plateau (17 min) for TEM analysis. (e) TEM image of a 1:1 mixture of wild type IAPP and NMe-G24, NMe-I26-IAPP in the presence of heparan sulfate. An aliquot was removed at the end of the reaction (333 min) for TEM analysis. Scale bars represent 100 nm. The kinetic experiments were conducted in 20 mM Tris-HCl (pH 7.4), 2% HFIP (v/v) with continuous stirring at 25 °C. The concentration of wild type IAPP was 16 μ M. Heparan sulfate, when present, was at 2.6 μ M.

Figure 11.

Heparan sulfate interferes with the ability of EGCG to inhibit IAPP amyloid formation. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. Black, IAPP in the presence of heparan sulfate; grey, a 1:1 mixture of IAPP and EGCG in the absence of heparan sulfate; red, a 1:1 mixture of IAPP and EGCG in the presence of heparan sulfate. (b) TEM image of an aliquot removed at the end of the experiment represented by the black curve in panel A, at the time point corresponding to the black diamond. (c) TEM image of an aliquot removed at the end of the experiment represented by the grey curve in panel A, at the time point corresponding to the grey circle. (d) TEM image of an aliquot removed from the sample represented by the red curve in panel A, at the time point corresponding to the red star. (e) TEM image of an aliquot removed at the end of the experiment represented by the red curve in panel A, at the time point corresponding to the red triangle. Scale bar represent 100 nm. Kinetic experiments were conducted under experimental conditions with 20 mM Tris-HCl, no HFIP and no stirring at 25 °C. The concentration of IAPP was 16 μ M. Heparan sulfate, when present, was at 1.3 μ M.

Figure 12.

EGCG can dissociate amyloid fibrils formed by IAPP in the presence of heparan sulfate. (a) Results of fluorescence-monitored thioflavin-T binding assays are displayed. Black, IAPP in the presence of heparan sulfate; red, a 1:1 mixture of IAPP and EGCG in the presence of heparan sulfate, EGCG was added at the time point indicated by the red arrow. (b) TEM image of an aliquot taken at the end of the experiment represented by the red curve in panel A, at the time point corresponding to the red triangle. Scale bar represent 100 nm. Kinetic experiments were conducted under experimental conditions with 20 mM Tris-HCl, no HFIP and no stirring at 25 °C. The concentration of IAPP was 16 μ M. Heparan sulfate was at 1.3 μ M.