Analysis of the Minimal Amyloid-forming Fragment of the Islet Amyloid Polypeptide

AN EXPERIMENTAL SUPPORT FOR THE KEY ROLE OF THE PHENYLALANINE RESIDUE IN AMYLOID FORMATION

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The development of type II diabetes was shown to be associated with the formation of amyloid fibrils consisted of the islet amyloid polypeptide (IAPP or amylin). Recently, a short functional hexapeptide fragment of IAPP (NH2-NFGAIL-COOH) was found to form fibrils that are very similar to those formed by the full-length polypeptide. To better understand the specific role of the residues that compose the fragment, we performed a systematic alanine scan of the IAPP "basic amyloidogenic units." Turbidity assay experiments demonstrated that the wild-type peptide and the Asn1 → Ala and Gly3 → Ala peptides had the highest rate of aggregate formation, whereas the Phe2 → Ala peptide did not form any detectable aggregates. Dynamic light-scattering experiments demonstrated that all peptides except the Phe2 → Ala form large multimeric structures. Electron microscopy and Congo red staining confirmed that the structures formed by the various peptides are indeed amyloid fibrils. Taken together, the results of our study provide clear experimental evidence for the key role of phenylalanine residue in amyloid formation by IAPP. In contrast, glycine, a residue that was suggested to facilitate amyloid formation in other systems, has only a minor role, if any, in this case. Our results are discussed in the context of the remarkable occurrence of aromatic residues in short functional fragments and potent inhibitors of amyloid-related polypeptides. We hypothesize that π-π interactions may play a significant role in the molecular recognition and self-assembly processes that lead to amyloid formation.

Amyloid fibril formation is a central feature in a variety of unrelated pathological situations. A partial list includes Alzheimer’s disease, prion diseases, diabetes mellitus (type II diabetes), familial amyloidosis, and light-chain amyloidosis (for review see Refs. 1–4). Islet amyloids are found in more than 95% of the patients with type II diabetes mellitus and are most likely an important factor in the development of β-cells failure (5–8). The islet amyloid fibrils consist predominantly of the islet amyloid polypeptide (IAPP or amylin), a 37-amino acid polypeptide hormone that is produced by pancreatic β-cells (5, 9–15). IAPP plays a central role in glucose homeostasis in its soluble form (16). Although the molecular mechanism of IAPP amyloidogenesis in vivo is not fully understood, the in vitro mechanism has been studied extensively. The 37-amino acid IAPP was shown to form amyloid fibrils in vitro (5, 10, 11, 16, 17). These fibrils were shown to be cytotoxic to pancreatic β-cell culture and thus are assumed to play a major role in the diabetes mechanism (18, 19). The kinetics of amyloid formation by IAPP as determined by turbidity assay is consistent with a nucleation-dependent mechanism of polymerization (20–23).

Recently, a six-residue peptide fragment of the human IAPP (with the amino acid sequence NFGAIL using the 1-letter code) was shown to form amyloid fibrils that are very similar to those formed by the full-length polypeptide (24). Furthermore, rodent IAPP, which does not form amyloid in vitro, is almost identical to human IAPP apart from a seven-amino acid block that includes most of this hexapeptide motif (Fig. 1A). Therefore, this six-amino acid motif seems to serve as the “basic amyloidogenic unit” of the human IAPP polypeptide. A shorter five-residue fragment (FGAIL) also forms ordered amyloid fibrils. However, those fibrils are somewhat different in their morphology as compared with the full-length peptide (24). A shorter peptide corresponding to the GAIL sequence did not form any fibrils at all (24).

Although different amyloid-related sequences do not reveal any sequence homology, they all share similar ultrastructural and physicochemical properties (reviewed in Refs. 1, 4, and 25). Amyloid deposits are characteristic of fibrils rich with β-sheet sheet structures, which are on average 7–10 nm in diameter and of varying length. Another well-known characteristic of amyloid fibrils is the observation of a green birefringence after staining of the amyloid with Congo red (CR) dye (26, 27). Amyloid formation is different from a simple process of nonspecific aggregation because amyloid fibrils show ordered structures that also have a characteristic x-ray diffraction pattern (1, 4). All of the above suggest that a specific pattern of molecular interactions, rather than nonspecific hydrophobic interactions, would lead to such an ordered process. Nevertheless, common structural elements that mediate the interactions that lead to these organized structures were not identified yet. The determination of such interactions, which underlay molecular recognition and self-assembly, is crucial for profound understanding of the amyloid formation process. Furthermore, such knowledge is valuable for the future design of drugs that can block these interactions and thus have a key clinical importance.

Here we determine the role of each residue of the six amino acids that comprise the basic amyloidogenic unit of the IAPP by performing a systematic alanine scan of the motif followed by a structural and functional analysis of the various peptides. We
studied the aggregation kinetics of the various peptides using a turbidity assay followed by dynamic light-scattering assays to estimate the size of aggregates that were formed by the different peptides. Finally, we determined the fibrillogenic nature of the different structures formed by the peptides using ultrastructural analysis by electron microscopy and CR staining.

**EXPERIMENTAL PROCEDURES**

**Peptides Synthesis**—Peptide synthesis was performed by Peptigenic Research & Co. Inc. (Livermore, CA). The correct identity of the peptides was confirmed by ion spray mass-spectrometry using a PerkinElmer Sciex API I spectrometer. The purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography on a C18 column using a linear gradient of 10 to 70% acetonitrile in water and 0.1% trifluoroacetic acid.

**Kinetic Aggregation Assay**—Freshly prepared stock solutions were prepared by dissolving lyophilized form of the peptides in Me2SO at a concentration of 100 mM. To avoid any pre-aggregation, fresh stock solutions were prepared for each and every experiment. Peptide stock solutions were diluted into assay buffer in enzyme-linked immunoosorbent assay plate wells as follows: 2 μL of peptide stock solutions were added to 98 μL of 10 mM Tris, pH 7.2 (hence the final concentration of the peptide was 2 mM in the presence of 2% Me2SO). Turbidity data were collected at 405 nm. Buffer solution containing the same amount of Me2SO as the tested samples was used as the blank. Turbidity was measured at room temperature over several time points.

**Dynamic Light Scattering**—Dynamic light scattering was used to estimate and compare the mean particle size for the aggregates that formed in each of the peptides solutions. Experiments were conducted with a laser-powered Noninvasive Back Scattering High Performance Particle Sizer (ALV, Langen, Germany). Freshly prepared stock solution with a concentration of 10 mM peptide in Me2SO was diluted into 10 mM Tris buffer, pH 7.2 (which was filtered through a 0.2-μm filter) to a final concentration of 100 μM peptide and 1% Me2SO. Autocorrelation data were fitted using the ALV-NIBS/HPPS software to derive average apparent hydrodynamic diameters.

**Congo Red Staining and Birefringence**—A suspension of peptide fibrils in 10 mM Tris buffer, pH 7.2 (aged for 4 days), was allowed to dry on a glass microscope slide. Staining was performed by the addition of a solution of 1 mM CR in 10 mM Tris buffer, pH 7.2, for about 1 min followed by rinsing with double-distilled water to remove excess CR and then drying. Alternatively, for the peptides that did not form aggregates well (and thus washing resulted in disattachment of the peptide from the slide) we used a saturated solution of CR in 80% ethanol (v/v) for staining and drying (without washing). Birefringence was determined with a WILD Makroskop m420 equipped with a polarizing stage.

**Electron Microscopy**—A solution of peptides (2 mM) in 10 mM Tris buffer, pH 7.2, was incubated overnight at room temperature. Fibril formation was assessed using a 10-μL sample placed on 200-mesh copper grids covered by carbon-stabilized Formvar film. After 20–30 s, excess fluid was removed, and the grids were then negatively stained with 2% uranyl acetate in water. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

**RESULTS**

The minimal amyloid-forming fragment of IAPP provides a unique case of an extremely short peptide fragment that contains all of the structural information needed to mediate the molecular recognition and self-assembly processes that lead to amyloid formation. To pinpoint the role of each amino acid in the formation of amyloid fibrils by this short fragment, a systematic substitution of the amino acid residues of the basic amyloidogenic unit with alanine was performed. We decided to substitute the various amino acids with alanine in order to specifically change the molecular interface of the peptides without dramatically changing their hydrophobicity and tendency to form β-sheet structures. The alanine scan was performed in the context of the block that is unique to human IAPP (Fig. 1A). This block includes two serine residues that follow the NFGAIL motif in the full-length polypeptide. These eight amino acid peptides were used to increase the solubility of the short peptides studied that are rather hydrophobic. Fig. 1B shows a schematic representation of the chemical structure of the wild-type peptide, and Fig. 1C indicates the amino acid substitu-
Although the physical size of the various structures may be somewhat different from the apparent hydrodynamic diameter because of irregularity of the amyloid structure, it certainly gave a clear indication of the order of magnitude of the structure formed and provided us with a quantitative criterion to compare the average sizes of the structures formed by the various peptides. The apparent hydrodynamic diameter of the structures formed by the various peptides seemed to be generally consistent with the results obtained by the turbidity assay with some subtle differences. As with the turbidity assay, the wild-type peptide and G3A peptide behaved in a very similar way (Fig. 3). Both peptides formed particles of very similar hydrodynamic diameters. Smaller structures were detected within the derivative peptides: N1A, I5A, and L6A (Fig. 3).
ments clearly suggested that no large particles are formed by the F2A peptide under the experimental conditions (Fig. 3).

**Congo Red Staining and Birefringence**—Next we studied whether the structures formed by the various peptides are indeed amyloid fibrils. One of the best known characteristics of amyloid fibrils is their ability to show typical birefringence upon binding of CR dye. The CR staining method combined with detection using cross-polarizers was applied to test the amyloidogenicity of the IAPP peptide fragment and its derivatives. Amyloid fibrils are known to bind CR and exhibit a gold-green birefringence under polarized light (26, 27). The occurrence and characteristics of the amyloid fibrils is their ability to show typical birefringence under the experimental conditions (Fig. 5) all indicate the essential role of the phenylalanine residue in the ability of the short peptide to form amyloid fibrils. These results are consistent with the observation of extremely slow kinetics, a solution of the peptide showed no detectable difference between this negative control and the staining of F2A peptide deposits.

To study whether the F2A peptide cannot form amyloid fibrils whatsoever or whether the undetectable CR binding is a result of extremely slow kinetics, a solution of the peptide under the same experimental conditions was incubated for 2 weeks. Although some degree of aggregation was observed after 2 weeks of incubation of the F2A peptide, CR staining showed no amyloid structure (results not shown). As a positive control, we incubated the wild-type peptide under the same conditions, and a typical CR birefringence was observed.

**Electron Microscopy**—We went further to carry out ultrastructural visualization of the structures formed by the various peptides. The occurrence and characteristics of the amyloid fibrils formed by the various peptides were studied by electron microscopy using negative staining. For this aim, we used peptide solutions (with the concentration of 2 μM) that were incubated overnight in 10 mM Tris buffer, pH 7.2, at room temperature. Filamentous structures were observed for all the peptides except F2A (Fig. 5). Appearance of fibrils formed by the I5A and L6A peptides (Fig. 5, E and F, respectively) was at a lower frequency as compared with the wild-type (Fig. 5D), N1A, and G3A peptides (Fig. 5, A and C, respectively). The solution containing peptide F2A consisted of amorphous aggregates only (Fig. 5B). In the case of peptides F2A, I5A, and L6A, the electron microscopy pictures do not exhibit a representative field but rather rare ones. Although those results support the quantitative results presented in the previous sections, it provides a qualitative evidence for the morphology of the fibrils.

**Discussion**

Despite the key clinical importance of the process of amyloid formation by diverse peptides and proteins, there is a profound lack of understanding regarding the structural elements that specifically mediate this process of self-assembly and molecular recognition. The short minimal active fragments of IAPP (Fig. 1) provide an excellent model system to address this question in the context of a very short amyloidogenic motif. The results presented in this article clearly indicate that a specific pattern of molecular recognition, rather than nonspecific hydrophobic interactions, directs this process of self-assembly in the case of IAPP fibrils formation. A major finding presented here is the key role of the phenylalanine residue in the process of amyloid formation by the short active fragment of IAPP. The turbidity assay (Fig. 2), dynamic light scattering (Fig. 3), CR binding experiments (Fig. 4), and electron microscopy examination (Fig. 5) all indicate the essential role of the phenylalanine residue in the ability of the short peptide to form amyloid fibrils. These results are consistent with the observation of

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**Table I**

| Name of parent peptide | Pathological or physiological condition | Short active sequence* | Reference |
|------------------------|----------------------------------------|------------------------|-----------|
| IAPP fibrilization inhibitor | Alzheimer's disease | FGAIL | (24), this study |
| IAPP fibrilization inhibitor | Alzheimers disease | QKLVF | (33) |
| IAPP fibrilization inhibitor | Listeria monocytogenes | LVPFD | (34, 35) |
| Lactadherin | Aortic medial amyloidosis | NFGGVQEV | (44) |
| Gelsolin | Finnish hereditary amyloidosis | SFNGOCGFILID | (45) |
| Serum amyloid | Chronic inflammation amyloidosis | SFFPSLGEAFPD | (46) |
| PrP | Creutzfeldt-Jakob disease | PHGGG | (2, 47) |
| Sup35p | Yeast prion protein | PGGGGQ | (48, 49) |

* Aromatic residues are underlined.

* The minimal active fragment may be shorter.

* Consensus sequence of six tandem repeats. In one of the repeats, Y is replaced by F.
dramatic reduction in the rate of aggregation of a longer IAPP fragment when the phenylalanine residue was changed to a proline or leucine (15, 23).

The isoleucine and leucine residues in the C terminus of the IAPP recognition element have an important role in the kinetics of amyloid formation (Fig. 2). However, unlike the F2A peptide, the I5A and L6A peptides will eventually form typical amyloid structures (Figs. 4 and 5), albeit with significantly slower kinetics (Fig. 2). Nevertheless, it is possible that the slow kinetics have a pathological significance, as in vivo amyloid formation of wild-type proteins is a very slow process (and thus amyloid diseases are generally associated with old age). It may be that under normal physiological conditions no fibrilization will occur upon the substitution of isoleucine or leucine to alanine in the context of a full-length IAPP.

The role of the asparagine residue as determined in this study is consistent with the study of the hexa- and pentapeptides (NFAGAIL and FGAIL, respectively (24)). We found that the presence of an alanine residue instead of an asparagine at the N terminus of the peptide actually accelerates the kinetics of the aggregation process (Fig. 2), but the hydrodynamic diameter (Fig. 3) and the morphology (Fig. 5) of the fibrils are also somewhat different from the wild-type fragment. This is consistent with the findings that the FGAIL pentapeptide forms fibrils but with different morphology as compared with the NFAGAIL hexapeptide and the full-length IAPP (24). Taken together, it seems that the asparagine residue is not essential for amyloid formation but has a role in the kinetics of aggregation and modulation of the fine structure of the fibrils.

Another interesting finding presented here is the fact that the G3A peptide behaved in a manner very similar to the wild-type peptide. This observation suggests that the glycine residue does not play a significant role in the formation of amyloids by the IAPP peptides. This observation is not trivial, as a priori glycine seems to be a residue that may have importance in amyloid formation because of its structural flexibility (27, 29, 30). Nonetheless, our results here are consistent with the study of synthetic peptides corresponding to a wild-type Alzheimer’s β-amyloid (Aβ) peptide fragment and a corresponding peptide with an alanine to glycine mutation (the “Flemish mutation”). The study of the aggregation of the peptides in vitro showed no significant change in the rate amyloid aggregation of the two peptides (31, 32).

The major role of the phenylalanine residue in the formation of amyloid by the IAPP short fragment is consistent with the key role that was found for phenylalanine residues in the amyloid formation by the Aβ polypeptide. A short fragment of Aβ that contains two phenylalanine residues (QKLVF) was shown to bind specifically to the full-length peptide (33). Furthermore, this short peptide could inhibit amyloid formation by the full-length Aβ (33). Follow-up studies have shown that not only the QKLVF peptide (33) but also a LVVFA peptide and its derivatives (34, 35) and a LPFFD peptide (36) are all potent inhibitors of amyloid formation by the Aβ polypeptide. Comparing the sequences of the various peptides clearly indicate that the pair of phenylalanine residues (“FF motif”) is the key structural element that mediates binding of the short peptides to Aβ. As the formation of amyloid is first of all a process of molecular recognition and self-assembly, the high affinity and selectivity of such an FF motif seems to provide the molecular recognition element needed for such processes in the context of the full-length Aβ.

Furthermore, all the above is consistent with the frequent occurrence of aromatic residues in other short amyloid-related fragments. When we analyzed a variety of short (5–12 amino acids) functional fragments of such sequences, we could clearly observe a notable frequent occurrence of aromatic residues in the group of short functional fragments (Table I). Such a frequent occurrence of aromatic residues raise the possibility that those residues play a significant role in the amyloid formation process by serving as structural and functional elements that direct molecular recognition and self-assembly. This is in line with the well known central role of π-stacking interactions in chemistry and biochemistry (37, 38). The actual thermodynamic details of π-stacking is still under open debate (39, 40). It is generally agreed that π-stacking contributes modestly to the enthalpic change (∆H) in free energy of interaction (∆G). It is also suggested that π-stacking has an entropic (∆S) part. According to these suggestions, ordered water molecules are released from the aromatic ring driven by the so-called hydrophobic interaction. In this line of reasoning, it is possible that aromatic interactions may significantly reduce the energetic barrier for the formation of amyloids, which can result in orders of magnitude acceleration of the amyloidosis process.

A great challenge in the field of amyloid research is to develop drugs that will specifically block the amyloid formation process. Our line of reasoning here suggests that drugs that will block π-stacking interaction may hint at a direction for the rational design of amyloid inhibitors. Recent studies directed toward the control of amyloid formation of the Aβ polypeptide (41) have shown the effectiveness of small molecules that contain aromatic elements (e.g. Ro 47-1816/001 (42), see Fig. 6A, and 3-p-toluoyl-2-[4’-(3-diethylaminopropoxy)-phenyl]-benzo-furan (43)). Interestingly, the CR dye, the specific amyloid-binding dye that was mentioned in the introduction, is also a generic inhibitor of amyloid formation (19, 26). The CR dye (Fig. 6C) as well as thioflavin T (Fig. 6B), another amyloid-specific dye, contain several aromatic elements. Analysis of the physicochemical properties of CR suggests that the aromatic elements are major structural elements in this compound. This implies that a stacking of aromatic moieties may mediate the interaction of CR with amyloid fibrils. Therefore, it is most striking that CR was found to interact with Aβ at the same binding site as Ro 47-1816/001 (42). Further work should be done to determine the actual mode of interaction of CR with amyloid fibrils. The mode of interaction of CR, a relatively simple molecule and a universal amyloid-binding dye, may serve as a paradigm for drug design to control amyloid formation.

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