the FEBS Journal

MINIREVIEW

Mechanisms of amyloid fibril self-assembly and inhibition

Model short peptides as a key research tool

Ehud Gazit

Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv, Israel

Keywords
amyloid formation; molecular recognition; protein folding; protein misfolding; protein–protein interactions; self-assembly; stacking interactions

Correspondence
E. Gazit, Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel
Fax: +972 3 640 5448
Tel: +972 3 640 9030
E-mail: ehudg@post.tau.ac.il

(Received 2 June 2005, accepted 10 October 2005)

doi:10.1111/j.1742-4658.2005.05022.x

The formation of amyloid fibrils is associated with various human medical disorders of unrelated origin. Recent research indicates that self-assembled amyloid fibrils are also involved in physiological processes in several micro-organisms. Yet, the molecular basis for the recognition and self-assembly processes mediating the formation of such structures from their soluble protein precursors is not fully understood. Short peptide models have provided novel insight into the mechanistic issues of amyloid formation, revealing that very short peptides (as short as a tetrapeptide) contain all the necessary molecular information for forming typical amyloid fibrils. A careful analysis of short peptides has not only facilitated the identification of molecular recognition modules that promote the interaction and self-assembly of fibrils but also revealed that aromatic interactions are important in many cases of amyloid formation. The realization of the role of aromatic moieties in fibril formation is currently being used to develop novel inhibitors that can serve as therapeutic agents to treat amyloid-associated disorders.

The formation of well-ordered amyloid fibrils by the self-assembly of various proteins and polypeptides is associated with serious human medical disorders like Alzheimer's disease, prion disorders (bovine spongiform encephalopathy and Creutzfeldt–Jakob disease), type II diabetes, and many others. Currently, about 20 different known syndromes are associated with the formation of amyloid deposits [1–5]. Several reports have also documented the formation of typical amyloid fibrils by disease-unrelated proteins [6,7]. Moreover, the results of recent studies point to the involvement of self-assembled amyloid fibrils in the formation of biofilm and aerial hyphae by micro-organisms [8–10]. Thus, the amyloid state is much more common and significant than previously appreciated.

A notable property common to this large group of amyloid protein deposits is that fibrils of different origins show similar biophysical and ultrastructural characteristics. In all cases, amyloid fibrils are highly ordered molecular assemblies with a diameter of 7–10 nm, as reflected by a typical X-ray fibre diffraction pattern of 4.6–4.8 Å on the meridian [3]. Additionally, various spectroscopic methods have shown that all fibrillar amyloid assemblies are predominantly in β-sheet conformation [1–5]. Another characteristic common to all amyloid aggregates is a clear green–gold birefringence upon staining with Congo red dye.

The association of amyloid fibrils formation with various medical conditions, as well as the structure and role of such fibrils in disease pathology, has been extensively reviewed [1–5]. In this minireview, we will focus on the experimental use of short peptide fragments as an important research tool for investigating the molecular recognition and self-assembly mechanisms that promote the formation of fibrillar protein and polypeptide deposits. Such simple, yet indispensable,

Abbreviations
Aβ, amyloid β-peptide; FDA, Food and Drug Administration; IAPP, islet amyloid polypeptide; PrP, prion protein.

models have provided new and surprising insights that have not only revolutionized the comprehension of the mechanisms of amyloid fibril formation but also point to novel ways for designing inhibitors.

Amyloid fibril formation as a generic protein-folding state

Despite the similarities among the supramolecular structures formed, no simple homology is apparent among the amyloid-forming proteins and polypeptides. The similarity among the different amyloid deposits and their ubiquity suggest that such structures might represent a generic form or the noncovalent packing of polypeptide chains [6,7,11]. It may very well be that the aggregation into such well-defined, nano-ordered assemblies represents a state of an efficient minimal energy arrangement of polypeptide chains, as often observed with crystalline organic and inorganic materials. Indeed, Jarrett & Lansbury [12] denoted amyloid fibrils as 'one-dimensional crystals'. Yet, because the amyloid crystallization process occurs even at low, submicromolar concentrations, a very clear process of molecular recognition and self-assembly must occur to enable the formation of such well-ordered, supramolecular structures.

Short peptides as models for amyloid formation

As a result of the complexity and enormous structural space allowed, even in relatively short 30–40 amino acid polypeptides, determining the molecular basis of the recognition and assembly processes fostering amyloid-fibril formation is a very complicated task. Moreover, the synthesis of large peptides, especially aggregative peptides, is expensive and difficult. An important direction in studying amyloid formation has emerged from the use of remarkably short peptide fragments.

Much of the pioneering work on the use of peptide models for the study of amyloid fibril formation was carried out by Westermark and co-workers [13–15]. This group had already demonstrated, in 1990, that a short decapeptide fragment of the islet amyloid polypeptide (IAPP), a polypeptide associated with type II diabetes [13,16], can form amyloid fibrils that are highly similar to those formed by the full-length, 37 amino acid polypeptide [13]. Identification of the short peptide motif was based on the discovery of a polymorphism within IAPP protein sequences that can either form or not form amyloid fibrils in various mammalian species. The variable region within the molecule was indeed found to mediate a recognition process initiating the formation of typical amyloid fibrils. The small size of this peptide fragment enabled its synthesis by simple solid-phase techniques, thus providing the possibility of constructing various analogues for determining the role of individual amino acids in the process [13].

The results of a later study demonstrated that, like the full-length polypeptide, a hendecapeptide fragment of serum amyloid A protein, which is involved in the chronic inflammation amyloidosis, forms typical amyloid fibrils (see Table 1 for a list of short amyloidogenic peptides) [14]. A dodecapeptide fragment of Gelsolin, a protein associated with Finnish hereditary amyloidosis, can also form such fibrillar structures [17]. Similarly, an octapeptide fragment of the medin protein was shown to form fibrillar assemblies [15]. The latter protein is of special interest because aortic amyloid fibrils composed of the medin protein are found in virtually all individuals above the age of 60 years [15]. It was subsequently revealed that even a minimal hexapeptide fragment of medin could promote the formation of typical amyloid deposits [18].

Table 1. Typical amyloid fibril formation by remarkably short aromatic peptide fragments[a].

Name of parent peptide	Pathological or physiological condition	Amyloidogenic sequence	Reference
Islet amyloid polypeptide	Type II diabetes	NFGAIL	[19]
		NFLVH	[22]
Amyloid β-peptide	Alzheimer's disease	KLVFFAE	[20]
Medin	Aortic medial amyloid	NFGSVQ	[18]
Calcitonin	Thyroid carcinoma	DFNKF	[21]
Gelsolin	Finnish hereditary amyloidosis	SFNNGDCCFILD[b]	[17]
Serum amyloid A	Chronic inflammation amyloidosis	SFFSFLGEAFD[b]	[14]
β2-microglobulin	Dialysis-associated renal amyloidosis	DWSFYLLYTEFT[b]	[57]
Designed peptide	None	KFFE	[23]

[a] Aromatic residues are underlined. [b] The minimal active fragment may be shorter.

A recent study carried out by Kapurniotu and co-workers [19] paved the way towards the present understanding of the mechanism of amyloid self-assembly. The authors first discovered that a hexapeptide fragment of human IAPP could form typical amyloid fibrils with ultrastructural and biophysical properties similar to those of the full-length 37 amino acid polypeptide. Moreover, the finding that both the short peptide and the full-length IAPP assemblies had similar cytotoxic activity showed, for the first time, that a peptide as small as a hexapeptide can form a well-ordered and functional amyloid structure. The authors then realized that even a pentapeptide fragment could form ordered fibrillar structures, although with a slightly different morphology than that found in canonical amyloid structures. In an independent work, Tycko and co-workers [20] demonstrated that a heptapeptide fragment of the amyloid β-peptide (Aβ) involved in Alzheimer's disease has the capacity of forming typical amyloid fibrils *in vitro*. Using solid state NMR, the authors further determined the structure of the formed deposits.

Following these studies, Reches *et al.* [21] and Mazor *et al.* [22] reported that other short fragments, such as the pentapeptide fragments of the human calcitonin peptide and IAPP (Table 1), rapidly and efficiently form typical amyloid fibrils. Despite having a diameter larger than that of the full-length protein, the tetrapeptide fragment of the calcitonin polypeptide formed ordered fibrillar structures [21]. Another group [23] reported that a short-designed tetrapeptide has the same clear ultrastructure, birefringence and secondary structure as that described for typical amyloid fibrils. Finally, a recent study revealed that a dipeptide fragment of the Alzheimer's Aβ peptide forms self-assembled nanotubular structures that are different from typical amyloid but show spectral signature and birefringence properties similar to those of the native peptide [24].

The role of aromatic interactions

Taken together, the results of these peptide studies indicate that very simple motifs contain all the molecular information required for the molecular recognition and self-assembly mediating the formation of amyloid fibrils. The small size of the peptides has reduced the complexity of the amyloid formation enigma while providing the ability to gain physicochemical insight into the mechanism of fibril formation.

One striking feature of the similarities among the short peptides that can form amyloid fibrils is the high occurrence of aromatic residues (Table 1). This observation is not trivial because aromatic moieties are among the less frequent amino acids found in proteins. It was suggested that stacking interactions have been suggested to provide an energetic contribution, as well as order and directionality, in the self-assembly of amyloid structures [25]. This view is in line with the well-known central role of aromatic-stacking interactions in general self-assembly processes in chemistry and biochemistry. One key example of the association of peptides into large ordered deposits is the spontaneous self-assembly of short aromatic peptides into ordered polymeric β-sheet tapes [26]. Such peptide polymers are partially stabilized by the noncovalent intersheet aromatic stacking that allows the ordered positioning of the assembled chains [26].

Accordingly, the systematic analysis of certain short amyloidogenic fragments using site-directed modification revealed that aromatic residues indeed play a crucial role in the fibrillization process [27]. The results of a systematic alanine-scan of a shorter IAPP fragment (Table 1) indicated that other than phenylalanine, any amino acid within the fragment could be replaced by alanine without losing the ability to form amyloid fibrils. When phenylalanine was replaced with alanine, however, no fibril formation occurred. Similarly, it was found that exchanging the phenylalanine residue with the calcitonin pentapeptide fragment completely abolishes the ability to form amyloid fibrils [21]. Similar results were obtained when the phenylalanine residue of the medin hexapeptide fragment was replaced with alanine or with the more hydrophobic amino acid, isoleucine [18]. The overall results of these studies pinpoint the central role of aromatic amino acids in the fibril formation process.

A hint for the role of aromatic residues in the formation of amyloid fibrils is also suggested by the analysis of peptide repeats that are involved in prion formation [25]. Both animal and yeast prion proteins are characterized by the occurrence of aromatic peptide repeats. The importance of these repeats is shown by the fact that many cases of inherited human prion disorders involved the addition of one to nine extra peptide repeats in addition to the five in normal prion protein (PrP) [28]. The role of the peptide repeats in the aggregation of yeast prion proteins was demonstrated by the observation that the conjugation of the repeats to heterologous nonaggregative protein induced its aggregation [29].

The suggested role of aromatic interactions in fibril formation is related to findings made by several groups that the structure of amyloid fibrils resembles β-helix architecture [30,31]. One main feature of the β-helix is

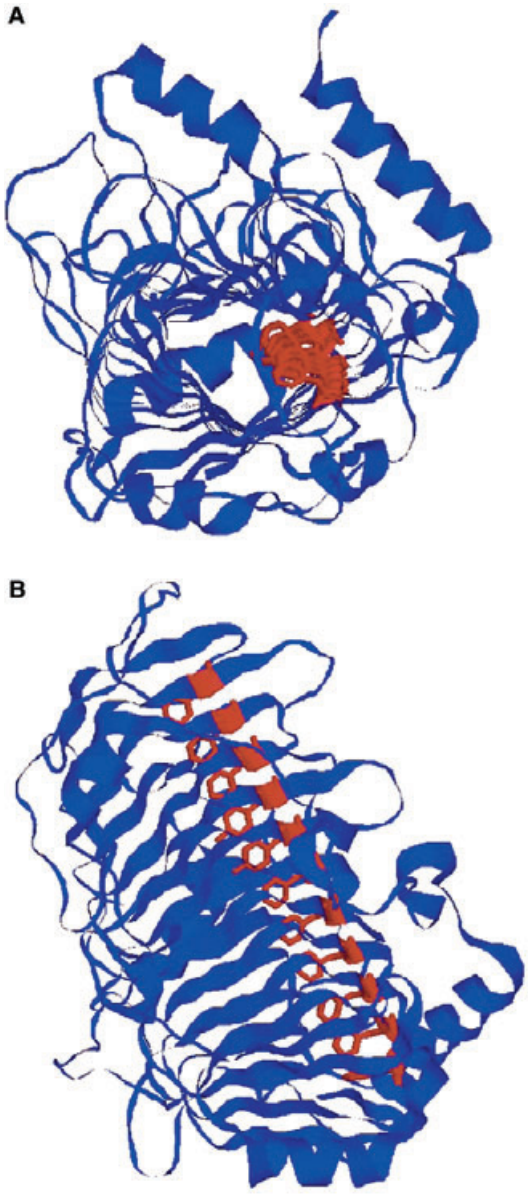

Fig. 1. Stacking interactions, a main characteristic of β-helical structures. (A) and (B) Ribbon view from two directions of chondroitinase B from *Flavobacterium heparinum* determined at a 1.7 Å resolution [31]. The stacked aromatic residues are shown in red display.

the stacking of similar residues on a flat β-sheet [33]. Figure 1 visualizes a stack of aromatic residues in the crystal structure of chondroitinase B from *Flavobacterium heparinum* [33]. The hypothesis and experimental results regarding the possible role of aromatic stacking in the process of amyloid formation by short peptide elements therefore provides further support to the theory relating amyloid fibrils to β-helix structures.

Recent structural and theoretical support to the aromatic interactions hypothesis

Two recent high-resolution structural studies provided direct evidence for the role of aromatic interactions in amyloid fibril formation [34,35]. A solid state NMR study of the calcitonin hormone, mentioned above, demonstrated that the aromatic moieties of its central phenylalanines are aligned on the same side of the β-sheet and stabilize the β-sheet conformation by forming aromatic interactions between the strands [34]. A more recent study provided an even higher-resolution analysis of the role of aromatic moieties in amyloid fibril formation. By combining the X-ray and electron diffraction studies, the authors determined the high-resolution structure of a crystalline preparation amyloidogenic dodecapeptide [35]. The high-resolution 1 Å diffraction revealed that the β-strands of the crystalline assemblies are zipped together by aromatic interactions between adjacent phenylalanine residues [35].

Theoretical studies also provided important information of the role of aromatic moieties in amyloid fibril formation [36–41]. A parameter-free model based on the mathematical analysis of many peptide fragments and their analogues had clearly suggested aromaticity as one of the key parameters for predicting the rate of the fibrillization process [36]. Molecular dynamics simulations of the stability of preformed amyloid fibrils clearly demonstrated the role of aromatic moieties in the *in silico* stabilization of such noncovalent assemblies. Significant stabilization mediated by aromatic moieties was observed in both IAPP fragments [37,38] and calcitonin [39]. Similar results were obtained when the assembly of IAPP peptides was simulated using molecular dynamics [40,41]. Simulations of the IAPP peptide with explicit solvent by two independent groups revealed the aggregative behavior of the peptide, with the aromatic moieties showing a key role in this interaction.

Amyloid formation by nonaromatic peptides

Worth mentioning is the fact that amyloid fibrils can also be formed by nonaromatic peptides. Such structures are formed by much larger peptides (e.g. including a domain of 42 or more amino acids in the case of Huntington-related polyglutamine repeats) or formed over a longer timescale (days and weeks compared with minutes in the case of the aromatic peptides). Moreover, the extent of amyloid formation by

most aromatic peptides studied is very high, with most of the material being converted into insoluble amyloid deposits. These observations – together with the concept of amyloid as a generic form of peptide aggregation – suggest that aromatic interactions are not essential for amyloid formation but can significantly aid in overcoming the energetic barriers that are necessary to form the structural assemblies. That any or most proteins will form amyloid fibrils at infinite time is likely, yet the presence of aromatic residues in specific structural contexts can accelerate this process by several orders of magnitude. Therefore, from the practical point of view, understanding of the mechanism of amyloid formation that is indeed associated with the interaction of aromatic moieties has a direct clinical importance.

Also important to stress is that the existence of aromatic moieties per se is not sufficient for amyloid fibril formation because not every aromatic pentapeptide or hexapeptide can form amyloid deposits. The limited number of short peptides shown in Table 1 provides certain structural clues. Apparently, the existence of opposite electrostatic charges and/or amide side-chains (glutamines and asparagines) is an important factor in the formation of efficient amyloidogenic short peptide fragments. More research should be undertaken to define the exact combinatorial chemical rules that mediate efficient fibrillization by such short peptide fragments.

Inhibition by short peptides

Analyzing short peptide fragments is crucial for developing small peptide inhibitors of this amyloidogenic process. Using peptide array technology in the case of the Alzheimer's Aβ peptide, a central region that mediates the intermolecular interactions between Aβ monomers to form amyloid fibrils was pinpointed [42]. This major recognition region is a pentapeptide element containing two phenylalanines, KLVFF, which indeed inhibited full-length Aβ-induced amyloid formation [43]. Such inhibition is probably based on recognition between the aromatic moieties, on the one hand, and electrostatic repulsion by the positively charged lysine residue on the other.

The KLVFF motif has served as a key platform for developing peptide and peptidomimetic inhibitors of Aβ fibrillization [43–48]. Hundreds of derivatives of this pentapeptide fragment have been investigated. The results of studies of the various derivatives clearly indicate that the presence of a central phenylalanine residue within the compounds is a key feature that must be preserved to achieve efficient and specific inhibition. This observation further supports the notion that aromatic residues play an important role in molecular recognition and assembly events.

A similar peptide array technique was also applied to identify the molecular recognition and self-assembly domains within IAPP molecules [22]. As before, in this

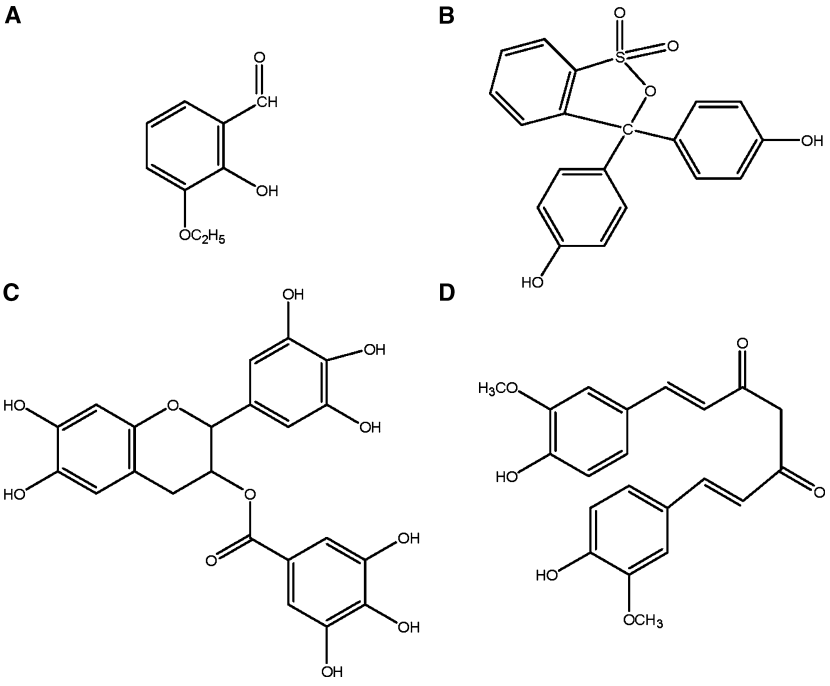

Fig. 2. Inhibition of amyloid fibril formation by small aromatic molecules. (A) 2-Hydroxy-3-ethoxy-benzaldehyde. This simple substituted benzene ring efficiently inhibited amyloid formation by amyloid β-peptide (Aβ) [49]. (B) Phenol red, the nontoxic model drug, tissue culture pH indicator, and clinically used reagent efficiently inhibited amyloid formation by islet amyloid polypeptide (IAPP) [50] and various amyloidogenic peptides. (C) Epigallocatechin gallate, the major polyphenolic component of green tea inhibits Aβ [51] and IAPP. (D) Curcumin, a phenolic yellow curry pigment shown to inhibit amyloid formation by Aβ *in vitro* and *in vivo* using model mice [52].

case a central aromatic region was identified by the nonbiased peptide scan [22]. Interestingly, the same motif was independently identified when analyzing peptide fragments [49]. As with the Aβ polypeptide, the IAPP recognition motif allowed the development of specific peptide molecules that inhibit IAPP formation [50].

The concept of aromatic interactions as a driving force for amyloid formation has a role also in the development of small molecule inhibitors. Aitken *et al.* [51] demonstrated that various polycyclic compounds inhibit amyloid fibril formation. Furthermore, even much simpler aromatic compounds, like 2-hydroxy-3-ethoxy-benzaldehyde (Fig. 2A), appeared to be extremely potent inhibitors of amyloid formation by Aβ [52]. Although the structural basis is not clear, one can reasonably speculate that interaction between the aromatic moieties and the aromatic recognition interface inhibits the further growth of the fibril. An interesting observation regarding this mode of small molecule inhibition was the discovery of the inhibitory properties of phenol red (Fig. 2B), a very simple nontoxic molecule [53]. This extensively studied drug model, approved by the United States Food and Drug Administration (US FDA) for intravenous injection into humans for imaging purposes, but not for therapeutic purposes, is a very potent inhibitor of IAPP-induced amyloid fibril formation. Other interesting nontoxic edible polyphenols that inhibit amyloid fibril formation are the catechins in green tea (Fig. 2C), curcumin (Fig. 2D), and other natural polyphenols [54,55]. These food ingredients appear to be safe, even after extensive human use, but have not yet undergone a rigorous study according to US FDA regulations.

An interesting point is that no pharmacophoric common denominator could be found among the various aromatic inhibitors. This finding further supports the idea of a rather simple recognition interface that can be interrupted by aromatic intercalation. This situation is very similar to aromatic DNA-intercalating agents. Although DNA is the most important biological assembly stabilized by aromatic interactions [56], a diverse group of planar aromatic compounds can intercalate between its bases with no clear sequence specificity.

Conclusions

Although the formation of amyloid fibrils is associated with major human diseases and has a clear physiological role in micro-organisms, the precise mechanism of its formation is not fully understood. As most amyloid-related diseases are correlated with advanced age, they are already becoming a major public health concern of the 21st century because of a gradual increase in life expectancy. The genuine understanding of the mechanisms leading to the formation of amyloid fibrils and its inhibition is therefore of high clinical importance. Recent studies using short peptide have paved the way towards understanding this life-threatening process. Extremely short aromatic peptides form amyloid fibrils readily and efficiently. The importance of aromatic moieties in the molecular recognition and self-assembly process of certain, very short, peptide fragments has been precisely defined, both experimentally and theoretically. Experiments ranging from nonbiased peptide arrays and peptide analogues to diffraction studies and molecular dynamics revealed the key role of aromatic moieties in amyloid formation. Hence, the interaction between aromatic moieties, in the context of either peptides or small organic molecules, provides a future therapeutic direction for treating amyloid-related diseases.

Acknowledgements

The author would like to acknowledge the excellent scientific editing by Dr Virginia Buchner and the financial support from the Israel Science Foundation.

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