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PROTEIN FOLDING SYMPOSIUM #2

Amyloidogenic hexapeptide fragment of medin: homology to functional islet amyloid polypeptide fragments

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KEY WORDS: Amyloid formation, aortic medial amyloid, protein misfolding, self-assembly, stacking interactions

ABBREVIATIONS: IAPP = Islet Amyloid Polypeptide; DMSO = Dimethyl Sulfoxide; NIH = National Institute of Health; RP-HPLC = Reverse Phase High-Pressure Liquid Chromatography; CR = Congo red; TEM = Transsmision Electron Microscope

Abstract

Medin is the main constituent of aortic medial amyloid that occurs in virtually all individuals older than sixty. It is derived from a proteolytic fragment of lactadherin, a mammary epithelial cell expressed glycoprotein that is secreted as part of the milk fat globule membrane. It was previously demonstrated that an octapeptide fragment of medin (NH_2-NFGSVQFV-COOH) forms typical well-ordered amyloid fibrils. To obtain further insights into the molecular determinants that mediate this process by such a short peptide fragment, we examined the amyloidogenic potential of its truncated forms and analogues. Our results clearly indicated that a truncated fragment of medin, the hexapeptide, NFGSVQ can form typical amyloid fibrils. A shorter pentapeptide fragment, NFGSV, self-assembled into a gel structure that exhibited a network of fibrous structures. The amyloid forming NFGSVQ hexapeptide is noticeably similar to the short amyloidogenic peptide fragments of the islet amyloid polypeptide (IAPP), NFGAIL and NFLVH. Moreover, the substitution of the phenylalanine residue with either alanine or isoleucine significantly reduced the amyloidogenic potential of the peptide fragment. Taken together, the results are consistent with the assumed role of stacking interactions in the self-assembly processes that lead to the formation of amyloid fibrils. The results are discussed in the context of models for the mechanism of fibril formation and ways to design inhibitors.

Introduction

The formation of amyloid fibrils is associated with a variety of diseases of unrelated origin. A partial list includes Alzheimer's disease, type II diabetes, prion diseases, and various familial amyloidosis diseases[1-6]. In all these cases, soluble proteins and polypeptides undergo a self-assembly process that leads to the formation of well-ordered fibrillar structures. As most amyloid-related diseases are correlated with advanced age, it seems that they will become one of the major public health concerns of the 21st century

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due to gradual increase in life expectancy. As an illustration, it is estimated by the National Institute of Health (NIH) that about three percent of men and women at ages 65 to 74 have Alzheimer's disease, yet nearly half of those aged 85 and older may have the disease (http://www.alzheimers.org/generalinfo.htm). The genuine understanding of the mechanism that lead to the formation of amyloid fibrils and its inhibition is therefore of the highest clinical importance.

The fibrillization process is linked to a structural transition of the proteins and polypeptides from their normal monomeric fold into a fibrillar assemblies with a predominant β-sheet secondary structure. In spite of the key medical importance of the process of amyloid self-assembly, the molecular mechanism by which amyloid fibrils are being formed is not fully understood. As the process of amyloid formation is basically a case of self-assembly into a very well-defined structure ('one-dimensional crystallization' as coined by Jarrett and Lansbury)[7] that can occur at concentrations as low as nanomolar, it is most likely that specific molecular recognition elements mediate this phenomenon. The study of very short peptide fragments that retain all the molecular information needed to mediate the process of amyloid fibril formation is therefore a very useful reductionist model to understand the specific interactions that mediate this process.

The NFGAIL hexapeptide fragment of the islet amyloid polypeptide (IAPP) is one of the shortest peptide fragments that can form well-ordered amyloid fibrils[8]. The morphology of the fibrils formed by this peptide have comparable ultrastructural properties to those formed by its correspondent full-length parent molecule. A shorter fragment, FGAIL, also formed fibrilar structures, but those structures had significantly larger diameters as compared to longer amyloidogenic peptides[8]. Amyloid-fibrils composed of the IAPP are found in the postmortem examinations of nearly all patients with Type II diabetes[9-11]. We previously demonstrated, using a systematic sequential substitution of each amino-acid with alanine ('alanine scan'), the importance of the phenylalanine residue in amyloid fibrils formation by a minimal amyloid forming fragment of IAPP, the NFGAIL peptide motif. The substitution of phenylalanine, but not of any other residue, by an alanine, abolished the ability of the fragment to form amyloid fibrils in vitro[12]. Based on this observation, the remarkable occurrence of aromatic residues in other short amyloid related sequences and the well-known role of stacking interactions in processes of self-assembly, we suggested that stacking of aromatic residues may play a role in the acceleration of the process of amyloid fibrils formation[12,14,15]. Motivated by this hypothesis, the role of charged residues in the fibrillization of human calcitonin, and the analysis of conformationally constrained analogues of the hormone, we identified an even shorter pentapeptide fragment of human calcitonin, DFNKF, that can form well-ordered amyloid fibrils similar to those formed by the full-length human calcitonin[16]. This was the first report of a peptide as short as a pentapeptide that can form amyloid fibrils with the same ultrastrucutral properties as much longer polypeptides. We further demonstrated that short aromatic dipeptides contain all the molecular information to self-assemble into well-ordered nanostructures that are structurally related to amyloid fibrils[17].

We therefore became highly interested in the study of the short amyloidogenic octapeptide (*NH2*-NFGSVQFV-*COOH*) derived from medin[18]. Medin is the main constitute of aortic medial amyloid deposits[18]. It is derived from a proteolytic fragment of lactadherin, a glycoprotein that was previously shown to be expressed by mammary epithelial cells as a cell surface protein and that is secreted as part of the milk fat globule membrane[18]. Previous studies revealed the existence of aortic medial amyloid in 97% of the subjects above the age of 50[19]. However, the pathological role of those amyloid deposits is still unknown. It was suggested that these amyloid deposits play a role in the diminished elasticity of aortic vessels that is related to old age[18,19]. While the study clearly identified the octapeptide NFGSVQFV as an amyloidogenic peptide[18], the minimal sequence of the peptide that is still amyloidogenic and the molecular determinants that mediate the amyloid formation process were not determined. Such information is critical not only for true understanding of the fibrillization process in the specific case of medin but also as a paradigm for the process of amyloid fibrils formation in general.

Here, we study the structural basis for the activity of the medin fragment, by structural and functional study of truncated analogues derived from the active octapeptide. We studied the kinetic of aggregation of the various peptides by turbidity assay. We then determined the fibrillogenic nature of the different structures formed by the peptides by CR staining and ultrastructural analysis by electron microscopy. The potential role of the aromatic residue present in the truncated fragment was studied by substitutions of either alanine or the more hydrophobic isoleucine. Our results are further discussed in the context of the attempts to develop efficient amyloid formation inhibitors.

Materials and Methods

Peptide synthesis

Peptide synthesis was performed by Peptron, Inc. (Taejeon, Korea) and Genemed Synthesis, Inc. (San

Francisco, CA). The correct identity of the peptides was confirmed by ion spray mass-spectrometry and the purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography (RP-HPLC). Freshly prepared stock solutions were prepared by dissolving the lyophilized form of the peptides in DMSO at a concentration of 100 mg/ml. To avoid any pre-aggregation, fresh stock solutions were prepared for each and every experiment.

Kinetic aggregation assay

Freshly prepared stock solutions were prepared by dissolving the lyophilized form of the peptides in DMSO at a concentration of 100 mg/ml. Peptides stock solutions were diluted as follows: 10 μl of peptide stock solution were added to 90 μl of double distilled water. Turbidity data were collected at 405 nm. Solution containing the same amount of DMSO as the tested samples was used as blank.

Congo red staining and birefringence

A 10 μl aliquot of the suspension of peptide was allowed to dry on a glass microscope slide. Staining was performed by the addition of a solution of 80% ethanol saturated with Congo red and NaCl. Birefringence was determined with a SZX-12 stereoscope (Olympus, Hamburg, Germany) equipped with a polarizing stage.

Electron microscopy

Samples were placed on 200 mesh copper grid, covered with carbon-stabilized formvar film (SPI Supplies, West Chester PA). After 1 min, excess fluid was removed and the grid was then negatively stained with 2% uranyl acetate in water. After 2 min, excess fluid was removed from the grid. Samples were viewed in JEOL 1200EX electron microscope operating at 80 kV.

Results

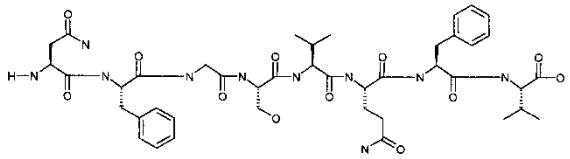
The studied peptides

It has been recently shown that a minimal fragment of medin (NFGSVQFV) contains all the structural information needed to mediate the self-assembly processes that lead to formation of well-ordered amyloid fibrils[18]. In order to get further insight into the structural elements of medin that retain the molecular information needed to mediate this process of molecular recognition and self-assembly, we studied the ability of truncated peptide fragments and analogues of medin to form amyloid fibrils in vitro. Figure 1A shows a schematic representation of the chemical structure of the largest peptide fragment studied and Figure 1B indicates the various peptide fragments and analogues that were used in the study.

Kinetic aggregation

For getting first insights into the aggregation potential of the various peptides studied, turbidity assay was performed. Freshly-made stocks of the amyloidogenic octapeptide and its truncated analogues were prepared in DMSO. The peptides were then diluted to aqueous solution and turbidity was monitored by following the absorbance at 405 nm as a function of time (Figure 2). The peptide that exhibited the highest degree of aggregation within minutes of incubation was the NFGSV pentapeptide (Figure 2A). Physical examination of solution indicated that the peptide formed a gel structure. The aggregation kinetics of the NFGSVQFV octapeptide was too fast to be measured, as turbidity was observed immediately with the dilution into aqueous solution (Figure 2A, 2B). Similar fast kinetics were observed with the NFGSVQ hexapeptide and the GSVQ tetrapeptide, although to a significantly lower turbidity levels (Figure 2B). The truncated FGSV and GSVQ tetrapeptides showed a gradual increase of turbidity over $\sim$30 min (Figure 2B) which was followed by a slight decrease into turbidity (probably due to sedimentation of large aggregates). Such kinetics and turbidity values are similar to those previously observed with amyloidogenic peptides of similar size[12].

A



B

Pep1	NH_2-NFGSVQFV-COOH
Pep2	NH_2-NFGSVQ -COOH
Pep3	NH_2-NFGSV -COOH
Pep4	NH_2- FGSVQ -COOH
Pep5	NH_2- GSVQ -COOH
Pep6	NH_2- FGSV -COOH
Pep7	NH_2-NAGSVQ -COOH
Pep8	NH_2-NIGSVQ -COOH

FIGURE 1. The studied peptides. (A) The chemical structure of the previously found amyloidogenic fragment of medin (NFGSVQFV). (B) The list of the synthetic peptide sequences that were synthesized and studied.

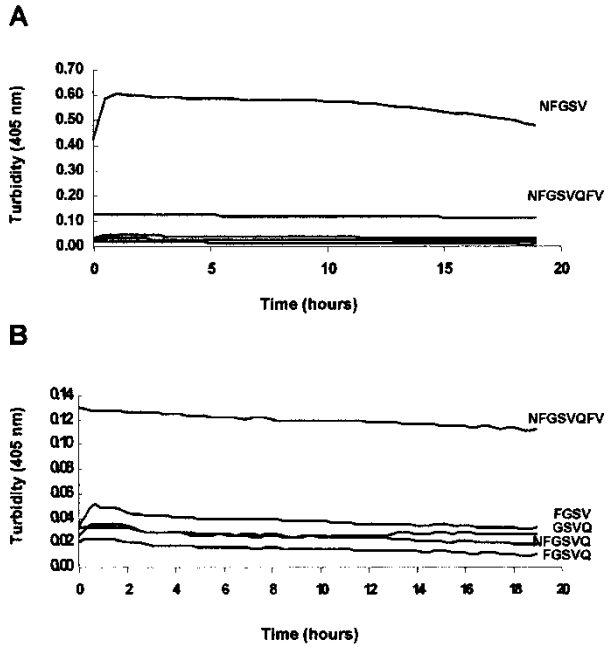


FIGURE 2. Turbidity assay. (A) Kinetics of aggregates formation of medin peptide fragment and its derivatives as followed by turbidity at 405 nm. Turbidity of peptide solution in aqueous solution in the presence of 10% DMSO, prepared as described in 'Methods' was followed for 20 h. (B) Scale up of panel A.

Identification of a minimal fibrils forming fragment of medin

Next, we studied whether the aggregative structures formed by the various peptides are indeed amyloid fibrils. The fibrillization potential of the peptide fragments was first examined by electron microscopy (EM) using negative staining (Figure 3). Stock solutions of the peptide fragments were suspended and incubated at room temperature for 4 days. Fibrillar structures were clearly seen in solutions that contained both the NFGSVQFV octapeptide (Figure 3A) and the truncated NFGSVQ (Figure 3B). In both cases the structures were similar to those observed with much longer polypeptides, such as the IAPP and the β-amyloid (Aβ) polypeptides. The shorter gel-forming NFGSV pentapeptide did not form a typical amyloid structure, but a network of fibrous structures (Figure 3C). Fibrous networks were recently observed upon gelation of the glutathione peptide[20]. No typical fibrils could be detected in solutions that contained the FGSVQ pentapeptide, the GSVQ tetrapeptide, or the FGSV tetrapeptide in spite of extensive search (Figures 3D, 3E and 3F, respectively). In the case of the FGSVQ peptide (Figure 3D) somewhat fibrillar and ordered structure could be seen (although significantly different than those formed by typical amyloidogenic peptide).

Congo-red staining and birefringence

Next, we wanted to study whether the structures formed by the various peptides show the typical birefringence that is observed with amyloid fibrils upon binding of the Congo-Red (CR) dye. The NFGSVQ hexapeptide binds CR and exhibited a characteristic bright and strong green-gold birefringence (Figure 4B). The NFGSVQFV octapeptide also exhibited significant birefringence (Figure 4B), although less typical than that observed with full-length polypeptides. The gel-forming NFGSV peptide deposits exhibited a very low degree of birefringence (Figure 4C). The FGSVQ and FGSV peptide showed no birefringence upon staining with CR (Figures 4D and 4F, respectively). There was clearly no significant difference between those two peptides and a negative control. An unexpectedly high level of birefringence was observed with the GSVQ tetrapeptide (Figure 4E). While the morphology of the structures formed by the GSVQ peptide (Figure 3E) is clearly different from that of amyloid fibrils, these structures may have a significant degree of order that is reflected in strong birefringence.

The effect of phenylalanine to alanine and phenylalanine to isoleucine substitutions

It was previously assumed that during amyloid fibrils formation hydrophobic patches that are normally buried in the protein hydrophobic core are being exposed to the solution due to partial unfolding. This process leads to molecular recognition between the protein monomers through hydrophobic interaction and hence to creation of amyloid fibrils. Johansson *et al*. studied the ability of tetrapeptides to form amyloid fibrils and suggested that hydrophobic interactions are not sufficient for fibril formation[21]. We recently showed, using short fragments of amyloid forming protein that there is no correlation between the hydrophobicity of a peptide and its amyloidogenic potential[16]. In order to get further insight regarding the role of aromatic interaction in fibril formation process, we substituted the phenylalnine residue in the hexapeptide fragment with hydrophobic alanine and isoleucine residues. As both substituted residues are hydrophobic and β-sheet formers they serve as a good model to alter specifically the molecular interface of the peptides without dramatically changing their hydrophobicity or tendency to form β-sheet structures.

The alanine and isoleucine substituted peptides were prepared and examined in the same way as described for the various fragments of medin. A significantly lower turbidity was observed with the alanine- and isoleucine-substituted peptides in comparison to the amyloidogenic

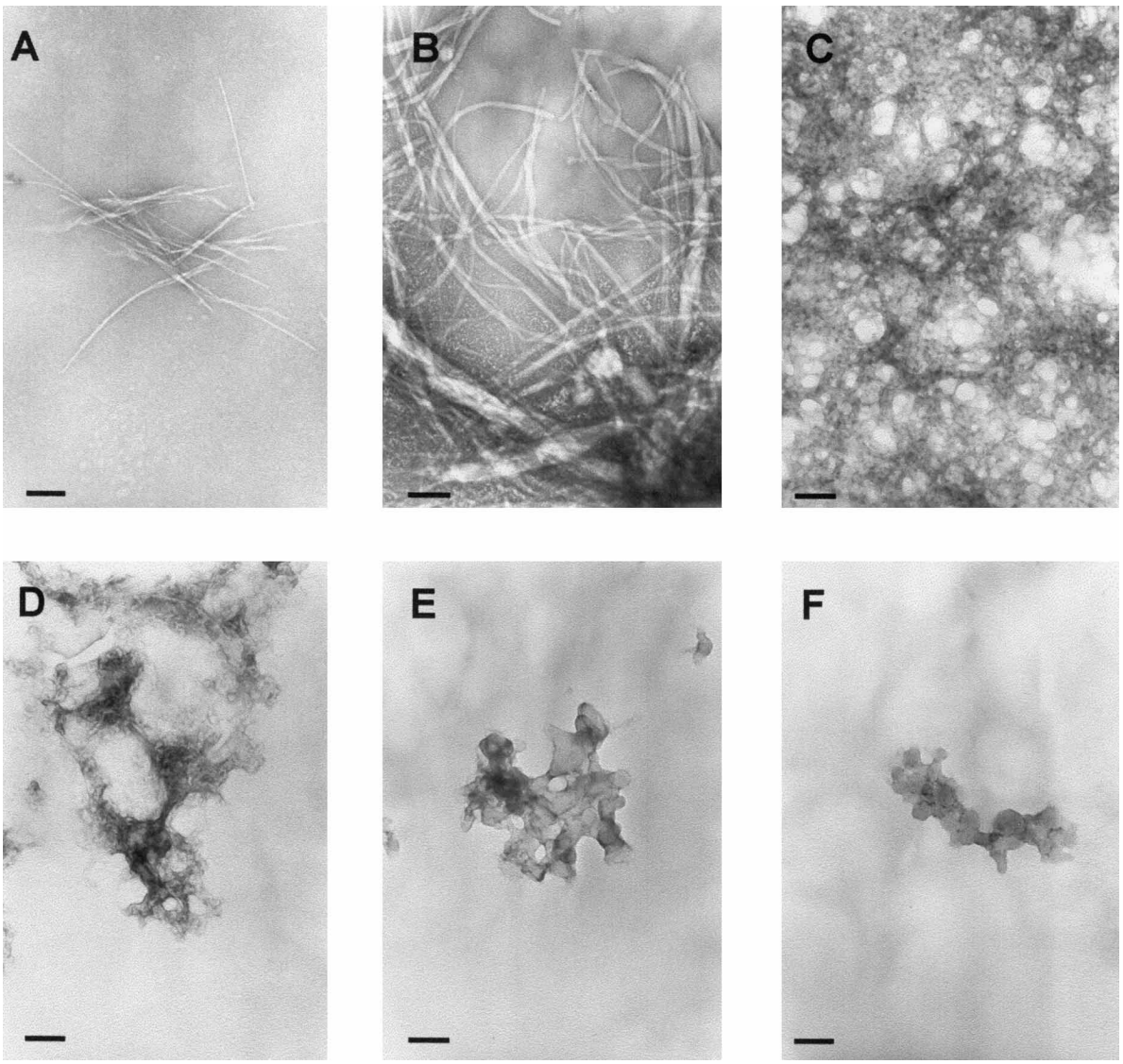


FIGURE 3. Electron microscopy. Electron microscopic examination of insoluble aggregates formed by the human medin peptide fragments. The scale bar represents 100 nm. Peptide solutions were used as described under 'Methods'. (A) NFGSVQFV, (B) NFGSVQ, (C) NFGSV, (D) FGSVQ, (E) GSVQ, (F) FGSV.

NFGSVQ hexapeptide (Figure 5A). When aggregated solution of the NAGSVQ and NIGSVQ peptides were visualized by TEM, no clear fibrillar structures could be detected (Figure 5B and 5C, respectively). This is in complete contrast to the high abundance fibrillar structures seen with the wild-type hexapeptide (Figure 3B). Furthermore, the structures that were visualized did not show any degree of order (as observed with the NFGSV and FGSVQ peptides as described above, Figures 3C and 3D), but were very similar to the completely non-fibrillar structures as were observed with the FGSV tetrapeptide (Figure 3E). While no birefringence was observed for the NIGSVQ peptide (Figure 5E), some degree of birefringence could be detected (Figure 5C) with the alanine-substituted peptide (as observed with the GSVQ peptide, Figure 4E). This may raise further doubt regarding the use of CR staining as a sole indicator of amyloid formation[22].

Discussion

The process of amyloid fibril formation has a key medical importance as it is involved in the pathogenesis of a variety of diseases of unrelated origin[1-6,14]. Amyloid fibril

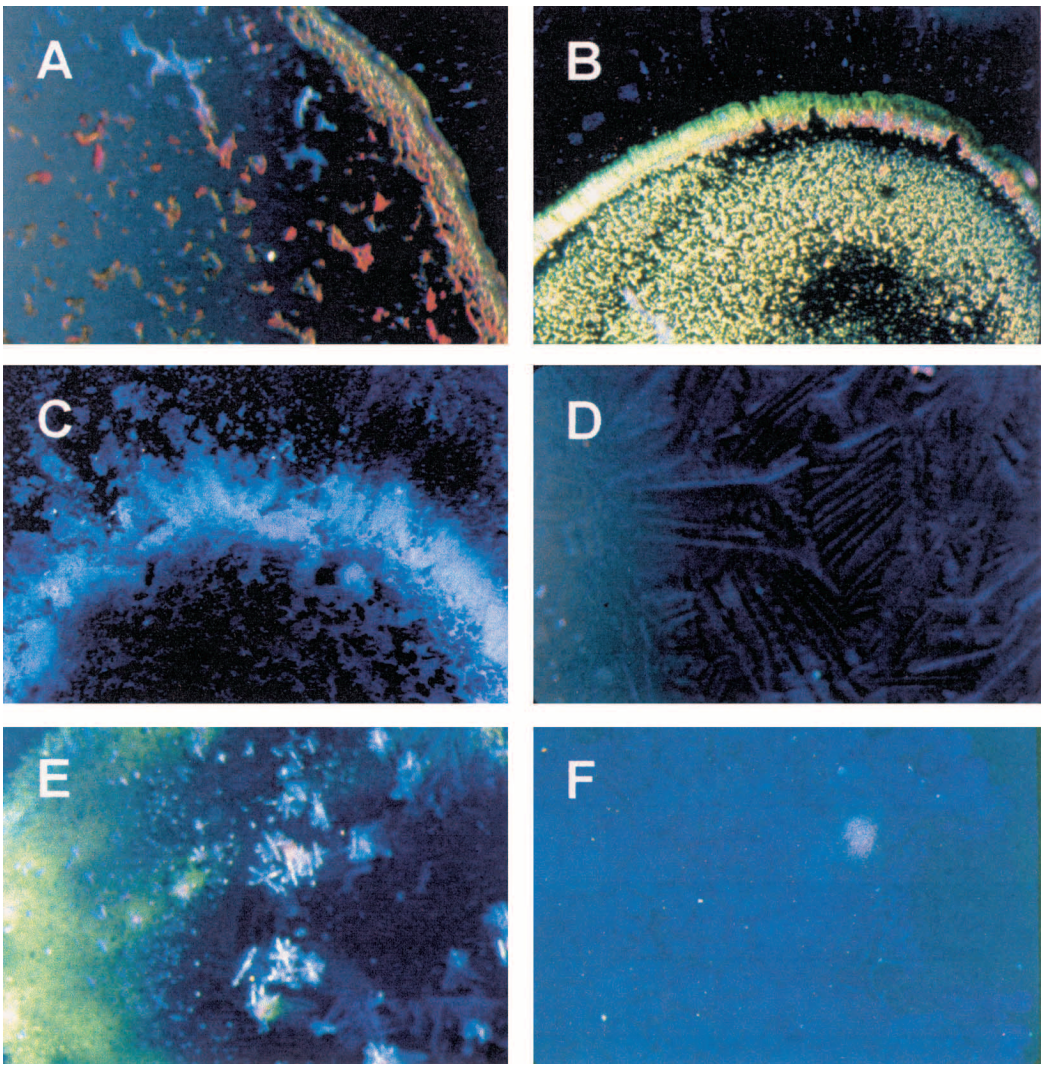


FIGURE 4. Congo red birefringence. Examination of the amyloidogenic nature of medin fragments by Congo red binding. Microscopic examination under polarized light following staining with CR. Peptide solutions were used as described under 'Methods'. (A) NFGSVQFV, (B) NFGSVQ, (C) NFGSV, (D) FGSVQ, (E) GSVQ, (F) FGSV.

formation by medin is of special interest due to its universal existence in the older population[18]. In spite of the significant medical importance of the process of amyloid fibril formation, its molecular mechanism is not fully understood. True mechanistic understanding of the process may be helpful in the development of inhibitors to control the formation of amyloid fibril. The identification of novel very-short amyloidogenic fragments is very important in that sense, since it reduces the complexity of analysis of molecular determinants of the fibrillization process.

Our study identifies a novel amyloid forming hexapeptide, the NFGSVQ peptide fragment of medin. The turbidity assay results (Figure 2B), the ultrastrucutral analysis by EM (Figure 3B), and the clear and strong CR birefringence (Figure 4B), are all consistent with a process of self-assembly of well-ordered amyloid fibrils. The similarity between the new identified hexapeptide amyloidogenic fragment and the NFGAIL fragment of IAPP[8] is evident. Both peptides share the NFGXXX motif and in both cases further truncation of the hexapeptide structure abolished the ability of the peptides to form typical amyloid structures. The shorter NFGSV pentapeptide is also clearly capable to aggregate (Figure 2A) into well-ordered fibrous structures (Figure 3C), although these structures are different than the typical pathologically relevant amyloid fibrils.

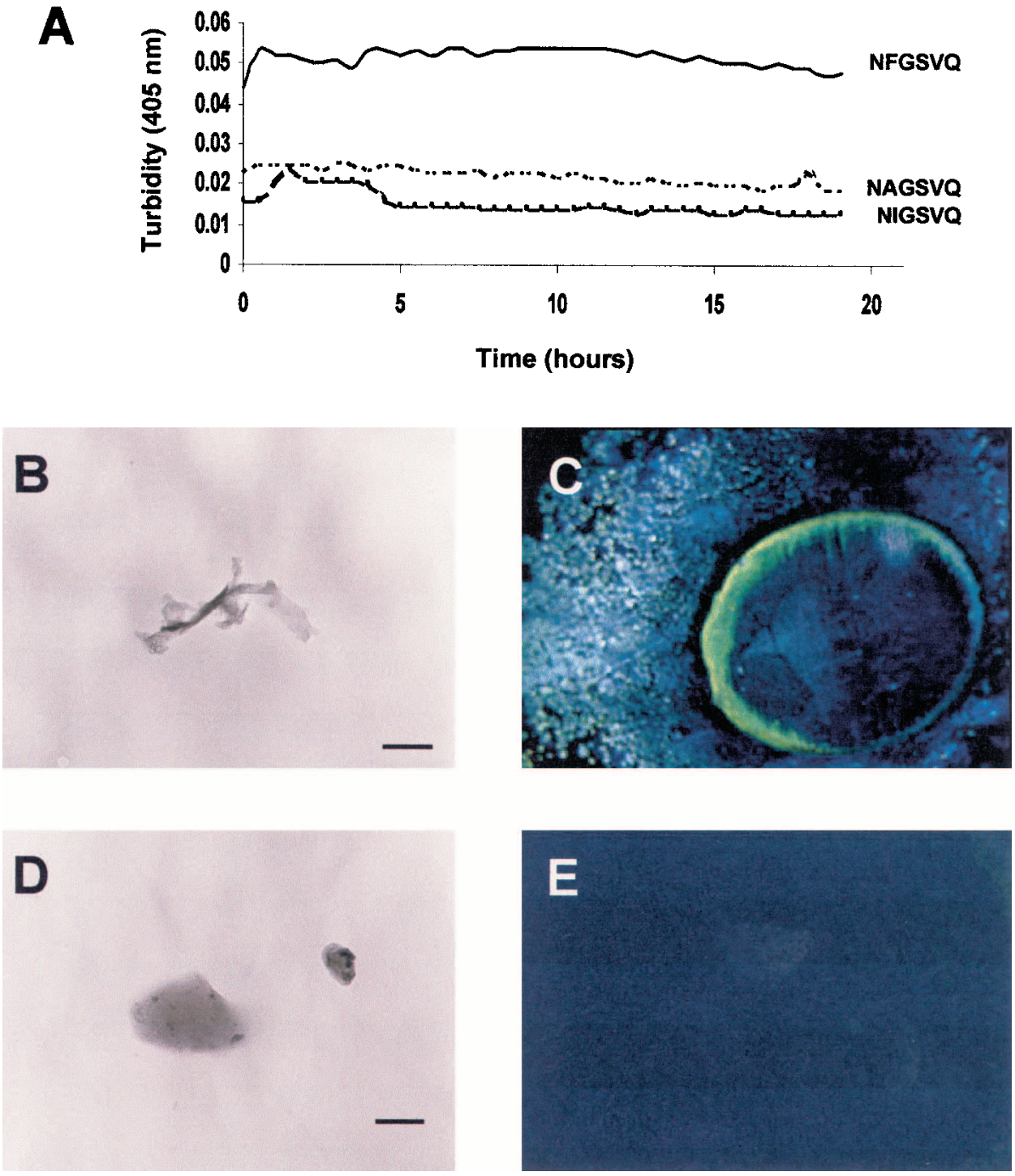


FIGURE 5. Analysis of the NAGSVQ and the NIGSVQ substituted peptides. (A) Turbidity of the NAGSVQ and NIGSVQ peptides as compared to the amyloidogenic hexapeptide fragment. (B) Electron microscopic examination of insoluble aggregates of the peptide, NAGSVQ, formed in solution. The scale bar represents 100 nm. (C) Congo red binding assay. Examination of the amyloidogenic nature of the peptide, NAGSVQ, by Congo red birefringence. (D) Electron microscopic examination of insoluble aggregates of the peptide, NIGSVQ, formed in solution. The scale bar represents 100 nm. (E) Congo red binding assay. Examination of the amyloidogenic nature of the peptide, NIGSVQ, by Congo red birefringence.

The current results further support our hypothesis regarding the possible role of aromatic residues in the acceleration of the amyloid formation process[12,14,15]. It is also related to the suggestion, made by several groups, that the structure of amyloid fibrils may resemble that of the β-helix architecture[23-25]. One of the main features of β-helixes is the stacking of similar residues on a flat β-sheet[26]. Our 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 that relates amyloid fibrils and β-helix structures. This possible arrangement of amyloid fibrils suggests a possible mechanism of inhibition by small intercalating aromatic molecules. Indeed, various studies demonstrated the ability of remarkably simple unrelated aromatic molecules, such as nicotine[27], acetylsalicylic acid[28], tetracycline[29], and bis-1-anilinonaphthalene-8-sulfonate[30], to inhibit amyloid fibril formation.

It is interesting that medin deposits are found in virtually all individuals older than 60 years. This is in contrast to the situation with Aβ, IAPP, and other amyloidogenic polypeptides, in which amyloid deposits are found only in subgroups of the population. It is assumed that the non-aggregated states of polypeptides such as the Aβ and IAPP are kinetically-trapped metastable states ('kinetic solubility' as described by Jarrett and Lansbury[7]) and that the aggregated states actually represent the true energetic minimum for an ensemble of these amyloidogenic proteins or even of any protein[6]. It is possible that medin represents a case in which the energetic barrier that keeps the protein kinetically soluble is lower than in the other cases mentioned above. Alternatively (or complementary), as amyloid fibril formation is a nucleation process whose rate is highly dependent on protein concentration[7], it is possible that the physiological concentrations of medin are higher than those of other aggregation-prone proteins and polypeptides.

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