Amyloid Fibril Formation by Pentapeptide and Tetrapeptide Fragments of Human Calcitonin*

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The process of amyloid fibril formation by the human calcitonin hormone is associated with medullary thyroid carcinoma. Based on the effect of pH on the fibrilization of human calcitonin, the analysis of conformationally constrained analogues of the hormone, and our suggestion regarding the role of aromatic residues in the process of amyloid fibril formation, we studied the ability of a short aromatic charged peptide fragment of calcitonin (NH2-DFNKF-COOH) to form amyloid fibrils. Here, using structural and biophysical analysis, we clearly demonstrate the ability of this short peptide to form well ordered amyloid fibrils. A shorter truncated tetrapeptide, NH2-DFNK-COOH, also formed fibrils albeit less ordered than those formed by the pentapeptide. We could not detect amyloid fibril formation by the NH2-FNKF-COOH tetrapeptide, the NH2-DFN-COOH tripeptide, or the NH2-DANKA-COOH phenylalanine to the alanine analogue of the pentapeptide. The formation of amyloid fibrils by rather hydrophilic peptides is quite striking, because it was speculated that hydrophobic interactions might play a key role in amyloid formation. This is the first reported case of fibril formation by a peptide as short as a tetrapeptide and one of very few cases of amyloid formation by pentapeptides. Because the aromatic nature seems to be the only common property of the various very short amyloid-forming peptides, it further supports our hypothesis on the role of aromatic interactions in the process of amyloid fibril formation.

The process of amyloid fibril formation is associated with a large number of diseases of unrelated origin. A partial list includes Alzheimer's disease, Parkinson's disease, Type II diabetes, Prion diseases, Familial British dementia, and several familial amyloidosis (1–7). All of these diseases are characterized by the transformation of soluble proteins into aggregated fibrillar deposits in different organs and tissues. The pathological significance of amyloid fibril formation is not completely understood in all cases. One such example is aortic medial amyloid deposits that occur virtually in all individuals older than 60 years, but its medical consequence is not known yet (8).

Although different amyloid-forming proteins do not share clear sequence homology, the fibrillar structures that are being formed have very similar physicochemical and ultrastructural characteristics as determined by transmission electron microscopy x-ray fiber diffraction, and other biophysical techniques (1–7). Furthermore, amyloid fibrils are being formed in vitro also by disease-unrelated proteins (9–13). This finding suggests that amyloid fibrils may serve as a generic structural form of aggregated proteins (1–7). Nevertheless, despite the significant medical importance of the amyloid fibril formation process and its consideration as a universal structural form, the exact mechanism that leads to the self-assembly of polypeptides into ordered fibrils is not fully understood.

Human calcitonin (hCT) is a 32-amino acid polypeptide hormone (Fig. 1) that is being produced by the C-cells of the thyroid and is involved in calcium homeostasis (14–16). Amyloid fibrils composed of hCT were found to be associated with medullary carcinoma of the thyroid (17–19). It was also found that synthetic hCT can form amyloid fibrils in vitro with a similar morphology to the deposits found in the thyroid (17–23). The in vitro process of amyloid formation is affected by the pH of the medium (23). Electron microscopy experiments have revealed that the fibrils formed by hCT are ~80 Å in diameter and up to several micrometers in length. The fibrils are often associated with one another. Calcitonin has been used as a drug to treat a various diseases including Paget's disease and osteoporosis (14–16). However, the tendency of hCT to associate and form amyloid fibrils in aqueous solutions at physiological pH is a significant limit for its efficient use as a drug (14–16). Salmon CT, the clinically used alternative to hCT, causes immunogenic reaction in treated patients because of low sequence homology. Therefore, understanding the mechanism of amyloid formation by hCT and controlling this process are highly important not only in the context of amyloid formation mechanism but also as a step toward improved therapeutic use of calcitonin.

CD studies have shown that in water, monomeric hCT has little ordered secondary structure at room temperature (19). However, studies of hCT fibrils using circular dichroism, fluorescence, and infrared spectroscopy revealed that fibrillated hCT molecules have both α-helical and β-sheet secondary structure components (21). NMR spectroscopy studies have shown that in various structure-promoting solvents like trifluoroethanol/H2O, hCT adopts an amphipilic α-helical conformation predominantly in the residue range of 8–22 (24–25). In Me2SO/H2O, a short double-stranded antiparallel β-sheet is being formed in the central region made by residues 16–21 (26). Recent work indicates a critical role of residues 18–19 for the oligomerization state and bioactivity of hCT (27).

In this study, we investigated the ability of short fragments of hCT (hCT15–19, hCT16–19, hCT15–18, and hCT15–17) to form...
Amyloid fibrils in vitro. We performed ultrastructural analysis by electron microscopy, determined the properties of the different structures formed by the peptides using Congo Red (CR) staining, and determined the secondary structure of the peptides by FT-IR spectroscopy. Our results demonstrate a remarkable amyloidogenic potential of the hCT15–19 pentapeptide. We also reveal for the first time the ability of a peptide as short as a tetrapeptide to form ordered fibrillar structures. Taken together, our results provide further experimental supports for our hypothesis on the role of aromatic interactions in the process of amyloid formation (28–30).

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptide synthesis was performed by Peptron, Inc. (Taejeon, Korea). 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. Human synthetic calcitonin was purchased from Sigma. Freshly prepared stock solutions were prepared by dissolving lyophilized form of the peptides and protein in Me2SO at a concentration of 100 mg/ml. To avoid any pre-aggregation, fresh stock solutions were prepared for each and every experiment.

Electron Microscopy—Peptides stock solutions were diluted into 0.02 M NaCl, 0.01 M Tris, pH 7.2. Fibril formation was assessed using 10 μl of sample aged for 2 days placed on a 200-mesh copper grid covered by carbon-stabilized Formvar film. 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.

Congo Red Staining and Birefringence—Birefringence was determined using the same solutions of peptides that were used for electron microscopy (EM) experiments. A 10-μl aliquot of the suspension of peptide aged for 2 days 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.

Fourier Transform Infrared Spectroscopy—Infrared spectra were recorded using a Nicolet Nexus 470 FT-IR spectrometer with a deuterated triglycine sulfate (DTGS) detector. Samples of aged peptide solutions taken from EM and CR assays were suspended on a CaF2 plate and dried by vacuum. The peptide deposits were resuspended with D2O and subsequently dried to form thin films. The resuspension procedure was repeated twice to ensure maximal hydrogen to deuterium exchange. The measurements were taken using a 4 cm−1 resolution and averaging of 2000 scans. The transmittance minimal values were determined by the OMNIC analysis program (Nicolet).

RESULTS

Selection of Peptides to Be Studied—Previous studies have demonstrated that the ability of hCT to form amyloid fibrils is reduced by acidic pH (22). Therefore, we assumed that negatively charged amino acids that undergo protonation at low pH may play a key role in the process of amyloid formation. The only negatively charged amino acid in hCT is Asp15 (Fig. 1A).

Furthermore, a critical role for residues Lys18 and Phe19 in the oligomerization state and bioactivity of hCT was recently shown (27). Therefore, we decided to study the ability of short peptide fragments of hCT in the 15–19 region to form amyloid fibrils in vitro. Another factor in our assumption of the role of the hCT15–19 region in the process of amyloid fibril formation was the fact that the region contains two phenylalanine aro-
Identification of Fibril-forming Fragments of Calcitonin—The fibrillization potential of the peptide fragments was first examined by EM. Stock solutions of the peptide fragments were suspended in 0.02 M NaCl, 0.01 M Tris, pH 7.2, aged for 2 days, and negatively stained. Fibrillar structures similar to those formed by the full-length polypeptide (19–23) were clearly seen with high frequency in solutions that contained the DFNKF-pentapeptide (Fig. 2A). The shorter DFNK-tetrapeptide also formed fibrillar structures (Fig. 2B). However, the structures formed were less ordered as compared with those formed by the DFNKF-pentapeptide. The amount of fibrillar structures formed by DFNK was also lower as compared with that of the DFNKF-pentapeptide. No clear fibrils could be detected using solutions that contained the FNKF-tetrapeptide and the DFNK-tripeptide despite extensive search. In the case of the FNKF-tetrapeptide, only amorphous aggregates could be found (Fig. 2C). The DFNK-tripeptide formed more ordered structures (Fig. 2D) that resembled the structure formed by gel-forming tripeptide (31). To study whether the FNKF-tetrapeptide and the DFNK-tripeptide peptide can or cannot form fibrils or whether the observation is a result of slow kinetics, a solution of the DFNK-tripeptide peptide (31) was used as described under “Experimental Procedures.” A, DFNKF. B, DFNK. C, FNKF. D, DFN.

Secondary Structure of the Formed Deposits—Amyloid deposits are characteristic of fibrils rich with β-pleated sheet structures. To get quantitative information regarding the secondary structures that were formed by the various peptide fragments, we used FT-IR spectroscopy. Aged peptide solutions were dried on CaF$_2$ plates forming thin films as described under “Experimental Procedures.” The DFNKF-pentapeptide exhibited a double minima at 1639 and 1669 cm$^{-1}$ (Fig. 4), an amide I FT-IR spectrum that is consistent with antiparallel β-sheet structure and is remarkably similar to the spectrum of the amyloid-forming hexapeptide fragment of the islet amyloid polypeptide (32). The amide I spectrum observed with the DFNK-tetrapeptide (Fig. 4) is less typical of a β-sheet structure. Although the spectrum exhibited a minimum at 1666 cm$^{-1}$, which may reflect an antiparallel β-sheet, it lacked the typical minimum at ~1620–1640 cm$^{-1}$ that is typically observed with β-sheet structures. The FNKF-tetrapeptide exhibited a FT-IR spectrum that is typical of a non-ordered structure (Fig. 4) and is similar to the spectra of the short non-amyloidogenic fragments of the islet amyloid polypeptide (32). The DFNK-tripeptide exhibited a double minima at 1642 cm$^{-1}$ and 1673 cm$^{-1}$ (Fig. 4), an amide I FT-IR spectrum that is consistent with a mixture of β-sheet and random structures. This
Procedures.

Aged peptide solutions were used as described under "Experimental Procedures." Congo Red birefringence and microscopic examination of the amyloidogenetic nature of the peptide DANKA formed in an aged solution as assessed by FT-IR. The FT-IR spectrum of the DANKA-pentapeptide was similar to that of the FNKF-tetrapeptide and the other short non-amyloidogenic peptide, typical of non-ordered structures (32). Taken together, the effect of the phenylalanine to alanine substitution is very similar to the effect of such a change in the context of a short amyloid-forming fragment of the islet amyloid polypeptide (28).

**DISCUSSION**

The understanding of the molecular mechanism of amyloid fibril formation has a key medical importance. This is attributed to the involvement of this process in numerous cases of human diseases. Some of these cases such as the Alzheimer’s disease and Type II diabetes will become more and more common in correlation with the extended life expectancy of the western population. Short amyloidogenic peptides serve as excellent model systems to study amyloid formation. This is attributed to the fact that the fragments contain all of the structural information needed to mediate the molecular recognition and self-assembly processes that lead to amyloid formation. Thus, the study of very short peptide fragments significantly reduces the complexity of the analysis of the process of amyloid fibril formation.

Here, we clearly demonstrate the ability of a peptide as short as a pentapeptide to form well ordered amyloid fibrils. The typical fibrillar structure as seen by electron microscopy visualization (Fig. 2A), the very strong green birefringence upon staining with CR (Fig. 3A), and the typical antiparallel β-sheet structure (Fig. 4A) all indicate that the DFNKF-pentapeptide is a very potent amyloid-forming agent. This is only one of very few pentapeptides that was shown to form amyloid fibrils. The other cases are that of the pentapeptide fragments of the islet amyloid polypeptides FGAIL (32), NFVLH, and FVLHS (33). Yet, in terms of the degree of birefringence and electron microscopy morphology, the hCT fragment seems to be the pentapeptide with the highest amyloidogenic potential. We do not fully understand why the potential of the DFNKF-peptide is so high as compared with other pentapeptides. It is possible that electrostatic interactions between the opposing charges on the lysine and aspartic acids direct the formation of ordered antiparallel structure. In that sense the newly discovered pentapeptide fragment may resemble the potent amyloidogenic fragment of the β-amyloid polypeptide KLVFFAE (34). We also do not fully understand why the DFNK-polypeptide has a significantly lower amyloidogenic potential as compared with the DFNKF-peptide. It is possible that a pentapeptide is a lower limit for potent amyloid former. This hypothesis is consistent with our recent results that demonstrate that two pentapeptides of islet amyloid polypeptide, NFVLH, and FVLHS can form amyloid fibrils, but their common denominator, the FLVH-tetrapeptide, could not form such fibrils (33).

It was previously assumed that hydrophobic interactions may play a significant role in the mechanism of amyloid fibril formation. One of the models for the process of amyloid formation by large cellular proteins suggests that partial unfolding exposes hydrophobic patches that are normally located in the hydrophobic core of the protein and thus allows intermolecular interaction between those patches that eventually lead to the formation of large amyloid structures. However, our current...
work demonstrates that short hydrophilic sequences or "patches" in the context of large proteins are capable of amyloid formation at least as good as hydrophobic sequences.

When we analyzed the correlation hydrophobicity and the amyloidogenic potential of various short peptides that were studied by our group and others (Fig. 6), no correlation could be observed. The only apparent indication for potential amyloid fibril formation in this group of peptides seems to be a combination of aromatic nature and minimal length. It is worth mentioning that a similar pattern was observed when other hydrophobicity scales (36–38) were used. This is consistent with our recent hypothesis regarding the role of aromatic residues in processes of amyloid formation. According to our hypothesis that is based on the analysis of a handful of short amyloid-related peptides, specific orientation of thermodynamically favored aromatic interactions may provide specificity and directionality as well as the energetic contribution needed for the amyloid formation process.

Taken together, the current study represents another advancement in the direction of the development of tools for the prediction and identification of very short amyloidogenic motifs. As the process of amyloid formation is one of self-assembly and molecular recognition, the identification of very short amyloidogenic sequences, that contain all the information needed to mediate the process, is highly important. Such short molecular recognition motifs may serve as a starting point for the design of inhibitors that block the amyloid formation process (39).

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