Inhibitor Design

Inhibition of Amyloid Fibril Formation by Peptide Analogues Modified with α-Aminoisobutyric Acid**

*Sharon Gilead and Ehud Gazit**

The accumulation of amyloid deposits is observed in diseases of unrelated origin, such as Alzheimer's disease, Parkinson's disease, type II diabetes, and prion disorders.[1] An increasing body of evidence supports the hypothesis that the assembly of amyloid fibrils is a central factor in the development of the clinical symptoms of these diseases. A key step in amyloid formation is the transition of a protein from its native structure to a β-sheet arrangement.[1] Upon formation of amyloid fibrils, the protein molecules that compose the fibrils lose their native conformation and adopt ordered, stacked β-sheet structures. This observation suggests that prevention of the ability of amyloidogenic proteins to adopt a β-sheet conformation would be useful as a way to interfere with the amyloid self-assembly process.[2,3] The use of β-breaker elements is a promising approach to the development of fibrillization inhibitory drugs. A milestone in the search for proof of this concept was provided by Soto et al., who demonstrated that incorporation of β-breaker elements into short peptides composed of the recognition sequence of the amyloidogenic protein results in inhibition of amyloid formation.[1e,2b,2d] The proline residue is widely used in such research since it has greater β-breaking potential than the other natural amino acids. Another approach involves N-methylation of peptide inhibitors that prevent β-sheet stacking by interfering with the intermolecular backbone hydrogen bonds needed to form the structure.[2g]

α-Aminoisobutyric acid (Aib) is a unique β-sheet breaker. This achiral amino acid has two methyl residues attached to the C_α atom and strongly favors helical conformations.[4] The presence of a methyl group instead of the hydrogen atom found in the natural amino acids greatly affects the steric properties of the residue. Alanine has a wide range of allowed ϕ, ψ torsion angles, but Aib, which is α-methylated alanine, has an extremely limited conformational space. The conformational map of Aib is derived by superposing the Ramachandran plots of L-alanine and D-alanine (Figure 1).[4b,c] As shown in the figure, the allowed angles are restricted to small regions that are clearly much more suitable for an α-helical conformation than for a β-strand structure. Comparison of the map for Aib with that for proline clearly demonstrates that Aib has the potential to be a better β-breaker than proline (Figure 1).

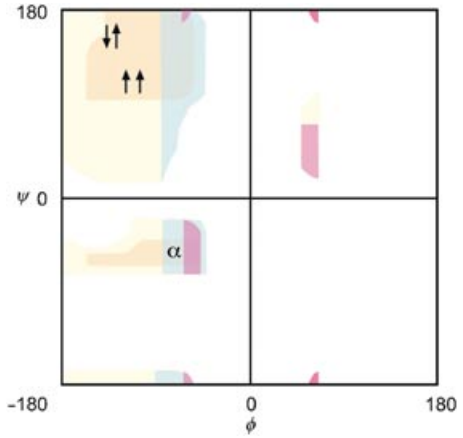

Figure 1. Conformational restriction of Aib. The Ramachandran plot shows the sterically allowed regions for all natural amino acids (yellow for fully allowed, orange for partially allowed), for L-proline (blue), and for the achiral Aib residue (magenta). ↑↓: antiparallel β sheet, ↑↑: parallel β sheet, α: α helix. The figure was prepared by merging separate plots from ref. [4c].

The effect of Aib incorporation on the conformation of short peptides has been studied extensively over the last two decades. The Aib residue has been shown to have a very high tendency to induce helical conformations and to disrupt β-sheet structures in a large number of peptides.[4] A study independent from that described herein recently demonstrated that the incorporation of an Aib residue into a fully protected Alzheimer's β-amyloid fragment ($A\beta_{17-21}$) induces

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helical structure in the fragment in organic solvents.[5] We previously suggested the use of Aib as a conjugated β-breaker element for the inhibition of amyloid fibril formation.[3] To test this concept experimentally, we assayed the ability of Aib-containing peptide analogues to inhibit amyloid formation by human islet amyloid polypeptide (hIAPP). This 37 amino acid hormone peptide accumulates in amyloid deposits in the pancreas of individuals with type II diabetes.[6,7]

A growing body of evidence suggests that hIAPP amyloid fibril formation may play an important role in the loss of β-cell mass and the progressive decline in insulin secretion characteristic of type II diabetes.[6] We recently reported the use of a nonbiased peptide array scan to identify a novel region within hIAPP that appears to mediate the molecular recogntion processes that lead to amyloid fibril self-assembly.[7] Peptide fragments of the $hIAPP_{14-20}$ domain are highly amyloidogenic and have greater affinity for full-length hIAPP than does the widely studied $IAPP_{20-29}$ peptide. Herein, we use the $hIAPP_{14-20}$ domain as a target for the inhibition of hIAPP fibrillization by designed peptide-based inhibitors. We used the native peptide as a template and introduced small chemical modifications that maintain the recognition properties of the fragment but abolish its ability to assemble into amyloid fibrils. When added to hIAPP, the new peptides bind to the recognition domain, block it, and prevent its assembly into amyloid fibrils.

We designed three peptide inhibitors by substituting hydrophobic alanine and leucine residues in the sequences ANFLVHSS, ANFLVH, and ANFLV (corresponding to residues 13–17, 13–18, and 13–20 of hIAPP) with Aib residues. The resulting potential inhibitors have the sequences Aib-NF-Aib-VHSS, Aib-NF-Aib-VH, and Aib-NF-Aib-V, respectively. Since these changes are quite conservative in terms of the chemical properties of the recognition surface, the breakage elements can be integrated into the molecular recognition sequence. Conjugation of disturbing residues to molecular recognition motifs produces larger peptides, which have reduced potential as drug leads.

We first examined the ability of the Aib-modified peptides to form amyloid fibrils, compared to that of native peptides. Although the overall changes in the chemical structures are minor, we observed critical differences between the abilities of the native and modified peptides to form amyloid fibrils. The amyloidogenic nature of the peptides was determined by using electron microscopy (EM), Congo red (CR) birefringence, and FTIR spectroscopy assays. All the native peptides, ANFLVHSS, ANFLVH, and ANFLV, formed fibrillar structures that could be seen under the electron microscope. The ANFLVH peptide formed dense bundles of long, coiling fibrils that closely resemble the amyloid fibrils formed by full-length hIAPP. No fibrillar structures were observed for the Aib-containing peptides Aib-NF-Aib-VHSS, Aib-NF-Aib-VH, and Aib-NF-Aib-V. Even after a longer period of incubation, only a small amount of amorphous aggregates was detected (Figure 2a). The abundance of the amorphous material on the microscope grid was extremely low compared to the high abundance of ordered fibrillar structures observed with the unmodified peptides.

Upon staining with Congo red, the ANFLVH and ANFLV peptides exhibited a typical yellow-green birefringence. The ANFLVHSS peptide exhibited less intense birefringence. The Aib-NF-Aib-VHSS, Aib-NF-Aib-VH, and Aib-NF-Aib-V peptides exhibited no birefringence and thus appear to have no potential to form amyloid fibrils, which is in agreement with the results of the EM studies (Figure 2a). FTIR spectra were recorded to determine the internal conformation of the observed ultrastructures. Modification of the native peptides to form Aib-containing peptides produced a significant change in the infrared spectra. The ANFLVHSS, ANFLVH, and ANFLV peptides have spectra typical of β-sheet structures, with minima at 1631–1633 cm^{-1}. The ANFLVHSS peptide has a second band at 1678 cm^{-1}, which might indicate

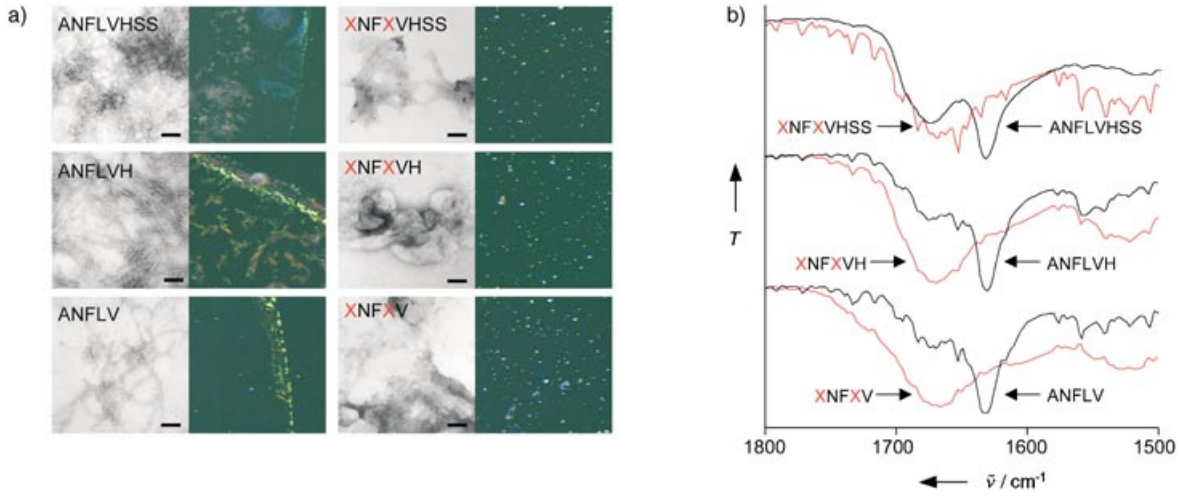

Figure 2. Amyloidogenic properties of native and Aib-modified peptides. a) The studied peptides were examined with an electron microscope and with a light microscope under cross-polarizers after staining with Congo red. The corresponding EM (left) and CR (right) photographs of each peptide are shown. b) Analysis of the secondary conformation of insoluble structures formed in aged solutions, as assessed by FTIR spectroscopy (*T*: Transmittance). The arrows show which spectrum corresponds to each peptide. Peptide solutions were aged for various periods of time, as described in the Experimental Section. X: Aib.

the presence of non-β elements. The Aib-NF-Aib-VHSS, Aib-NF-Aib-VH, and Aib-NF-Aib-V peptides have minima at 1666–1670 cm^{-1}. These band frequencies are typical for Aib-containing peptides and indicate the presence of 3_{10} helices (Figure 2b).[8] We conclude that there is a fundamental difference between native peptides and their Aib-containing analogues. Native peptides are highly amyloidogenic, whereas their Aib-modified analogues are unable to form amyloid fibrils. The observation that this very minor chemical modification results in such a dramatic effect on the amyloidogenic potential of the peptide is consistent with structural models of the effect of Aib incorporation on conformational flexibility.[4]

The modified peptides display a recognition surface that is almost identical to that of the native sequence but lacks any amyloidogenic potential. These peptides are thus excellent candidates for the inhibition of amyloid self-assembly. We investigated the ability of the modified peptides to inhibit full-length hIAPP amyloid fibril formation by using the thioflavin-T (ThT) binding assay. This method provides quantitative information on amyloid fibril growth. hIAPP was allowed to form amyloid fibrils either in the presence of a 10-fold excess of peptide inhibitor or with no inhibitor. The fibrillization process was monitored and when a plateau was reached, the fluorescence intensity was recorded. The formation of amyloid fibrils was significantly reduced in the presence of the various Aib-containing peptides, as shown by the observed fluorescence intensities (Figure 3a). In contrast, when hIAPP was incubated in the presence of a 10-fold excess of the wild-type peptide ANFLVH in a control experiment, no significant inhibition was observed; the average ThT fluorescence was $97.1 \pm 7.4\%$ (four independent repeats) relative to that observed upon incubation of hIAPP in the absence of added peptides. These results clearly indicate that Aib-containing peptides have great potential as inhibitors of hIAPP amyloid fibril formation.

Since the Aib-NF-Aib-VH peptide exhibited the strongest inhibitory effect, we investigated the kinetics of hIAPP fibrillization in the presence and absence of this inhibiting peptide. In both cases, hIAPP fibrillization followed a typical nucleation-dependent path.[9] However, fibrillization occurred with strong exponential growth in the absence of inhibitor, whilst the fluorescence plot corresponding to fibrillization in the presence of inhibitor has only a small slope. The fluorescence intensity observed when the process had reached a plateau was dramatically lower for fibrillization under inhibitory conditions than in the absence of inhibitor (Figure 3b), as in the experiment described above. A clear difference was also observed upon staining with Congo red. Without inhibition, intense gold-green birefringence was observed; in the presence of the Aib-NF-Aib-VH peptide, only weak green birefringence was seen (Figure 3c).

Taken together, the results of our study suggest a new direction for the development of amyloid formation inhibitors: use of an Aib moiety as a β-breaker. Incorporation of such elements within a recognition motif rather than adjacent to it appears to be a powerful tool for the design of peptide inhibitors. This approach allows the creation of a wide range of molecules with highly specific chemical properties. In the last two years we have used a variety of experimental and theoretical techniques to demonstrate the key role of aromatic residues in self-assembly processes such as amyloid formation.[10] Herein, we have shown that the combination of an Aib modification and a native aromatic residue in a short

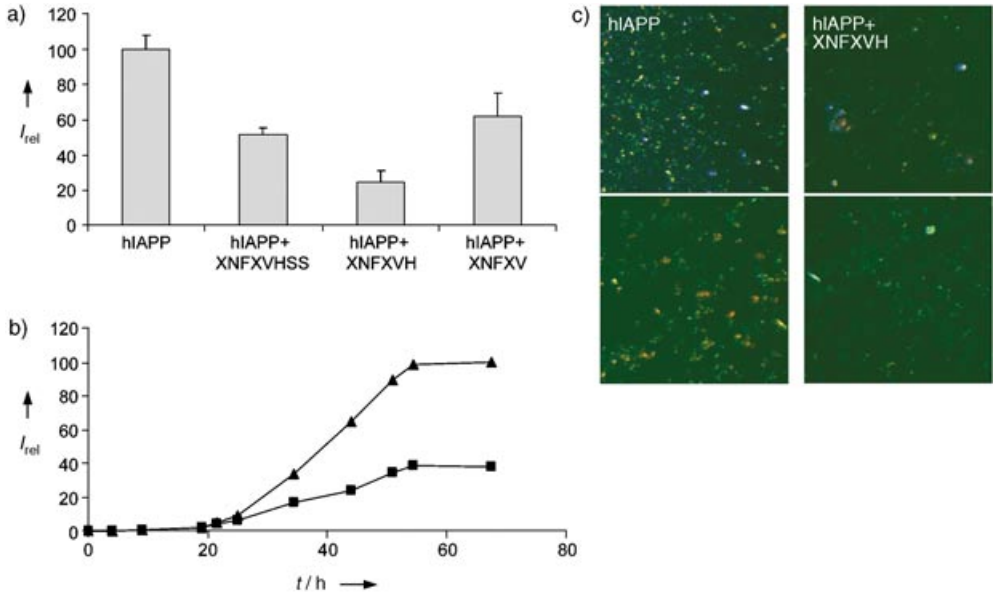

Figure 3. Inhibition of hIAPP amyloid formation. Quantitative representation of amyloid fibril formation by hIAPP in the absence and in the presence of Aib-containing peptide inhibitors, as assessed by the ThT binding assay. Peptide inhibitors were added in 10-fold excess. a) Relative fluorescence intensity I_{rel} of ThT added to hIAPP solutions aged for 3 days. Four independent repeat experiments were conducted. b) The kinetics of fibril formation by hIAPP alone (▲) and by hIAPP in the presence of the Aib-NF-Aib-VH peptide inhibitor (■). ThT was added to aliquots of hIAPP solutions over a period of 3 days, as described in the Experimental Section. c) Congo red staining and birefringence of hIAPP alone and in the presence of the Aib-NF-Aib-VH peptide inhibitor. Staining was performed after incubation for two (upper panel) and four days (bottom panel).

recognition motif generates the molecular basis for significant inhibition properties, which include prevention of β-sheet formation and a high affinity for the hIAPP molecule.

Experimental Section

Peptide synthesis and solutions: Peptide synthesis was performed by Peptron, Inc. (Taejeon) using solid-phase methods. The identities of the peptides were confirmed by ion-spray mass spectrometry on a HP 1100 series LC/MSD instrument. The purity of the peptides was confirmed by reverse-phase high-pressure liquid chromatography on a C_{18} Vydac column. Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM. To avoid pre-aggregation, fresh stock solutions were prepared for each experiment. Peptide stock solutions were diluted with tris(hydroxymethyl)amino-methane buffer (10 mM, pH 7.2) to a final concentration of 5 mM (5% DMSO).

Congo red staining and birefringence: A suspension (10 μL) of peptide solution (5 mM) aged for 10 days was allowed to dry overnight on a glass microscope slide. A sample (100 μL) from the ThT assay, aged for 2 or 4 days, was used for hIAPP. Staining was performed by adding a suspension of saturated Congo red (10 μL) and NaCl in 80% ethanol (v/v) to the sample. Birefringence was determined with a SZX-12 stereoscope (Olympus) equipped with cross polarizers.

Transmission electron microscopy: A sample (10 μL) aged for 4 (native peptides) or 10 days (modified peptides) was placed on a 400-mesh copper grid (SPI supplies) covered with carbon-stabilized formvar film. After 1 minute, excess fluid was removed and the grid was negatively stained by treatment with 2% uranyl acetate in water for 2 minutes. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

Fourier transform infrared spectroscopy: A sample (30 μL) of peptide solution aged for 2 weeks was suspended on a CaF_2 plate and dried in vacuo. The peptide deposit was resuspended in D_2O then dried. The resuspension procedure was repeated twice to ensure maximal hydrogen/deuterium exchange. Infrared spectra were recorded on a Nicolet Nexus 470 FT-IR spectrometer with a DTGS detector.

Thioflavin T binding fluorescence: Lyophilized synthetic hIAPP (Calbiochem) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to produce a 400-μM stock solution. This stock solution was added to solutions of the inhibiting peptides (40 μM) in sodium acetate buffer (20 mM, pH 6.5; 10-fold excess of inhibiting peptide), or to sodium acetate buffer (20 mM) alone to a final concentration of 4 μM. Immediately after dilution, the samples were centrifuged for 20 minutes at 18000 rpm in an Optima LE 70 ultracentrifuge (Beckman) equipped with a Ti 70.1 rotor at 4 °C. After centrifugation, the pellet was removed. 500-μL aliquots of the reaction solutions were added to sodium acetate buffer (500 μL) containing ThT (3 μM). Fluorescence values were measured with excitation at 450 nm and emission at 480 nm. The end-point experiment was repeated four times. Figure 3 shows average values with bars indicating standard deviations.

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