Reversible Mechanical Unzipping of Amyloid β-Fibrils*

Miklós S. Z. Kellermayer‡§, László Grama‡, Árpád Karsai‡, Attila Nagy‡, Amram Kahn‡, Zsolt L. Datki§, and Botond Penkek

From the ‡Department of Biophysics, University of Pécs, Faculty of Medicine, Pécs H-7624, Hungary and the §Department of Medicinal Chemistry and Protein Research Group of the Hungarian Academy of Sciences, University of Szeged, Szeged H-6720, Hungary

Amyloid fibrils are self-associating filamentous structures, the deposition of which is considered to be one of the most important factors in the pathogenesis of Alzheimer’s disease and various other disorders. Here we used single molecule manipulation methods to explore the mechanics and structural dynamics of amyloid fibrils. In mechanically manipulated amyloid fibrils, formed from either amyloid β (Aβ) peptides 1–40 or 25–35, β-sheets behave as elastic structures that can be “unzipped” from the fibril with constant forces. The unzipping forces were different for Aβ1–40 and Aβ25–35. Unzipping was fully reversible across a wide range of stretch rates provided that coupling, via the β-sheet, between bound and dissociated states was maintained. The rapid, cooperative zipping together of β-sheets could be an important mechanism behind the self-assembly of amyloid fibrils. The repetitive force patterns contribute to a mechanical fingerprint that could be utilized in the characterization of different amyloid fibrils.

Amyloid fibrils are self-associating filamentous structures formed from the 39–43-residue-long amyloid β-peptide (Aβ) or its subfragments (1). The deposition of amyloid oligomers (2) and fibrils is considered to be one of the most important factors in the pathogenesis of Alzheimer’s disease (3) and other disorders (4). The structure of Aβ-fibrils has for long been enigmatic because of insoluble aggregate formation that precludes the use of standard structural methods such as x-ray crystallography and solution NMR. Recent data from site-directed spin labeling (5), and particularly from solid-state NMR experiments (6, 7), have formed the basis of a high resolution model of the Aβ1–40 fibril: β-hairpins lying perpendicular to the fibril axis are associated into β-sheets that line up to form protofilaments, which are then assembled parallel into fibrils. Protofilaments are thus thought to represent an ~2–3 nm-diameter structural unit within the amyloid fibril (1). During amyloidogenesis the formation of fibrils is preceded by the appearance of globular aggregates that are thought to fuse, by not fully understood mechanisms, into fibrillar structures (8). Recently, curved, beaded, ~200 nm-long and ~6–8 nm-wide fibrillar precursors were described to appear in the amyloidogenic pathway, which were called protofibrils (9–12). The protofibrils are thought to go through a structural transition on their way to forming the amyloid fibril. The exact nature of structural dynamics within the amyloid fibril and related to amyloidogenesis, however, remains to be resolved.

Single molecule manipulation experiments have in the recent past provided unique and unprecedented insights not only into the structure and elasticity but also into mechanically driven transitions of molecular systems (13–21). In the present work we mechanically manipulated amyloid fibrils formed from either Aβ1–40 or Aβ25–35 peptides. We showed that filamentous entities most likely corresponding to β-sheets can be “unzipped” from the fibril with constant forces in an equilibrium process, indicating that during mechanical relaxation the β-sheets rapidly rebind to the fibril surface. This rapid, mechanical zipping together of β-sheets could be an important mechanism behind cooperative amyloid fibril formation.

**Experimental Procedures**

**Samples**—Amyloid peptides were prepared by solid phase synthesis (22). Aβ25–35 and Aβ1–40 peptides contained amino acid residues 25–35 and 1–40 of the β-peptide, respectively. Fibrils were generated by dissolving the peptides in PBSA buffer (10 mM K-phosphate, pH 7.4, 140 mM NaCl, 0.02% NaN3) at 0.5 mg/ml concentration. Aβ1–40 and Aβ25–35 fibrils were grown in solution at 25–37 °C for several days.

**Surface Adsorption of Amyloid Fibrils**—Depending on the type of experiment, different surfaces and methods were employed for attaching amyloid β-fibrils. 1) For AFM imaging under ambient conditions, mica surface was used. A 20-μl sample (0.5 mg/ml concentration) was pipetted onto a freshly cleaved mica surface and incubated at room temperature for 10 min. Subsequently, the surface was rinsed with MilliQ water and dried with a stream of clean N2 gas. 2) For molecular force spectroscopy measurements, fibrils were either adsorbed non-specifically to a precleaned glass surface or attached covalently to a glass coverslip. The two methods gave similar results. Glass coverslips were cleaned by sonication in acetone, followed by rinsing with MilliQ water and drying in a stream of clean N2 gas. A 20-μl sample (0.5 mg/ml concentration) was pipetted onto the glass surface and incubated at room temperature for 10 min. Unbound fibrils were washed away by rinsing with PBSA buffer. Rinsing was carried out by repetitively (5 ×) adding and removing 100 μl of buffer solution. The surface was then left covered with PBSA solution. For covalent modification, precleaned coverslips were incubated in toluene vapor containing 2% 3-glycidoxypropyl-trimethoxysilane (Fluka) for 12 h at room temperature. Attaching of fibrils to the covalently modified surface was carried out as described above, with an additional step: following the attachment of fibrils, the surface was incubated with PBSA buffer of pH 9 to facilitate covalent binding to the amino-modified surface.

**Single Molecule Force Spectroscopy**—Amyloid fibrils were stretched with an AFM (Asylum Research MFP-3D or MPFM) by first pressing the cantilever (Veeco Microlever or Olympus BioLever) tip against the surface and then pulling the cantilever away with a constant, preadjusted rate. The experiments were carried out under aqueous buffer conditions (PBSA buffer, pH 7.4). Stiffness was determined for each cantilever by using the thermal method (23).

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
Atomic Force Microscopy—Non-contact mode AFM images of amyloid fibrils bound to freshly cleaved mica were prepared with the MFP3D AFM instrument using silicon cantilevers (Olympus AC160, typical resonance frequency ~300 kHz). 512 x 512 images were collected at a typical scanning frequency of 1 Hz.

In Situ Force Spectroscopy—In situ force spectroscopy was carried out by first scanning (under aqueous buffer conditions) the glass-bound sample surface, pressing the cantilever tip to targeted surface locations identified on the image, and finally rescanning the surface to test for drift, the same area was analyzed in several cycles of scanning. To correct for drift, the same area was analyzed in several cycles of scanning and mechanical probing.

Data Analysis—In the case of non-linear mechanical responses, the relaxation curves were fitted with the worm-like chain equation as shown in Equation 1 (25).

\[ f_A = \frac{z}{k_BT} \left[ L - \frac{4}{z(L+z)} - \frac{1}{z} \right] \]

(Eq. 1)

Force plateaus were analyzed by measuring the distance between the average plateau force and the force baseline (Fig. 2A, i, inset). Force steps were analyzed by measuring the distance between the average force values of consecutive plateaus (Fig. 2C, inset).

Modeling and Simulation—Amyloid fibril unzipping was simulated with a simple elastically coupled two-state model (26, 27) in which the activation kinetics are influenced by the mechanical load and the shape of the interaction potential holding the amyloid fibril together. In the model, a β-sheet was gradually pulled off the fibril surface. The apparent contour length of the simulated β-sheet was reduced by the presence of a set of bonds spaced 4.7 Å apart. As the chain was extended, force was generated according to the wormlike chain equation (25). This entropic force is counterbalanced with the unzipping (or desorption) force, which may include electrostatic and non-electrostatic (e.g. hydrophobic interactions) components (28). In each polling interval (dt) the probability of bond rupture (unzipping) at the given force (f) was calculated according to Equation 2,

\[ P_f = w_d e^{-\frac{E_{zz}}{k_BT}} \]

(Eq. 2)

where \( w_d \) is attempt frequency set by Brownian dynamics (29), \( k_BT \) is thermal energy, \( E_{zz} \) is activation energy of unzipping, and \( \Delta L \) is distance between the bound and transition states along the unzipping reaction coordinate. Similarly, in the same polling interval the probability of binding (zipping) was calculated according to Equation 3,

\[ P_z = w_d e^{-\frac{E_{zz}}{k_BT}} \]

(Eq. 3)
sponding to the smallest plateau forces appeared at 33 pN (±7 S.D., n = 258) and 41 pN (±7 S.D., n = 76) for Aβ1–40 and Aβ25–35, respectively. The rest of the peaks appeared at forces that are integral multiples of the 33- or 41-pN unit forces.

We found that the plateau transition was reversible and the force staircases were repeatable. There were several manifestations of reversibility. If the pulling experiment was reversed prior to reaching the end of the force plateau, or if the plateau was preceded by non-linear elastic response (Fig. 3A), then the relaxation force curve followed the stretch force curve. The plateau force was independent of stretch rate, and the lack of hysteresis was maintained across two orders of magnitude of stretch velocity (Fig. 3B). Reversible force staircases, with no hysteresis, were also observed (Fig. 3C). Furthermore, these reversible force staircases often persisted through successive stretch-relaxation cycles (Fig. 3C). In these experiments, fluctuations across force steps were also observed (Fig. 3C, inset), indicating that the rate of the underlying structural fluctuations exceeds the stretch (or relaxation) rate.

**Time-dependent AFM Imaging and Force Spectroscopy**—The force spectroscopy results suggested that during stretch elastic strands are pulled off the Aβ-fibril surface. To explore the nature of these strands, time-dependent AFM imaging and force spectroscopy experiments were performed. Fig. 4 shows the results for Aβ1–40 fibrils. Shortly after dissolution in PBSA buffer, globular aggregates were present, but filamentous structures were not observed (Fig. 4, Day 0). In the corresponding force spectra an occasional force sawtooth was observed, but force plateaus and complex force patterns were absent. The length of the captured molecular species was 20 nm. The observed mechanical behavior describes most likely the capture, unfolding, and release of oligomers of the Aβ peptide. Force plateaus, non-linear elastic responses with long contour lengths, and various combinations of these mechanical responses were observed only after the third day and became prominent in preparations more than 1 week old (Fig. 4, Day 7, lower panel). These preparations contained an abundance of mature Aβ fibrils. Thus, the time-dependent AFM imaging and force spectroscopic results allowed the tentative conclusion that the prominent mechanical responses (Fig. 1) are associated with mature Aβ-fibrils.

**In Situ Force Spectroscopy**—To further explore the origin of the elastic strand pulled off the fibril surface, we carried out...
in situ force spectroscopy experiments. A glass surface with covalently attached mature Aβ1–40 fibrils was first gently scanned in non-contact mode under aqueous buffer conditions. Subsequently, force spectroscopy measurements were carried out at specific target locations of the surface. Fig. 5 shows the results for Aβ1–40 fibrils. Repeated attempts of pulling at the control location (Fig. 5, A and B, spot 1), which was devoid of clearly identifiable amyloid fibrils, produced no characteristic force response. By contrast, if fibrils were targeted (spots 2 and 3), characteristic force plateaus and staircases appeared. Plateau forces exceeding 500 pN were often observed (Fig. 5B, ii and iii). Force plateaus longer than 250 nm were seen (Fig. 5B, iv), and force staircase patterns persisted through successive stretch-relaxation cycles (Fig. 5B, iv) in accordance with earlier observations (Fig. 3C). The fundamental force plateau of ~30 pN was observed to reach a length of >200 nm (Fig. 5B, v). AFM imaging performed following the mechanical perturbations revealed that the fibrils were not removed in toto by the pulling attempts (Fig. 5A, iii and iv). Furthermore, the gross helical structure of the fibrils was not perturbed either, at least not to an extent resolvable under the experimental conditions (Fig. 5C). Thus, the in situ force spectroscopy experiments indicated that the elastic strands pulled away from the Aβ-fibril represent subfibrillar components of the mature fibril.

**DISCUSSION**

**Identity of Strands Unzipped from the Aβ-Fibril**—In the present work we mechanically manipulated surface-attached Aβ-fibrils, formed from either Aβ1–40 or Aβ25–35 peptides, by using single molecule force spectroscopy and in situ force spectroscopy techniques. Various combinations of two main phenomena, non-linear elasticity (Fig. 1A) and force plateau (Fig. 1B), dominated the mechanical response of the fibrils. Our results suggested that the mechanical response is determined by the elasticity and interactions of an element within the structural hierarchy of the mature Aβ-fibril. There are three elements, the Aβ-fibril itself, the protofilament, or individual β-sheets, that may in principle explain the observed mechanics (31). First, an entire Aβ-fibril could, in principle, be manipulated and lifted off the substrate surface, resulting in the observed force responses. However, we excluded this possibility because the fibrils were attached firmly (covalently in many experiments) to the substrate, and in situ force spectroscopy experiments demonstrated that fibrils were not displaced in toto by the mechanical perturbations (Fig. 5, B and C). Second, protofilaments could in principle be lifted off the fibril surface, producing the observed mechanical behavior. We excluded this possibility as well, for two main reasons. 1) A single Aβ-fibril contains five or six protofilaments (1). By contrast, the high plateau forces (Fig. 5B, ii and iii) indicate that the number of strands pulled off the fibril may be up to at least nine (see also explanation below). 2) The gross helical appearance of the Aβ-fibril, which is thought to be determined by the protofilament structure and arrangement (32), is not altered significantly by the mechanical perturbations (Fig. 5C). Thus, we concluded that the force responses observed in mechanically manipulated Aβ-fibrils are determined by the elasticity and interactions of individual β-sheets.

**Non-linear Elastic Behavior**—The fully reversible, non-linear force response (Fig. 1A) most likely describes the elasticity of the β-sheet that has been liberated from its lateral confinement within the fibril but held firmly at its ends. We could fit the non-linear force curves with the wormlike chain model of entropic elasticity. The persistence length obtained from wormlike chain fits to force data obtained for 5-day-old Aβ1–40 samples was 0.38 nm (±0.06 nm S.E., n = 165), which is comparable with the persistence length of a fully unfolded protein chain (titin) (15). We assumed that the short calculated persistence length is an apparent value, which is underestimated by the mechanical behavior of the fibril (33). The β-sheet (or bundles
Reversible Mechanical Unzipping of Aβ-Fibrils

We observed a significant difference between the unit unzipping forces for Aβ1–40 and Aβ25–35 fibrils (p < 0.0001). The multiple peaks in the plateau force histograms (Fig. 2) were interpreted to arise from the simultaneous unzipping of strands containing different numbers of β-sheets. Accordingly, the smallest unit forces of 33 or 41 pN correspond to the unzipping of single Aβ1–40 or Aβ25–35 β-sheets from the fibril, respectively. It is important to point out that a single β-sheet can interact with the underlying substrate at both of its ends, so as to form a loop, which results in unzipping force that is twice the unit force (see also “Model and Simulation” below). With the unit unzipping forces it is possible to calculate the number of β-sheets involved in a given force plateau transition. For example, the prominent ~130-pN force plateau seen in Fig. 5B, iv is caused by the unzipping of a minimum of two (if both form loops) and a maximum of four β-sheets (if none forms a loop). At present we can only speculate about the origin of the differences between the unit unzipping forces of Aβ1–40 and Aβ25–35 β-sheets. Considering that amino acid side chain interactions determine the strength of interaction between parallel β-sheets, the origin of the unzipping force difference could be because of differences in the arrangement of the exposed side chains. One possibility is that Lys-28, which participates in forming a salt bridge with Asp-23 that stabilizes the hairpin structure of the Aβ1–40 peptide (6), is exposed in Aβ25–35 and may form a relatively strong interaction with Met-35 of the neighboring peptide (40), resulting in greater unzipping forces.

**Force Steps and Staircases**—The unzipping process continues until the entire β-sheet is liberated, which is marked by an abrupt decrease in force. Descending force staircase (Fig. 1C) arises during the simultaneous unzipping of several β-sheets followed by their gradual, one-by-one (or group-by-group) release from the underlying fibril surface. The presence of force staircase patterns suggests that the β-sheets terminate at different positions, probably because of a staggered arrangement within the fibril. The structural implications for a possible staggered β-sheet arrangement within the amyloid fibril are not fully understood. The position of the force step along the length axis allows the estimation of the length of a β-sheet within the fibril. We found unit unzipping force plateaus up to 220 nm long (Fig. 5B, v), indicating that the length of individual β-sheets within the fibril can be well in excess of 200 nm.

Considering that protofibrils, the putative precursors of amyloid fibrils, have a maximum observed length of 200 nm (11), our findings raised the intriguing possibility that during amyloidogenesis protofibrils fuse by the annealing of their component β-sheets. The observed long β-sheet unzipped from the fibril raises a topological problem. β-sheets are embedded in hierarchically wound helices with a pitch of ~46 nm (41, 42). Unzipping a β-sheet, the length of which exceeds the helix pitch, is expected to cause torsion or rotation of or within the

---

**FIG. 5.** In situ force spectroscopy of Aβ1–40 fibrils. **A**, non-contact mode AFM images acquired in PBSA buffer before (i and ii) and after (iii and iv) tapping and pulling at specific spots (labeled with red arrows and numbers). The fibrils were attached covalently to an amine-modified glass surface (for details see “Experimental Procedures”). Corresponding height- (i, iii) and amplitude-contrast (ii, iv) images are shown. Spot 1 is devoid of fibrils and is therefore considered control location for the mechanics experiments. **B**, force curves acquired at corresponding spots shown in the AFM images. **i**, force data recorded in five consecutive stretch-relaxation cycles at the control location (spot 1). ii–v, force data recorded at spots corresponding to clearly distinguishable amyloid fibrils. ii, example of descending force staircase recorded at spot 2. iii, example of descending force staircase recorded at spot 3. iv, force data recorded in four consecutive stretch-relaxation cycles at spot 3. v, low force plateau (plateau height ~30 pN) recorded at spot 3. **C**, magnified amplitude-contrast AFM images of the amyloid fibrils at spot 3 before (i) and after (ii) pulling. Red arrow marks the location of the mechanical perturbation (spot 3).
fibril. Considering that the rotation of the entire fibril was prevented by its firm attachment to the substrate surface, it may be possible that the component protofilaments possess a certain degree of torsional or rotational freedom within the fibril.

**Reversibility of Force Plateau and Force Step**—The lack of force hysteresis often seen in the force responses (Fig. 3) indicates that the mechanically perturbed amyloid fibril system passes through identical structural states during stretch and release and is in thermodynamic equilibrium at each point of extension. The absence of stretch-rate dependence of plateau force (Fig. 3B) indicates that the equilibrium is highly dynamic and the system fluctuates between the (associated and dissociated, or zippered and unzipped) states on a time scale that is much faster than that of the pulling experiment. Because of the equilibrium, the average mechanical energy invested in driving the transition reflects the associated free energy change (ΔG) (43). Considering the mean plateau forces for the single β-sheet and a 4.7 Å monomer spacing (1), the ΔG of lateral β-sheet binding is \(-1.6 \times 10^{-20}\) J and \(-2 \times 10^{-20}\) J/monomer (i.e. \(-9.6\) kJ mol\(^{-1}\) and \(-12\) kJ mol\(^{-1}\)) for Aβ1-40 and Aβ25-35, respectively.

The prerequisite of reversibility is coupling, via the β-sheet, between the bonds holding the β-sheet laterally within the fibril. In lieu of coupling the dissociated monomers would diffuse away and reassociation would be unlikely. Coupling is serial in the case of a single β-sheet that is gradually unzipped from the fibril surface. However, parallel coupling can also occur, as in the case of parallel-associated β-sheets. A tight coupling between parallel-associated β-sheets can result even in the reversal of the force staircase related to complete β-sheet dissociation, and a completely released β-sheet can rebind to the fibril in a reversible reaction (Fig. 3C). Because the complete release and rebinding of a β-sheet results in large force steps, the rapid fluctuation between the states becomes visible in the force traces (Fig. 3C, inset).

**Repeatability of Mechanical Response**—We found that the complex mechanical response of the amyloid fibril was repeatable through successive stretch-relaxation cycles. That is, the force patterns recorded in successive mechanical cycles were identical or very similar (Figs. 3C and 5B, iv). The observation indicates that the structural features of the manipulated strand (e.g. number and arrangement of β-sheets) are preserved and that the mechanical events occur not randomly, but in a sequential manner (44). Repeatability is easily reconciled if an elastic coupling persists between the cantilever tip and the fibril surface that may guide the β-sheet rebinding through consecutive mechanical cycles (Fig. 3C). However, if the bundle of β-sheets is completely removed by the tip between stretch-relaxation cycles (such as happened in Fig. 5B, iv), the β-sheets first need to be delivered back to the fibril surface and allowed to rebind in a fast reaction. Possibly, under these conditions the underlying fibril surface serves as a spatial guide for structurally correct reassociation. Considering the added complexities due to helical protofibril arrangement, much further work is required to understand the details of the process.

**Model and Simulation**—We have proposed the following model to explain our observations (Fig. 6A). During mechanical manipulation of surface-adsorbed Aβ-fibrils, β-sheets are lifted off the fibril surface (Fig. 6A, i). As the pulling progresses, the β-sheets become unzipped via the sequential rupture of side chain interactions, which convert the β-sheets from an associated to a dissociated state. Because each rupture event increases the length of the unzipped β-sheet with a uniform and very small distance, the unzipping process proceeds at an apparent constant force that results in a force plateau (Fig. 6A, i). Because the β-sheets within a bundle are parallel-coupled, the plateau force scales with the effective (i.e. two in case of a loop) number of β-sheets. If a β-sheet becomes unzipped entirely, the force drops in a unit step. Numerous sequential steps result in a descending force staircase (Fig. 6A, ii). If the β-sheet bundle is parallel-coupled to an elastic element (e.g. a β-sheet already liberated but held at its ends, Fig. 6A, iii), then the shape of the force staircase is distorted by the superimposed non-linear elastic behavior. If the β-sheet is allowed to retract, it reassociates to the underlying fibril in a rapid and an apparently cooperative process. The driving force of the rapid rebinding is the very high local concentration of the conjugate binding sites, which increases with each rebinding event. The rapid rebinding is probably further facilitated by the correct orientation of the conjugate binding sites defined by the structural order of the β-sheet. As a result, the
β-sheets are rapidly zipper together.

A simulation based on a simple elastically coupled two-state model (26, 33) using the deduced 2 × 10⁻¹⁰ J/monomer ΔG between the bound and dissociated states (for Aβ25–35) recovered the essential features of our findings (Fig. 4), including the ascending and descending force staircases, the effect of parallel coupling to a non-linear elastic element, and reversibility (at relatively low, <60 nm/s, stretch rates). At high stretch rates (>60 nm/s), however, force hysteresis appeared in the simulation and the unzipping/switching forces became loading rate-dependent (35). The loading rate dependence in the simulation is due to the lack of consideration for the high local binding site concentration.

Implications—We have mechanically manipulated individual amyloid β-fibrils and argued that their complex mechanical responses are most plausibly explained by a reversible unzipping of long elastic β-sheets from the fibril. The findings are thought to have important implications for understanding the structure, structural dynamics, and mechanisms of formation of amyloid fibrils. The reversible re-binding of β-sheets at high loads and loading rates to the underlying fibril surface indicates that the associated state is strongly favored and a mechanically perturbed amyloid fibril is rapidly recovered by zipper together the β-sheets. This or a similar process may be important in the structural rearrangements that are thought to occur during the final formation of the amyloid fibril. The repeatable force patterns provided a mechanical fingerprint of the Aβ-fibril. This fingerprint was observed to be different for Aβ1–40 and Aβ25–35 fibrils, probably because of underlying differences in the arrangement and interactions of their β-sheets. Although we tentatively excluded that protofibrils, the precursors of mature amyloid fibrils (9–12), have contributed to the observed mechanics, because of their relatively high β-sheet content (up to 50%) (12) they may also have a particular mechanical fingerprint that could be explored by our method. Ultimately, the mechanical fingerprinting method employed herein may provide a novel tool for exploring the mechanisms of amyloid structural dynamics, differences between various amyloids, and the mechanisms of action of chemicals (e.g. β-sheet breaker peptides) (45, 46) that interfere with amyloidogenesis.

Acknowledgment—We thank Ildikó Konrásd for assistance.
