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Noncooperative Dimethyl Sulfoxide-Induced Dissection of Insulin Fibrils: Toward Soluble Building Blocks of Amyloid[†]

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ABSTRACT: The enormous molecular weight complicates detailed structural studies of amyloid fibrils and obscures identification of biologically active forms of protein aggregates in amyloid-related diseases. Here we show that aqueous solutions of dimethyl sulfoxide (DMSO) solubilize insulin fibrils while maintaining their β-pleated structure. This is accompanied by a marked decrease in the fluorescence of thioflavin T. According to atomic force microscopy images and dynamic light scattering measurements, the partial DMSO-induced dissection of insulin fibrils favors formation of smaller soluble oligomers, which retain a limited capacity to induce daughter generation of fibrils through seeding to the native insulin, as well as the ability to reassemble into fibrils upon removal of DMSO through dialysis against water. These findings suggest that the DMSO-induced ensembles of insulin molecules are closely related to elementary building blocks of amyloid fibrils.

Spontaneous conversion of protein molecules into β-pleated linear aggregates, so-called amyloid fibrils, has been implicated in a number of degenerative disorders, such as Alzheimer's disease (*1*). Despite the urgency surrounding the clinical consequences of amyloidogenesis, the current understanding of this phenomenon is incomplete. To a large degree, this is caused by the scarcity of detailed three-dimensional structures of amyloids, which remain notably difficult to obtain due to the insolubility and noncrystalline character of the fibrils. Dimethyl sulfoxide (DMSO)[1] has been successfully employed in amyloid-oriented studies (*2*−*8*) as the ultimate denaturant converting aggregated β-sheets into a random coil conformation. While the solvent is not as commonly used for this purpose as guanidinium hydrochloride or urea, it has certain advantages over the two former chaotropes. First, the denaturing effect of DMSO is recognized to rely mostly on targeting a specific type of interaction stabilizing protein structure, namely hydrogen-bonded networks (*9*), and because of that, the DMSO action may be finely tuned (e.g., refs *3*, *4*, and *8*). Second, dimethyl sulfoxide's vibrational bands do not overlap with the amide I band of proteins, which enables use of FT-IR spectroscopy, one of the most revealing methods in protein aggregation studies, as a probe of conformational transitions accompanying assembly and dissociation of protein fibrils.

In this paper, we are presenting an overlooked aspect of the DMSO-induced destabilization of model amyloid fibrils obtained from bovine insulin: formation of kinetically stable oligomers with an amyloid-like secondary conformation. These findings suggest that the degree of oligomerization of non-native protein aggregates may be tuned with diluted DMSO to render them accessible to biophysical methods of separation and characterization, which are only applicable to low-molecular mass specimens.

MATERIALS AND METHODS

Preparation of Amyloid Fibrils. First-generation fibrils were obtained via a 60 °C, 48 h incubation of a 1 wt % solution of bovine insulin (from Sigma) in 100 mM NaCl and D_2O (pD 1.9) (adjusted with diluted DCl from Sigma-Aldrich). The thus obtained mother fibrils were briefly sonicated and used for seeding a native insulin solution [1 wt % in 100 mM NaCl and D_2O (pD 1.9)] at a 1:100 amyloid:native insulin weight ratio. Daughter fibrils obtained from a following 60 °C, 24 h incubation were used in further experiments. All protein samples were dissolved in heavy water to permit observation of the conformation-sensitive infrared amide I band (between 1600 and 1700 cm^{-1}). Mature fibrils were briefly sonicated prior to the following measurements.

FT-IR Spectroscopy. For most FT-IR measurements, a CaF_2 transmission cell equipped with 0.05 mm Teflon spacers was used. The temperature in the cell was controlled through an external water circuit connected to a thermostat controlled by a personal computer. All FT-IR spectra were recorded at 25 °C on a Nicolet NEXUS FT-IR spectrometer equipped with a liquid nitrogen-cooled MCT detector. Typically, for a single spectrum,

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[1]Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; FT-IR, Fourier transform infrared; ThT, thioflavin T.

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256 interferograms of 2 cm^{-1} resolution were co-added. During measurements, the sample chamber was continuously purged with CO_2-free dry air. From each sample's spectrum, corresponding buffer and water vapor spectra were subtracted. Spectra were baseline-corrected and normalized according to the integral intensity of the amide I band. Quantitative plots (e.g., Figure 2d) of the progress of the α-helix-to-β-sheet transition upon aggregation were calculated as $(I - I_\alpha)/(I_\beta - I_\alpha)$, where I_α is the spectral intensity at a wavenumber assigned to the β-sheet (ca. 1625 cm^{-1}) in native insulin (the first spectrum), I_β is the intensity after complete aggregation, and I is a transient intensity. Data processing was performed with GRAMS (ThermoNicolet). For all FT-IR experiments, protein concentrations were kept constant (either 1 or 0.3 wt %) while DMSO:D_2O ratios varied. All further experimental details were the same as specified previously (*5*).

Fluorescence of ThT, Static Light Scattering. Insulin fibrils were suspended in DMSO and D_2O at a concentration of 0.3 wt %, after which ThT was added to its final concentration of 10^{-4} wt %. After a brief incubation, fluorescence measurements were taken using 2 mm quartz cuvettes and an AMINCO Bowman Series 2 luminescence spectrometer (λ_{excit} = 450 nm; λ_{em} = 482 nm). Measurements of static light scattering at 350 nm were taken using the same cuvette and spectrometer setup in the absence of ThT (*5*−*7*).

Atomic Force Microscopy. Freshly prepared insulin amyloid samples (either aqueous or from DMSO/D_2O mixtures) were allowed to equilibrate for 2 h at room temperature prior to an abrupt 500-fold dilution with deionized water. Subsequently, the diluted samples were deposited on freshly cleaved mica and left to dry up at room temperature. After 24 h, AFM tapping-mode measurements followed (in both amplitude and height regimes, as indicated in the text and figure captions) using a Nanoscope III atomic force microscope from Veeco and TAP300-Al sensors (resonance frequency of 300 kHz) from BudgetSensors. Other experimental details of the AFM imaging were the same as those described previously (*10*).

Dynamic Light Scattering. The DMSO- and D_2O-diluted insulin amyloid samples were further diluted approximately 100 times with DMSO/D_2O solutions of the same composition. The measurements were taken at 25 °C on a Zetasizer 3000 apparatus from Malvern Instruments using the viscosity and refractive index data for water/DMSO mixtures from the published sources (*11*−*16*).

Filtration. DMSO was added dropwise to stirred samples of previously sonicated daughter fibrils to a final concentration of 75 wt %. After a brief incubation at room temperature, clear samples were transferred onto Eppendorf probe size Ultrafree-MC microcentrifuge Durapore PVDF filters with 0.22 μm pores (from Sigma-Aldrich). The samples were carefully centrifuged for 10 min at 1200 rpm, and both diffusate and retentate were collected for FT-IR measurements.

Dialysis. Freshly prepared daughter fibrils were characterized with AFM, ThT fluorescence, and light scattering. Subsequently, DMSO was added dropwise to a stirred suspension of fibrils to its final concentration of 75 wt %. Once the protein dissolved, ThT fluorescence and scattering measurements followed. The sample was continuously stirred at room temperature for 72 h, after which it was filtered through Ultrafree-MC microcentrifuge Durapore PVDF filters with 0.4 μm pores (from Sigma-Aldrich). Filtered samples were again checked for ThT fluorescence and light scattering and, subsequently, placed in benzoylated dialysis tubing (from Sigma-Aldrich, with a molecular weight cutoff of 2000) and dialyzed at room temperature against a 100-fold volumetric excess of 100 mM NaCl in H_2O (pH 1.9). After 24 h, aqueous dialysate was replaced with a new portion. Accordingly, the dialysis routine was repeated twice. After dialysis for a total of 72 h AFM, ThT fluorescence and light scattering measurements ensued. For FT-IR measurements, it was necessary to replace H_2O with D_2O, which was facilitated by the fact that insulin aggregates precipitated upon dialysis. Samples collected from dialysis bags were centrifuged, and pellets were resuspended in a ca. 10-fold volumetric excess of 100 mM NaCl in D_2O (pD 1.9). After brief vortex-assisted elution, the samples were centrifuged again and washed with a new portion of the D_2O-based solution. The procedure was repeated five times. Because of the reciprocal quadratic dependence of the amount of remaining H_2O on the total molar fraction of deuterium in a H_2O/D_2O system, such elution is very effective in terms of removal of the minor isotopic component (H_2O) overlapping the amide I band (*5*). Infrared spectra were collected twice: immediately after the elution of insulin aggregates with heavy water that was repeated five times and after a following 72 h incubation of the sample at room temperature.

RESULTS AND DISCUSSION

The infrared spectra shown in Figure 1 depict how the increasing concentration of DMSO perturbs secondary structures of native insulin (a) and insulin fibrils (b). The spectral changes concomitant with the transition of either the native, predominantly α-helical structure (a) or the aggregated β-sheets (b) to DMSO-solvated random coil consist of the amide I band shifting to 1663 cm^{-1} (*9*). Apparently, this process occurs in a more abrupt manner in the case of the amyloid fibrils (Figure 1c). Since, unlike for the native protein, the secondary structure of insulin amyloid appears to be stable even in the presence of 75 wt % DMSO, we have conducted parallel measurements of light scattering intensity and fluorescence of thioflavin T (ThT), an amyloid-specific marker. All samples were prepared in D_2O, while concentrations of both the protein and the fluorophore were adjusted so that all measurements could be conducted on identical samples and under the same conditions, except for varying optical path lengths, which varied between 0.05 and 2 mm. This approach has allowed us to determine simultaneously DMSO concentration dependencies of β-sheet content (probed by infrared spectroscopy), total fibril count (monitored by ThT fluorescence), and light scattering on insoluble protein particles. The corresponding plots are juxtaposed in Figure 1d. The striking lack of cooperativity of unfolding of the secondary and quaternary structures implies that smaller, β-pleated intermediate forms populate solutions of insulin fibrils in the 75 wt % DMSO/D_2O mixture. An even higher concentration of DMSO, routinely used in amyloid studies (*2*−*4*), leads to the complete dissociation and denaturation of fibrils yielding DMSO-solvated random coil conformation (*9*) (Figure 1b,d).

Certainly, the gradual DMSO-induced dissection of insulin amyloid represents a pathway of conformational transition quite distinct from a "reverse mode" amyloidogenic self-assembly of the protein. The conformational state of fibrils dissolved in 75 wt % DMSO may be related more closely to amyloid building blocks than to aggregation-competent intermediates of insulin (*5*). Because the β-sheet-rich conformer features sufficient kinetic stability (Figure 1c), its further characterization was

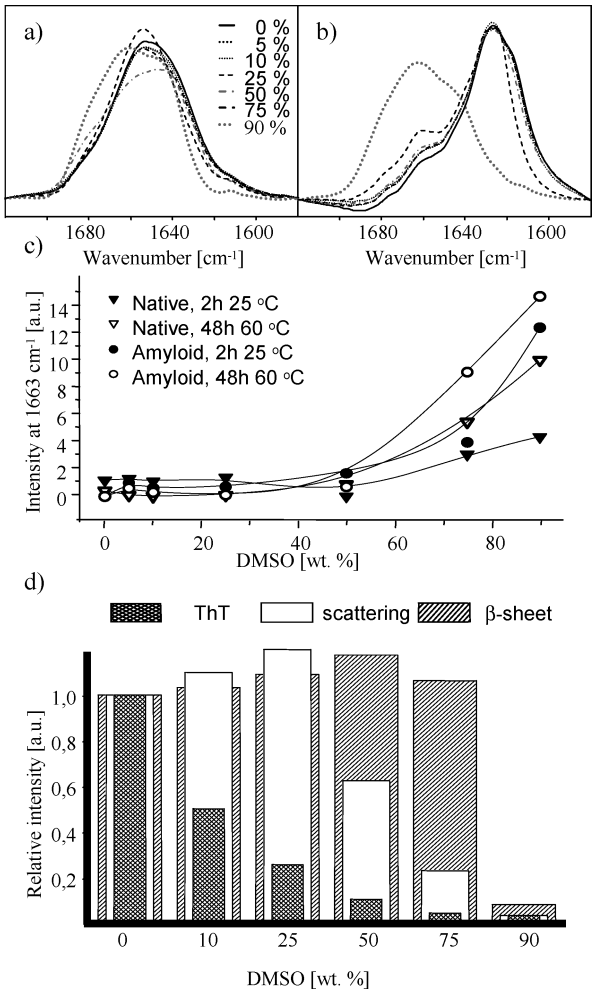

FIGURE 1: Influence of an increasing concentration of DMSO on the amide I band of insulin in the native (a) and amyloid (b) states. The spectra were recorded at 25 °C for fresh (2 h after preparation) 1 wt % protein samples in the mixtures of DMSO and 100 mM NaCl in D_2O (pD 1.9). The DMSO concentration dependencies of the intensity of the spectral component at 1663 cm^{-1} for native insulin and insulin fibrils were obtained for freshly prepared samples (2 h after preparation) and after incubation at 60 °C for 48 h (c). The noncooperative character of the dissection of DMSO-titrated insulin fibrils is reflected through changes in β-sheet content according to the infrared data, which are considerably decelerated compared to the changes in the total count of fibrils estimated from ThT fluorescence intensity and light scattering at λ_{350} (d). The spectra were recorded under ambient conditions approximately 2 h after the preparation of the samples.

possible. Figure 2a shows AFM images of insulin amyloid fibrils treated with DMSO. From these images, it is apparent that the presence of 75 wt % dimethyl sulfoxide results in a breakage of fibrils into shorter stretches, which slowly convert at room temperature to 5−12 nm large clusters (according to height measurements), a size that coincides with the diameter of mature insulin amyloid rather than of constituent protofilaments and protofibrils (*10*). Samples of the soluble β-sheet conformer matured for 24 h at room temperature may still contain an occasional fibrillar specimen (Figure 2a). AFM images of the insulin clusters are similar to the random coil deposits formed in the presence 90 wt % DMSO. As a complementary global probe of the size of particles, DLS has been employed in the subsequent experiments. The data shown in Figure 2b reveal a dramatic decrease in the average size of insulin aggregates once the DMSO concentration reaches 75 wt %. An even higher concentration of the solvent gives rise to an extremely narrow peak, which is likely to stem from singly dispersed denatured monomers. Thus, the combined static light scattering, AFM, and DLS data convey a strong argument that DMSO leads to fragmentation of insulin fibrils. At the same time, whether this process of dissection proceeds mostly via unbraiding of laterally aligned protofilaments or through cutting component fibrils into shorter stretches remains uncertain.

The problem of homogeneity of the soluble β-conformer has been independently addressed in a simple filtration experiment described in Materials and Methods in which spectra of the conformation-sensitive amide I band of insulin fibrils in 75 wt % DMSO were compared before and after filtration through a 0.22 μm polypropylene mesh (Figure 2c). The spectral outlook of either the main diffusate fraction or viscous retentate film left on the filter is not significantly different from that corresponding to mother fibrils and their fresh solution in 75 wt % DMSO, suggesting that the amyloid-like β-structure is the feature of the soluble phase.

As the hitherto examined characteristics of the β-sheet-rich phase of insulin amyloid in 75 wt % DMSO support the existence of soluble "building blocks" of insulin amyloid, it was crucial to check whether it possesses the capacity to induce daughter fibrils upon seeding to the native protein. The seeding experiment reported in Figure 2d shows that the soluble aggregates appear to accelerate growth of daughter fibrils, though the process is visibly retarded compared to the seeding with sonicated fibrils. Control seeding with sonicated fibrils in the presence of residual 0.75 wt % DMSO allowed us to exclude a possible decelerating effect of the cosolvent's traces brought into samples along with soluble "seeds" [after 100:1 dilution (Figure 2d)]. On the other hand, the possibility that traces of artifact fibrils surviving DMSO treatment and being incidentally transferred along with smaller oligomers contribute to the observed seeding kinetics cannot be ruled out entirely. Moreover, given the fact that DMSO appears to break and disperse fibrils more effectively and therefore create more "sticky ends" than sonication does, the relatively weak seeding effect of the soluble β-conformer is puzzling. It was shown by Weiss and colleagues that even amorphous aggregates of a covalent analogue of proinsulin still act as effective templates for fibrillation of wild-type insulin (*17*). Surprisingly, the tiny aggregates of insulin with an amyloid-like secondary fold obtained through the DMSO-induced fragmentation of fibrils do not act as equally efficient triggers of amyloidogenesis of the protein. At this stage, we may only hypothesize that as the disassembly of amyloid in DMSO/D_2O solution destabilizes the protein's tertiary and quaternary contacts, it may as well render sticky ends of insulin protofilaments frayed and dangling and therefore less receptive for incoming insulin monomers during elongation of fibrils. In other words, the core structure of insulin oligomers may be amyloid-like, but the surface may be far less rigid with fluctuating side chains, which would hamper "docking" of monomers. It is also likely that the surface is saturated with bound dimethyl sulfoxide molecules preventing "proper" interactions between protein chains coming into contact. Importantly, these two effects would also account for the rapid decrease in ThT fluorescence in the presence of DMSO. Namely, the increasing quantum yield of the fluorescence of ThT molecules upon binding to amyloid fibrils is attributed to the restricted intramolecular rotation of the fluorophore (*18*). Should the rigidity of ThT-trapping surface moieties be compromised by side chains becoming more "shaky" or

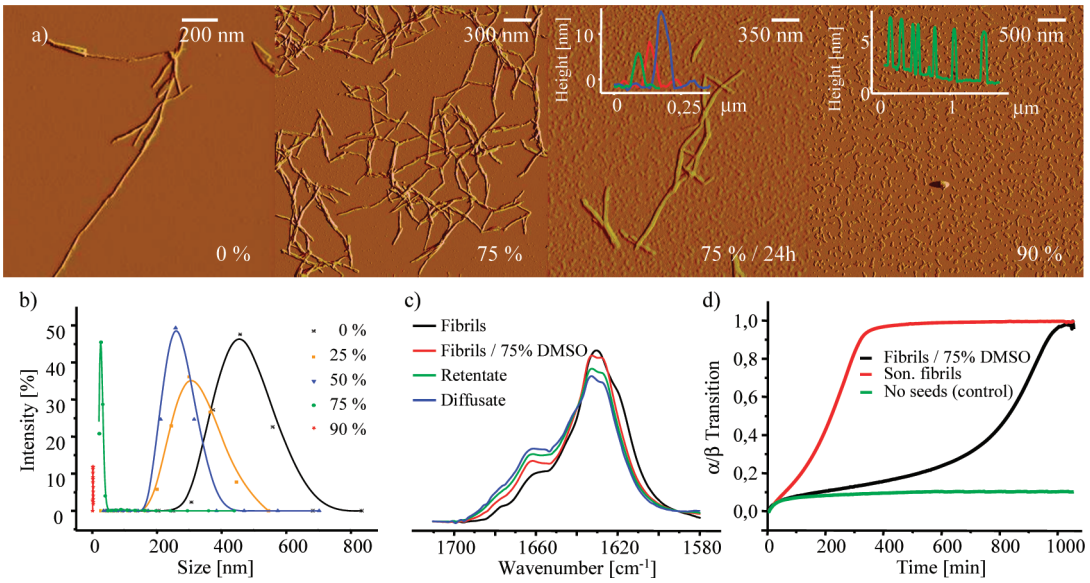

FIGURE 2: AFM amplitude images of insulin fibrils in the absence and presence of concentrated DMSO (a). The cross-section analysis indicated in the panels at the right corresponds to data collected in the height mode. DLS data reflect the rapidly decreasing size of particles of insulin amyloid with the increasing concentration of DMSO (b). Normalized infrared spectra show the amide I band of insulin fibrils (before and after dissolution in 75 wt % DMSO) and of the latter one (before and after filtering through a 0.22 μm filter) (c); spectra of both retentate and diffusate fractions were collected separately. (d) Kinetics of FT-IR-monitored aggregation of 1 wt % bovine insulin at 37 °C in 100 mM NaCl and D_2O (pD 1.9) induced by sonicated fibrils, and fibrils previously dissolved in 75 wt % DMSO (all at a 100:1 native protein:fibril weight ratio). All samples contained 0.75 wt % DMSO.

by intercalation of cosolvent molecules, this would inevitably lead to a decrease in the fluorescence intensity of thioflavin T. While a minor fluorescence quenching effect of DMSO is possible (at this high concentration), the parallel light scattering data and earlier reported DMSO-induced bleaching of the intensively violet inclusion complex of molecular iodine and insulin fibrils (where no quenching effect or chemical reaction may play a role) suggest that the topological instability of the DMSO-solubilized oligomers is a more adequate explanation (*6*).

To elucidate the reversibility of the DMSO-induced dissection of insulin fibrils, the cosolvent was removed from solubilized amyloid samples through an extensive, triplicate dialysis against acidified water. The AFM images shown in Figure 3 permit a comparison between pristine insulin fibrils and clumped aggregates obtained through a 72 h dialysis of fibrils previously dissolved in a 75 wt % DMSO/D_2O mixture against 100 mM NaCl in H_2O (pH 1.9) (control experiments with pH-neutral dialysate were conducted as well; see Materials and Methods and Supporting Information). The apparently reassembled fibrils are similar in length and height to the wild-type amyloid, which is confirmed by a qualitative image analysis of the corresponding height images (AFM data in the Supporting Information). The wild-type pristine fibrils were on average 600 nm long and 12 nm high, whereas dissolution in DMSO and the following reassembly in aqueous environment yielded predominantly 400 nm long and 15 nm high specimens. While these values are too large to call the reconstituted aggregates protofilaments (*10*), the diameter is likely to be considerably overestimated due to the persistent clumping of the aggregates. Thus, it appears probable that the elongated aggregates reassembled upon dialysis represent clumped hierarchically lower elements of the amyloid architecture, such as protofilaments or protofibrils (*10*). It is important to realize that using acidified, NaCl-containing dialysate has certain consequences: dimeric bovine insulin is soluble and aggregation-competent under such conditions. Thus, only misfolded aggregates may precipitate upon removal of DMSO. Should pH-neutral solution or pure water be used instead, it is likely that some insulin oligomers would precipitate instantly without much chance to form fibrils. However, control experiments showed that the altered pH has little effect on the course of changes observed upon dialysis [the same routine carried out at neutral pH (Supporting Information)].

Measurements of ThT fluorescence and static light scattering conducted at different stages of dialysis (Figure 3d) support the idea that replacing DMSO with water leads to a partial (60%) reassembly of ThT-detectable aggregates. The original amyloid samples were grown in D_2O and at a temperature sufficiently high to denaturate aggregating insulin monomers (*5*) (see Materials and Methods). This resulted in the complete solvent exchange and deuteration of the protein, reflected by the disappearance of the amide II band at 1550 cm^{-1}, which is indicative of the number of remaining unexchanged amide protons. Because H_2O was subsequently used as a dialysate, this provided an opportunity to trace the reassembly process through a reversed D−H exchange within the protein amide bonds. It was shown earlier that in the case of insulin fibrils the amide bonds may be sharply divided into two groups: a minority of surface-exposed and therefore fast proton-exchanging ones and a majority of nonexchanging ones trapped deeply within the fiber cores (*5*). Moreover, it is well-known that at high DMSO concentrations, H−D -exchange in proteins is effectively halted (*4*). The situation changes dramatically during dialysis, when the concentration of DMSO decreases and D_2O is simultaneously replaced with H_2O. The resulting reversed D−H exchange will be predominantly controlled by (i) the degree of initial solvent exposure of protein chains, which in the soluble oligomers is arguably much higher than in insoluble amyloid fibrils, and (ii) the rate of burying these chains within gradually reconstituting fibrils. Obviously, the latter process strongly depends on the rate of diffusion and, therefore, on the concentration of protein itself. The infrared spectra in Figure 3e show both the amide I and amide II vibrational regions. Interestingly, samples of

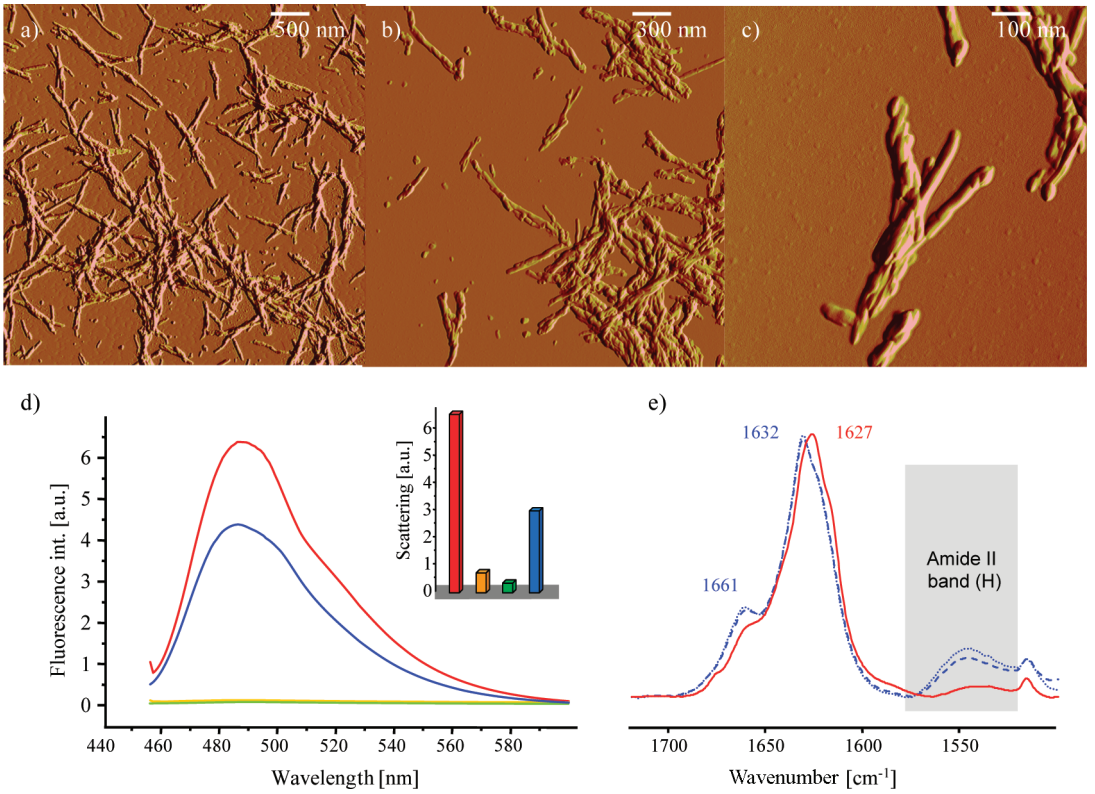

FIGURE 3: Dialysis of 75 wt % DMSO-dissolved samples of the β-pleated insulin oligomers against 100 mM NaCl in water (pH 1.9). Phase AFM images of sonicated insulin fibrils before dissolution in 75 wt % DMSO (a) and after the final stage of a 72 h dialysis (b and c). The corresponding ThT fluorescence data (d) and light scattering data at 350 nm (inset in panel d) were obtained for freshly sonicated fibrils (red), subsequently dissolved in 75 wt % DMSO and incubated at room temperature for 72 h (orange), subsequently filtered (green), and finally after a 72 h dialysis at room temperature (blue). (e) FT-IR spectra of the dialysis-recovered precipitate of insulin aggregates obtained immediately after extensive elution with 100 mM NaCl in D_2O (pD 1.9) (dotted line) and after an additional 72 h at room temperature (dashed line). Overlapped is a spectrum of freshly sonicated D_2O-grown fibrils before dissolution in DMSO (red line).

reassembled fibrils (as opposed to fibrils freshly grown in D_2O) do exhibit a pronounced spectral intensity at 1550 cm^{-1}, which proves that the reassembling fibrils solvent-exchanged with the surrounding environment. As the infrared spectra were acquired after extensive elution of precipitates with D_2O, the persistent presence of the band at 1550 cm^{-1} (even after incubation for 72 h in D_2O) demonstrates that the exchange process could only proceed transiently during the reassembly of solvent-exchange-prone β-pleated oligomers, but not later, once the rigid fibrous amyloid scaffold has been reconstituted. Along with the amide II band at 1550 cm^{-1}, a minor blue shift of the amide I band (from 1627 to 1632 cm^{-1}) is also detected. This is yet another spectral aspect of protons being trapped within amide groups. The small peak at 1661 cm^{-1} suggests a transient solvent exposure of less structured elements such as turns and loops (*5*).

In the low concentration range, DMSO acts as a more effective denaturant of the native insulin than of insulin fibrils (Figure 1a,b). We have conducted additional seeding experiments in the presence of varying concentrations of DMSO. This time, sonicated regular insulin fibrils without DMSO pretreatment were used. The results revealed a transient acceleration of the fibrillation process in the presence of 5−25 wt % DMSO (Figure 4a). These intermediate conditions promote the transition of the native conformation to an aggregation-competent intermediate state without a significant perturbation of amyloid seeds. Interestingly, at 75 wt % DMSO, the aggregation process is completely halted, even though the amyloid secondary structure remains intact (Figures 1d and 4a). This is likely to be one more aspect of the previously detected weak seeding properties of the aggregate coupled to possible consequences of strong hydrogen bond-mediated binding of DMSO molecules to solvent-exposed insulin main chains. This would prevent proper molecular recognition and docking of monomers onto amyloid templates. In conclusion, we have reported an intriguing noncooperative disassembly of mature insulin fibrils in a 75 wt % DMSO/water mixture, which leads to small soluble species that are amyloid-like secondary structure oligomers (Figure 4b). The relationship between these ensembles and building blocks of amyloid fibrils remains uncertain. The feasibility of obtaining finely dispersed (and therefore featuring enormous surface accessible area) amyloid samples may be useful in studies of the secondary nucleation scenarios, which has been proposed for insulin fibrillation (*19*, *20*). The particular composition of the solvent sustaining the soluble β-sheet coincides not only with the approximately equimolar ratio of water and DMSO but also with a number of physical anomalies in the DMSO/water system (*15*, *16*). The capacity of diluted DMSO [unseen for other amyloid-disrupting cosolvents such as trifluoroethanol (e.g.,, ref *21*)] to dissect amyloid fibrils into soluble elementary building blocks may facilitate making proteinaceous assemblies accessible to these biophysical methods of conformational characterization, whose application is strictly limited to lower-molecular weight species. On the other hand, in light of the ongoing debate about the molecular nature of neurotoxicity of protein aggregates [e.g., whether the most detrimental are smaller (*22*) or larger assemblies of misfolded protein molecules (*23*)], our results may be useful in the quest for new techniques of separation and fragmentation of amyloid fibrils, oriented at an

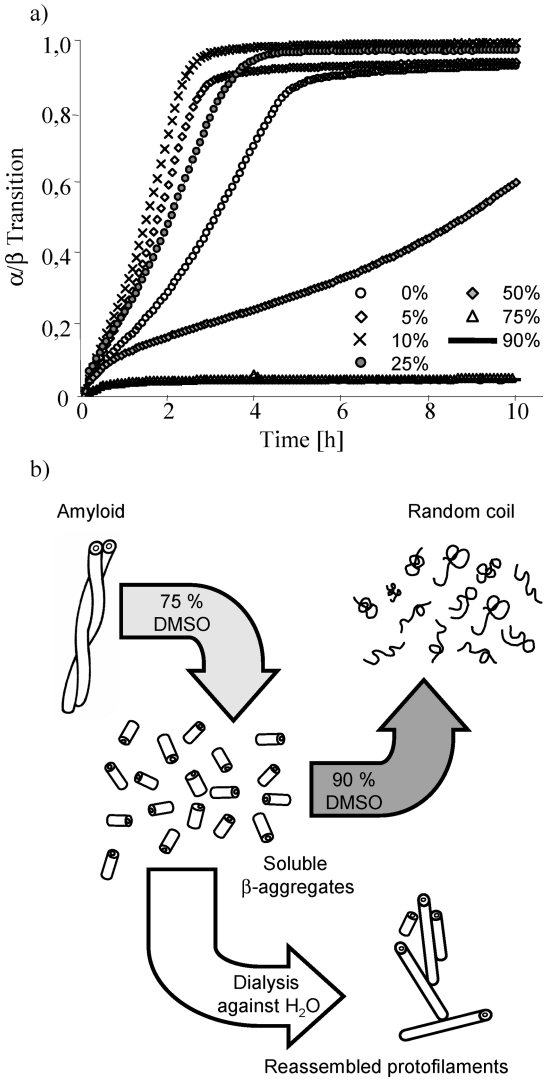

FIGURE 4: (a) FT-IR-monitored kinetics of seed-induced (at a 100:1 native protein:amyloid mass ratio) aggregation of 1 wt % bovine insulin at 37 °C in 0.1 M NaCl and D_2O (pD 1.9) in the presence of the indicated varying concentrations of DMSO (weight percent). (b) Schematic representation of the noncooperative dissection of insulin amyloid fibrils in the aqueous solution of DMSO occurring within the intermediate concentration range of the denaturant and the partial reassembly and aggregation of protofilaments upon dialysis against water.

unambiguous determination of the relationship between the degree of association of misfolded protein molecules and their biological activity.

SUPPORTING INFORMATION AVAILABLE

Kinetics of the dissection of insulin fibrils and additional dialysis and AFM data. This material is available free of charge via the Internet at http://pubs.acs.org.

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