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Dissolution of β_2-Microglobulin Amyloid Fibrils by Dimethylsulfoxide

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Increasing numbers of proteins have been found to aggregate into insoluble fibers, collectively referred to as amyloid fibrils. To address the conformational stability of amyloid fibrils, we studied the effects of dimethylsulfoxide (DMSO), 2,2,2-trifluoroethanol (TFE), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) on β_2-microglobulin amyloid fibrils by circular dichroism, thioflavin T fluorescence, light scattering, and electron microscopy. When measured by circular dichroism and thioflavin T fluorescence, HFIP, and TFE dissolved the fibrils, producing predominantly helical conformations. However, these alcohols did not dissolve the amyloid fibrils completely as monitored by light scattering and electron microscopy. On the other hand, DMSO completely dissolved the amyloid fibrils although a high concentration [*i.e.*, 80% (v/v)] was required. These results are consistent with the important role of hydrogen bonds in stabilizing amyloid fibrils.

Key words: amyloid fibrils, circular dichroism, dimethylsulfoxide, β_2-microglobulin, protein folding, β-structure, solvent effects.

Abbreviations: β_2-m, β_2-microglobulin; ThT, thioflavin T; CD, circular dichroism; EM, electron microscopy; EtOH, ethanol; TFE, 2,2,2-trifluoroethanol; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; DMSO, dimethylsulfoxide.

Many proteins and peptides form amyloid fibrils (*1*, *2*). Although most are related to diseases, it has been shown that several proteins (*3*, *4*), peptides (*5*) and, moreover, polyamino acids (*6*) can also form amyloid fibrils. Amyloid fibril formation is now recognized as a phenomenon common to many proteins and peptides. Understanding the properties of such fibrils is essential to obtaining further insight into the conformation and folding of proteins.

Among various amyloidogenic proteins, β_2-microglobulin (β2-m), a 99-residue protein with a molecular mass of 11.8 kDa, is a major target of intensive study because of its clinical importance (*7–9*) and appropriate size for biophysical studies (*10–16*). We have been studying the mechanism of amyloid fibril formation using the recombinant β_2-m expressed in *Pichia pastoris* (*17–21*). In the previous study using H/D exchange of amide protons followed by the dissolution of fibrils and multidimensional NMR analysis (*20*), we showed that most residues in the middle region of the molecule, including the loop regions in the native structure, form a rigid β-sheet core, explaining the remarkable rigidity and stability of amyloid fibrils. In the above study, we used dimethylsulfoxide (DMSO) to dissolve amyloid fibrils.

In studying effects of alcohols on proteins (*22–26*), we suggested three types of effects. First, alcohols can denature the native state of proteins by weakening hydrophobic interactions in the protein interior. Second, alcohols can induce already unfolded proteins and peptides to assume a helical structure of by strengthening hydrogen bonds. Third, alcohols can dissolve protein aggregates by weakening the hydrophobic interactions responsible for associations among protein molecules. Because amyloid fibrils can be considered to be a form of protein aggregate but with ordered structures, we anticipated that alcohols, as well as DMSO, can dissolve amyloid fibrils. However, we did not know which would be more effective for dissolution (or depolymerization) of amyloid fibrils.

To address the conformational stability of amyloid fibrils, we studied the effects of DMSO, ethanol (EtOH), 2,2,2-trifluoroethanol (TFE), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) on β_2-m amyloid fibrils. These organic solvents have all been used to dissolve amyloid fibrils (*27–30*). We chose conditions in the presence of 20 mM HCl at which amyloid fibrils of β_2-m are stable (*26*, *31*, *32*) and alcohol-denaturation does not induce protein aggregation (*22*). Our results show that, although HFIP and TFE dissolve amyloid fibrils, the effects are not complete with some remaining aggregates. On the other hand, DMSO completely dissolves amyloid fibrils, although high concentrations are required.

MATERIALS AND METHODS

β_2-Microglobulin—Recombinant β_2-m was prepared as described previously (*17*, *18*). Three species with different extensions (Glu-Ala-Glu-Ala-Tyr-Val-, Glu-Ala-Tyr-Val-, and Val-) added to the N-terminal Leu of intact β_2-m due to heterogeneous cleavage of the signal peptide were expressed in methylotrophic yeast *Pichia pastoris* and purified as described previously. In this study, we used β_2-m with one additional amino acid residue (Val).

Amyloid Fibrils—β_2-m amyloid fibrils were formed by the fibril extension method established by Naiki and coworkers (*31*, *32*). Sonicated β_2-m amyloid fibril seeds (final

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concentration: 5 μg/ml) were extended with 25 μM to 75 μM monomeric protein in 50 mM Na citrate buffer (pH 2.5) and 100 mM KCl. This solution was incubated overnight at 37°C in a temperature-controlled incubator.

Dissolution (*i.e.*, depolymerization) of β_2-m amyloid fibrils was carried out by adding fibrils to alcohol/water solutions. Typically, 100 μl of fibril solution was mixed into 390 μL of various concentrations of alcohols and 10 μl of 1.0 N HCl (final HCl concentration 20 mM) by pipetting and vortexing. Light scattering was measured immediately or other measurements such as ThT and CD were performed 30 min after the start of the depolymerization reaction.

CD—CD measurements were performed on a Jasco spectropolarimeter, model J-720, using quartz cells with a 1-mm path length. The temperature was controlled at 20°C with a Peltier thermostat (Jasco PTC-348WI). The final protein concentrations were 0.1 and 1.0 mg/ml for far-UV and near-UV CD measurements, respectively.

Fluorescence—Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model F-4500, at 20°C using a cell with a 10-mm light path. For ThT analysis, typically, 7.5 μl of the solution to be measured was added into 1.5 ml of 5 μM ThT in 50 mM glycine-NaOH (pH 8.5). The ThT fluorescence was measured at excitation and emission wavelengths of 445 nm and 485 nm, respectively. The slit was set at 5.0 nm for excitation and 10 nm for emission. Fluorescence was measured immediately after the mixture was prepared and was averaged for the initial 5 seconds.

Light Scattering—Light scattering measurements were performed with a Hitachi fluorescence spectrophotometer, model F-4500, at 20°C using a cell with a 10-mm light path. The wavelengths for excitation and emission were both set at 350 nm, and the slit length was 1.0 nm.

Electron Microscopy—For electron microscopy (EM), reaction mixtures were spread on carbon-coated grids, then negatively stained with 1% phosphotungstic acid (pH 7.0). The samples were examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 7.5 W.

RESULTS

Solvent Effects Measured by CD—As the standard experimental conditions, we chose 20 mM HCl (pH 2 in the absence of alcohol) and 20°C where the amyloid fibrils of β_2-m are stable (*17–19*, *31*, *32*) and alcohol-denaturation does not tend to induce aggregation of the proteins (*22*). First, we examined the effects of HFIP and TFE on β_2-m monomers in 20 mM HCl, where the protein is acid-unfolded. Additionally, the effect of EtOH was examined for comparison. We could not measure the effects of DMSO by CD because of its strong absorption in the far-UV region. At pH 2.0, the far-UV CD of β2-m monomers showed a spectrum with a minimum at 205 nm, consistent with substantial unfolding (Fig. 1a). Upon the addition of HFIP, β_2-m formed an α-helical structure as shown by the spectrum with two minima at 208 nm and 222 nm. The maximal ellipticity at 222 nm was about –18,000 and the helical content was estimated to be about 45% by the method of Chen *et al.* (*33*). Spectra in the presence of EtOH, TFE, and HFIP all showed similar

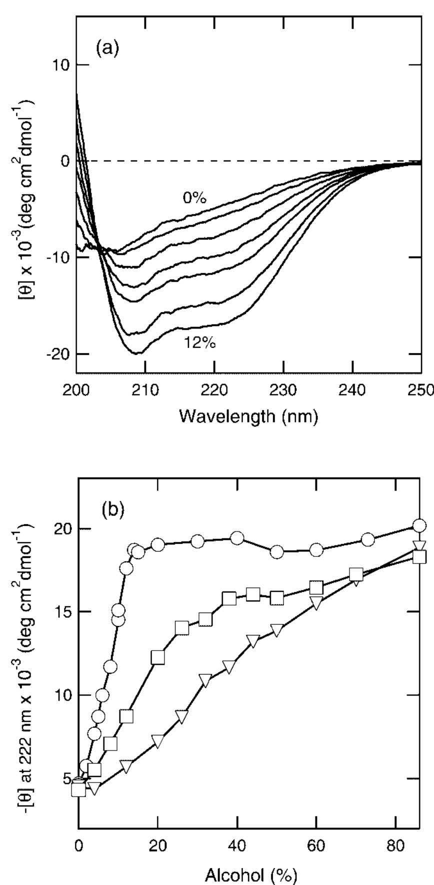

Fig. 1. **Alcohol-induced transition of acid-denatured β_2-m monomers as measured by CD.** (a) Far-UV CD spectra of acid-denatured β_2-m monomers in the presence of various concentrations of HFIP at 20 mM HCl and 20°C. HFIP concentrations from top to bottom at 222 nm: 0, 2, 4, 6, 8, 10, and,12% (v/v). (b) Conformational transition of acid-denatured β2-m monomers induced by various alcohols measured by the ellipticity at 222 nm in 20 mM HCl at 20°C. HFIP (circles), TFE (squares), EtOH (inverted triangles).

spectra (data not shown), but the transition from the unfolded structure to helical structure occurred at different alcohol concentrations.

Transition curves were constructed by plotting the intensity at 222 nm against alcohol concentration (Fig. 1b). The maximal ellipticities were similar among the three alcohols at around –18,000. The effectiveness of alcohols was HFIP > TFE > EtOH, consistent with the order found for the alcohol effects on the native state of proteins (*e.g.* β-lactoglobulin) and unfolded peptides (*e.g.* melittin) (*23–25*).

We then measured the effects of these alcohols on the far-UV CD of β_2-m amyloid fibrils (Fig. 2a). The far-UV CD spectrum of β_2-m fibrils has a maximum at 218 nm, characteristic of a β-sheet structure. With increasing HFIP concentration, the negative intensity at 218–220 nm became larger and another peak at 208 nm developed, showing the conversion from β-sheet to α-helix. The spectra of β_2-m fibrils at HFIP concentrations over 18% (v/v) were the same. Moreover, the spectra of β_2-m fibrils at HFIP concentrations over 18% (v/v) had the same shape and intensity as those of monomeric β_2-m in the corresponding HFIP concentrations (Fig. 1). This con-

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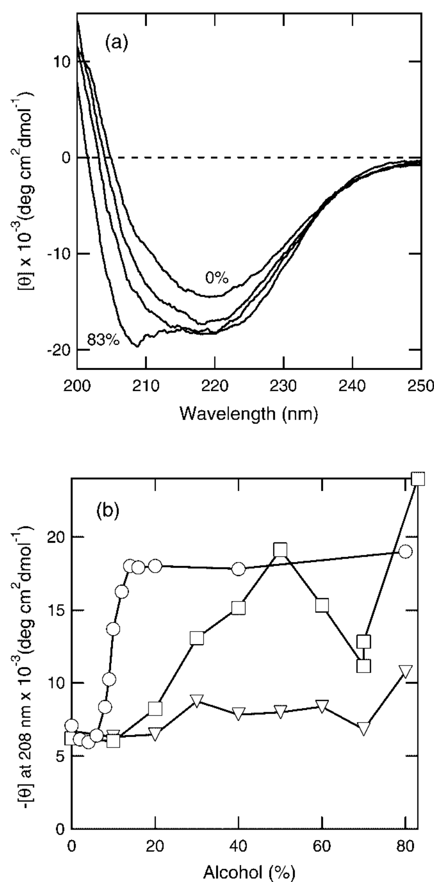

Fig. 2. **Alcohol-induced transition of β_2-m amyloid fibrils as measured by CD.** (a) Far-UV CD spectra of β_2-m amyloid fibrils in the presence of various concentrations of HFIP at 20 mM HCl and 20°C. HFIP concentrations from top to bottom at 208 nm: 0, 9, 10.5, and 83% (v/v). (b) Conformational transition of β_2-m amyloid fibrils induced by various alcohols measured by the ellipticity at 208 nm in 20 mM HCl at 20°C. HFIP (circles), TFE (squares), EtOH (inverted triangles).

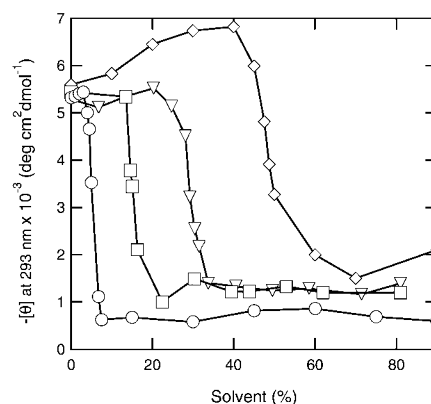

Fig. 3. **Conformational transition of β-lactoglobulin induced by various alcohols and DMSO.** Transitions were measured by the ellipticity at 293 nm in 20 mM HCl at 20°C. HFIP (circles), TFE (squares), EtOH (inverted triangles), and DMSO (diamonds).

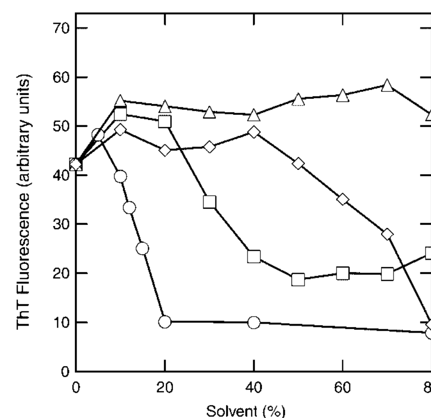

Fig. 4. **β_2-m amyloid fibril dissolution by various alcohols and DMSO as measured by ThT fluorescence.** The reaction mixtures were incubated in 20 mM HCl at 20°C for 30 min. HFIP (circles), TFE (squares), EtOH (inverted triangles), and DMSO (diamonds).

firms that the conformation at high concentrations of HFIP is almost independent of the original state, as far as the CD spectrum is concerned.

The effects of TFE and EtOH on β_2-m fibrils were also examined by CD (Fig. 2b). CD spectra in the presence of TFE concentrations up to 50% (v/v) showed a gradual increase in negative intensity at 218 nm with increasing TFE concentration. CD spectra of β_2-m fibrils in TFE concentrations over 50% (v/v) showed two minima at 208 and 218 nm with the latter being the larger (data not shown). In other words, the spectrum was different from the CD spectra of β_2-m at high concentrations of HFIP as shown in Fig. 1a and 2a, where the magnitude at 208 nm is larger than that at 222 nm. Moreover, CD spectra of β_2-m fibrils at high concentrations of TFE were unstable and often non-reproducible. The ability of EtOH to unfold the amyloid fibrils measured as by CD was negligible (Fig. 2b).

It would be informative to examine the effects of both alcohols and DMSO on the native state of β_2-m using a common measurement method. The native state of β_2-m is stable at neutral pH. However, the addition of alcohol to protein solutions at neutral pH frequently leads to unwanted aggregation, rendering conventional spectroscopic measurements difficult (*22*). Therefore, instead of β_2-m, we used bovine β-lactoglobulin (isomer A purchased from Sigma) as a model protein to examine the effect of alcohols and DMSO. β-Lactoglobulin assumes its native state at pH 2.0 and no aggregation is observed upon the addition of alcohols (*22–24*) or DMSO. We used near-UV CD instead of far-UV CD since DMSO does not adsorb in the near-UV wavelength region. HFIP, TFE, EtOH, and DMSO were added at various concentrations and CD at 293 nm was monitored (Fig. 3). Cooperative denaturation curves were observed for all solvents used. The transition curves in Figure 3 show that the order of effectiveness for denaturing the native state of β-lactoglobulin at pH 2.0 is HFIP > TFE > EtOH > DMSO. We do not know the exact conformation of the DMSO denatured state, however, heteronuclear NMR analysis (Masaru Hoshino and Y. Goto, unpublished results) suggests that the conformational state is close to random coil rather than the α-helix common for the alcohol-denatured state.

Solvent Effects Measured by Thioflavin T Assay—ThT assay using the large increase in fluorescence intensity upon binding to amyloid fibrils is commonly used to analyze amyloid fibrils (*31*, *34*). Figure 4 shows the ThT fluo-

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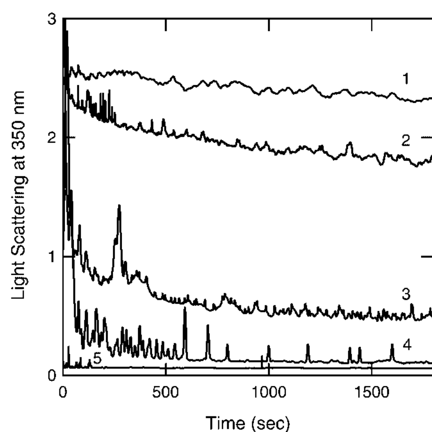

Fig. 5. **β_2-m amyloid fibril dissolution by alcohols and DMSO as monitored by light scattering.** Amyloid fibrils were added to 20 mM HCl (1), 40% (v/v) TFE and 20 mM HCl (2), 80% (v/v) HFIP and 20 mM HCl (3), and 80% (v/v) DMSO and 20 mM HCl (4). Line (5) shows light scattering of β_2-m monomer in water. The fibril dissolution reaction was carried out at 20°C.

rescence values for β_2-m fibrils in the presence of various concentrations of HFIP, TFE, EtOH, and DMSO measured 30 min after the addition of the alcohols or DMSO. The fluorescence intensity of monomeric β_2-m was around 7.0, as low as the fluorescence intensity of ThT without proteins. The ThT fluorescence value of β_2-m fibrils in the absence of organic solvents was around 40. A slight increase in fluorescence intensity was observed upon the addition of a low concentration of HFIP. Then, ThT fluorescence decreased at 10% (v/v) HFIP and the transition ended at 20% (v/v) HFIP. The ThT value after the transition (*10*) was close to that (7) of monomeric β_2-m, although not the same.

For TFE, after a slight increase at low concentrations, ThT values decreased at above 20% (v/v) TFE and the transition ended at 50% (v/v) TFE. However, the values above 50% (v/v) TFE were around 20, apparently higher than that of monomeric β_2-m. The effects of EtOH monitored by ThT fluorescence was negligible except for a slight increase in ThT fluorescence below 10% (v/v) EtOH.

The ThT fluorescence of β_2-m fibrils in the presence of various concentrations of DMSO is shown in Fig. 4. At concentrations up to 40% (v/v), DMSO produced no notable effects. At DMSO concentrations higher than 50% (v/v), the ThT values gradually decreased. At 80% (v/v) DMSO, the ThT value was similar to that of monomeric β_2-m, confirming that the dissolution was complete.

Light Scattering—Light scattering was used to monitor the time course of the dissolution reaction (Fig. 5). Since the size of monomers is very small relative to fibrils, a large decrease in light scattering intensity upon dissolution should be observed. The solutions were prepared carefully to prevent contamination by dust or other particles that could cause spike noise. Nevertheless, the noise level was higher than that of monomeric solutions. It is likely that the spike noise came from larger fibrils or clustered fibrils since it is difficult to prepare fibrils that are homogeneous in terms of size.

Light scattering in 80% (v/v) DMSO (line 4) decreased rapidly within the dead time of manual mixing to the level of the monomeric protein, indicating that high concentrations of DMSO dissolve amyloid fibrils completely. Light scattering in 80% (v/v) HFIP (line 3) also showed a

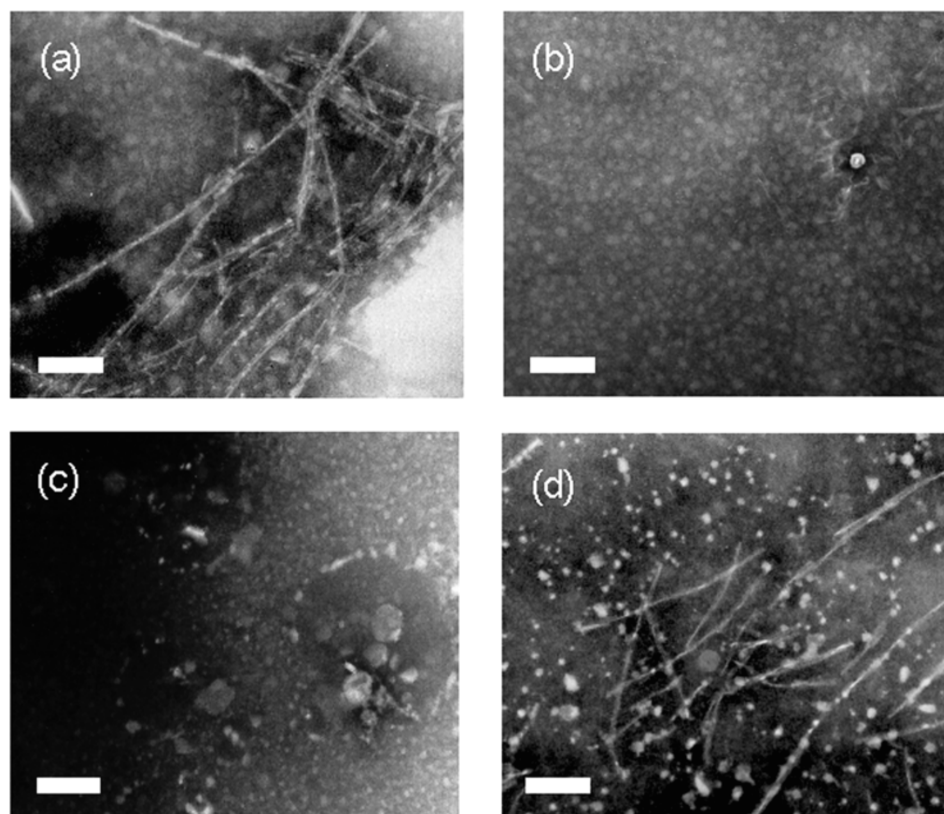

Fig. 6. **Electron micrographs of β_2-m amyloid fibrils.** Amyloid fibrils were added to 20 mM HCl (a), 80% (v/v) DMSO and 20 mM HCl (b), 80% (v/v) HFIP and 20 mM HCl (c), and 80% (v/v) TFE and 20 mM HCl (d). The bars indicate 200 nm.

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rapid decrease, followed by a slow decrease in intensity. However, it did not reach the level of monomeric proteins. The addition of 40% (v/v) TFE decreased the fluorescence value only by 20% compared to the original light scattering of the fibrils. Since 40% TFE is close to the concentration at which the transitions measured by ThT fluorescence or CD end, the results of light scattering indicate that the transition does not result in the complete depolymerization of amyloid fibrils, as suggested by the relatively high ThT value or intermediate CD intensity.

Electron Microscopy—The effects of alcohols and DMSO were examined by EM (Fig. 6). β_2-m amyloid fibrils prepared by the seed-dependent extension reaction were straight with a diameter of about 15 nm (Fig. 6a). After dissolution in 80% (v/v) DMSO, virtually no fibrils were observed, confirming that the dissolution process is complete (Fig. 6b). In the presence of 80% (v/v) HFIP, although no rigid fibrillar structure was observed, a variety of aggregates was often observed suggesting amorphous aggregates were induced by HFIP (Fig. 6c). In 80% (v/v) TFE, various amounts of fibrillar structures were observed, indicating that the dissolution by TFE is not complete (Fig. 6d). Amorphous aggregates were also observed in TFE.

DISCUSSION

Understanding the effects of DMSO on amyloid fibrils is important for various reasons. First, DMSO is used in clinical treatment (*35*, *36*). Treatment by ingestion, direct application to the skin or intravenous administration improves several clinical outcomes of amyloidosis, such as AL amyloidosis nephrosis, carpal tunnel syndrome, dermal amyloidosis and gastrointestinal symptoms. The mechanisms of the effects of DMSO in clinical treatment are not clear and any information concerning the mechanism may lead to better therapeutic methods. Second, as shown in our previous study (*20*), DMSO, $(CH_3)_2S{=}O$, without any exchangeable protons is a useful solvent for quenching the H/D exchange of amyloid fibrils with concomitant dissolution of the fibrils. This enables us to learn more about the elusive structure of amyloid fibrils. Studying amyloid fibrils is difficult owing their low solubility. Various solvents, including alcohols and DMSO, have been used to dissolve amyloid fibrils for further experiments. This study is unique in that it compares the effects of alcohols and DMSO on amyloid fibrils to elucidate the mechanism of dissolution.

The present study shows that, although the effect of DMSO is not so strong with respect to the concentration required for dissolution, it has the ability to completely dissolve β_2-m amyloid fibrils. In fact, we first expected that HFIP would be more effective than DMSO for dissolution because its ability to denature the native state and induce the helical conformation is much higher than that of DMSO. In accordance with this expectation, we observed the substantial dissolution of fibrils by HFIP when monitored by CD and ThT fluorescence. Nevertheless, light scattering indicated some remaining aggregates even at high concentrations of HFIP and EM showed some amorphous aggregates remaining in the mixtures. TFE, a commonly used solvent in studies of amyloid fibril and protein folding, was less effective in dissolving fibrils than HFIP. The CD transition suggested the formation of additional aggregates at high TFE concentrations. The ThT fluorescence value did not decrease to the level of monomers and light scattering at 40% (v/v) TFE decreased only slightly. EM showed remaining fibrils and unknown aggregates. It is likely that HFIP and TFE cannot completely dissolve very rigid fibrils and, moreover. may induce the formation of additional aggregates.

The difference between the effects of alcohols and DMSO might be interpreted in terms of their solvent properties. The perfluoroalcohols used here are considered to be non-polar solvents. The effects of these alcohols can be explained by their nonpolarity or hydrophobicity. By disrupting hydrophobic interactions, these alcohols denature the native state of proteins or dissociate protein aggregates and amyloid fibrils into smaller particles, or ultimately, into separate protein molecules. However, the strong nonpolarity of these solvents concomitantly strengthens hydrogen bonds, as revealed by helix formation in the presence of alcohol. The failure of these alcohols to completely dissolve amyloid fibrils might be the result of a strengthening of the hydrogen bond network in the fibrils. Moreover, it is known that this occasionally results in the formation of intermolecular hydrogen bonds, causing aggregation or amyloid fibril formation. The TFE-induced formation of amyloid fibrils has been reported by other groups (*5*, *37*).

On the other hand, DMSO is a polar solvent with a strong potential to become a proton acceptor (*38*). Its denaturing effect is mainly the result of the destruction of the hydrogen bond network, which is important for maintaining the native state of proteins as well as the suprastructure of amyloid fibrils. Thus, although high concentrations are required, DMSO does not stabilize or produce protein aggregates. Our previous study (*20*) indicates that the hydrogen bond network of the β-structure in fibrils is more extensive than that in the native globular state, conferring rigidity to amyloid fibrils. The observation that amyloid fibrils are completely dissolved by DMSO also reveals the importance of hydrogen bonds for the stability of amyloid fibrils.

In conclusion, HFIP or TFE can dissolve amyloid fibrils by weakening hydrophobic interactions. However, this effect is not sufficient to dissolve rigid fibrils. Moreover, depending on protein species and conditions, it is probable that the non-polar environment introduced by alcohols works adversely by strengthening the intermolecular hydrogen bonds, thus stabilizing amyloid fibrils or causing them to form amorphous aggregates. In contrast, DMSO, although the high concentration needed is a disadvantage, dissolves amyloid fibrils completely without such adverse effects.

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