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Mechanism for retardation of amyloid fibril formation by sugars in Vλ6 protein

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Abstract: Sugars, which function as osmolytes within cells, retard the amyloid fibril formation of the amyloidosis peptides and proteins. To examine the mechanism of this retardation in detail, we analyzed the effect of sugars (trehalose, sucrose, and glucose) on the polypeptide chains in 3Hmut Wil, which is formed by the mutation of three His residues in Wil mutant as a cause of amyloid light-chain (AL) amyloidosis, at pH 2, a pH condition under which 3Hmut Wil was almost denatured. Sugars caused the folding of 3Hmut Wil so that its polypeptide chains adopted a native-like rather than a denatured conformation, as suggested by tryptophan fluorescence, CD spectroscopy, and heteronuclear NMR. Furthermore, these sugars promoted the folding to a native-like conformation according to the effect of preferential hydration rather than direct interaction. However, the type of sugar had no effect on the elongation of amyloid fibrils. Therefore, it was concluded that sugar affected the thermodynamic stability of 3Hmut Wil but not the elongation of amyloid fibrils.

Keywords: Alzheimer's disease; AL amyloidosis; heteronuclear NMR analysis; sugar; preferential hydration

Introduction

Osmolytes are small organic solutes such as sugars, amino acids, methylamines, and polyol that are produced by the cells of most organisms, and serve to maintain the structure of cellular proteins during the exposure to various types of denaturing environmental stress.[1] They also significantly involved in several disorders, including protein conformational diseases, and many biological processes associated with protein misfolding/aggregation.[2] If an osmolyte could be shown to protect these proteins from conformational diseases, we could improve our understanding of the molecular relationships between osmolytes and conformational diseases.

Several sugars have been shown to inhibit the amyloid fibrillation of proteins.[3–5] Fibrillation of Aβ1-40 was depressed in the presence of 1.5*M* sucrose after incubating for 60 h under physiological conditions.[6] Moreover, a recent article showed that Aβ attenuated oligomer-induced membrane permeability in the presence of 250 m*M* sucrose.[7] The effects of sugars on proteins have been widely investigated.[8–11] In particular, sugars have been shown to stabilize proteins through preferential hydration.[9,12,13] It has been considered that sucrose may stabilize the native state of amyloid protein,

Abbreviations: AL, amyloid light-chain; Aβ, amyloid beta; CD, circular dichroism; HSQC, heteronuclear single quantum coherence; ThT, thioflavin T.

Additional Supporting Information may be found in the online version of this article.

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resulting in the inhibition of amyloid formation.[14] However, there is little information on the detailed mechanisms by which sugars retard the amyloid fibril formations of polypeptide chains.

Amyloid light-chain (AL) amyloidosis is a monoclonal plasma cell disorder associated with the overproduction and extracellular deposition of light chains such as Wil in the form of insoluble fibrils under physiological conditions.[15,16] We previously confirmed that the denatured structure of the pathogenic Wil was related with the formation of amyloid fibrils at pH 2, where the structure of the denatured state can be evaluated at the atomic level using heteronuclear NMR spectroscopy.[17] Moreover, we also found that 3Hmut Wil, in which His34, His53, and His93 are simultaneously mutated to Gln, Gln and Ser in Wil, respectively, formed amyloid fibrils earlier than Wil at pH 2.[18] Therefore, 3Hmut Wil is a convenient model to use in experiments investigating the retardation of amyloid fibril formation by sugars.

In this work, therefore, we investigated the effects of sugars on amyloid fibril formation of 3mut Wil at pH 2. The results showed that sugar stabilized the native protein by preferential hydration but did not inhibit fibril elongation, and thereby retarded the amyloid fibril formation.

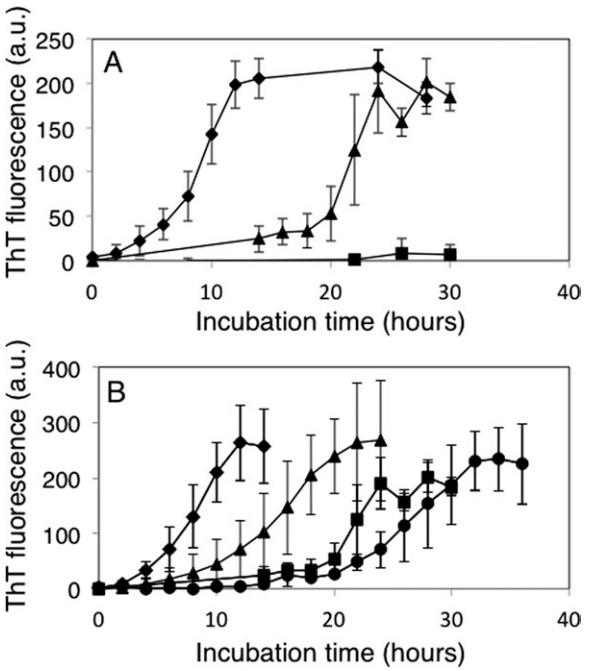

Figure 1. Amyloid fibrillation of the 3Hmut Wil protein (A) in the absence (filled diamonds) or presence of 0.5*M* (filled triangles) or 1.0*M* (filled squares) sucrose, and (B) in the absence (filled diamonds) or presence of 0.5*M* glucose (filled triangles), sucrose (filled squares), or trehalose (filled circles), at pH 2 and 37°C. Values are expressed as the means ± SE obtained for three experiments.

Results

Retardation of amyloid fibril deposits in 3Hmut Wil by sucrose at pH 2

ThT binds specifically to amyloid fibrils, and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the amount of amyloid formed. The use of ThT as a fluorescent marker is more specific than other methods, such as turbidity or sedimentation, for the semi-quantitative determination of amyloid-like aggregates.[19] As was previously reported, the amyloid fibril formation of 3Hmt Wil occurred at pH 2 and 37°C in the absence of sucrose.[18] In this study, with an increase in the concentration of sucrose, the formation of amyloid fibrils was drastically retarded, with the fibril formation being completely depressed in the presence of 1.0*M* sucrose under the conditions employed [Fig. 1(A)]. We confirmed that sucrose did not disturb the binding of ThT to the amyloid fibrils (Supporting Information Fig. S1) and that the pH was maintained under a high sugar concentration condition. These results indicate that sucrose retarded the amyloid fibril formation of 3Hmut Wil.

Unfolded 3Hmut Wil was refolded in the presence of sucrose

To examine the cause of amyloidosis retardation, we measured the state of the protein using fluorescence and CD spectroscopy. Fluorescence of tryptophan in 3Hmut Wil at pH 2 was measured in the absence or presence of sucrose [Fig. 2(A)]. In the absence of sucrose, the maximum wavelength of fluorescence was shown at 350 nm, indicating the unfolded conformation as described in the previous article.[18] With an increase in the sucrose concentration, we observed a quenching of fluorescence. Previous articles have suggested that Trp36 provides a convenient spectroscopic means for assessing the protein's conformational state.[17] The disulfide bond formed between Cys23 and Cys88 in the native state quenches the fluorescence of Trp36. Therefore, the fluorescence change suggested that unfolded 3Hmut Wil was refolded to the native-like conformation under a high sucrose concentration condition.

The CD spectra also provided a typical pattern for a random structure in the absence of sucrose (Fig. 3). However, with an increase in the sucrose concentration, the negative molar ellipticity around 200 nm was decreased, whereas we could show the negative 218 nm ellipticity under high sugar concentration condition, indicating a typical β-sheet-rich protein structure. The molar ellipticity of 3Hmut Wil in the presence of more than 1.5*M* sucrose was $\sim 4000^{\circ}\mathrm{cm}^2\ \mathrm{dmol}^{-1}$, which was similar to that of the folded rVλ6 proteins.[17]

Together, the CD and fluorescence results, suggested that the unfolded 3Hmut Wil at pH 2 changed to the native-like conformation by addition

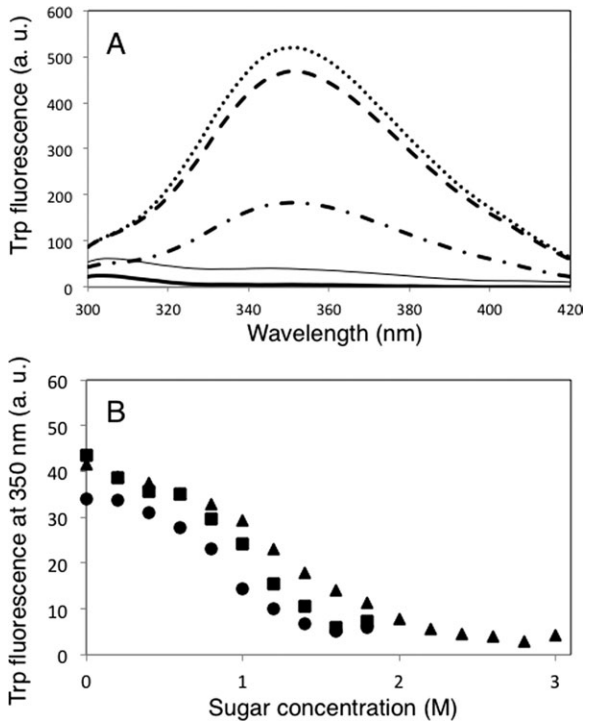

Figure 2. A: Tryptophan fluorescence spectra of 10 μ*M* 3Hmut Wil protein in the absence (dotted line) or presence of 0.5*M* (dashed line), 1.0*M* (dashed and dotted line), 1.5*M* (thin line), or 2.0*M* (thick line) sucrose at pH 2 and 37°C. Tryptophan fluorescence was excited at 280 nm. B: Sugar concentration-dependent fluorescence changes at 350 nm. One μ*M* 3Hmut Wil was analyzed at various concentrations of glucose (filled triangles), sucrose (filled squares), and trehalose (filled circles), respectively.

of a high concentration of sucrose. In the amyloidosis model, the denaturation of protein is a first step in the amyloid formation.[20–22] Therefore, we suspected that the sugar was induced to a native-like conformation of the polypeptide chain, resulting in the retardation of the amyloid fibril formation. In other words, the sugar shifts the thermodynamic equilibrium from a denatured state to a native-like one, and the less denatured state leads to the retardation of amyloid fibril formation.

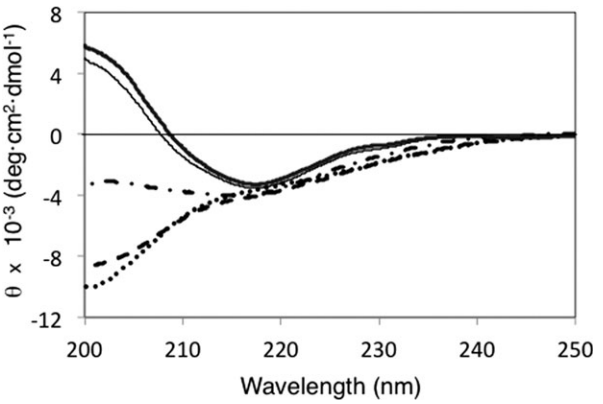

Figure 3. Far-UV CD spectra of the 3Hmut Wil protein in the absence (dotted line) or presence of 0.5*M* (dashed line), 1.0*M* (dashed and dotted line), 1.5*M* (thin line), or 2.0*M* (thick line) sucrose at pH 2 and 37°C.

Retardation of the amyloid fibrils by the other sugars

We further examined the effect of other sugars. Figure 1(B) shows the amyloid fibril formation in the presence of 0.5*M* glucose and trehalose. The results indicated that all sugars retarded the amyloid fibril formation. The fibrillation kinetics showed a quasi-sigmoidal behavior, consisting of a lag phase followed by a logarithmic growth phase. In the evaluation of a lag phase time of fibril formation in the presence of each sugar, glucose had less affect on retardation, whereas the effect of trehalose was a little greater or similar to that of sucrose. In the case of glucose, the protein was probably glycated, which in turn would have been responsible for amyloidosis.[23] Therefore, we examined the possibility of glycation of a protein by using MALDI-TOF MS of the final amyloid fibril in the presence of 0.5*M* glucose. However, protein glycation did not occur under the conditions employed here (data not shown).

We also examined the fluorescence spectra of Trp in 3Hmut Wil in the presence of trehalose and glucose. The Trp fluorescence in 3Hmut Wil was quenched by addition of glucose and trehalose, much as in the case of sucrose [Fig. 2(B)]. This suggested that the unfolded 3Hmut Wil was refolded to the native-like conformation in the presence of sugars. The decreases of fluorescence at 350 nm resulted in a typical sigmoid curve. Therefore, 3Hmut Wil possessed the two-state transition between the denatured and native-like conformation. From the sigmoidal curves, we calculated the efficient half concentration of each sugar in refolding to the native-like state. The concentrations were 0.9*M* (trehalose), 0.95*M* (sucrose), and 1.25*M* (glucose), respectively. The efficient half concentrations of trehalose were similar to or a little greater than those of sucrose, and that of glucose was smaller. This was the same order as for the efficient retardation of amyloid fibril formation.

Effects of sugars on the ^{1}H-^{15}N HSQC spectra of the denatured state of 3 Hmut Wil at pH 2

We next examined the state of 3Hmut Wil by analyzing the results of NMR spectrometry in detail. Dissolved 3Hmut Wil produced a well-dispersed ^{1}H-^{15}N HSQC spectrum just as in the previous results[16] [Fig. 4(A)]. Figure 4(B) shows the HSQC spectra of 3Hmut Wil at pH 2 in the presence of 0.5*M* sucrose. During NMR measurements, no aggregates were observed. Most of the cross peaks derived from amino acid residues in 3Hmut Wil were broadened, and the chemical shifts were slightly and equally shifted upfield in the presence of 0.5*M* sucrose [Fig. 4(D)].

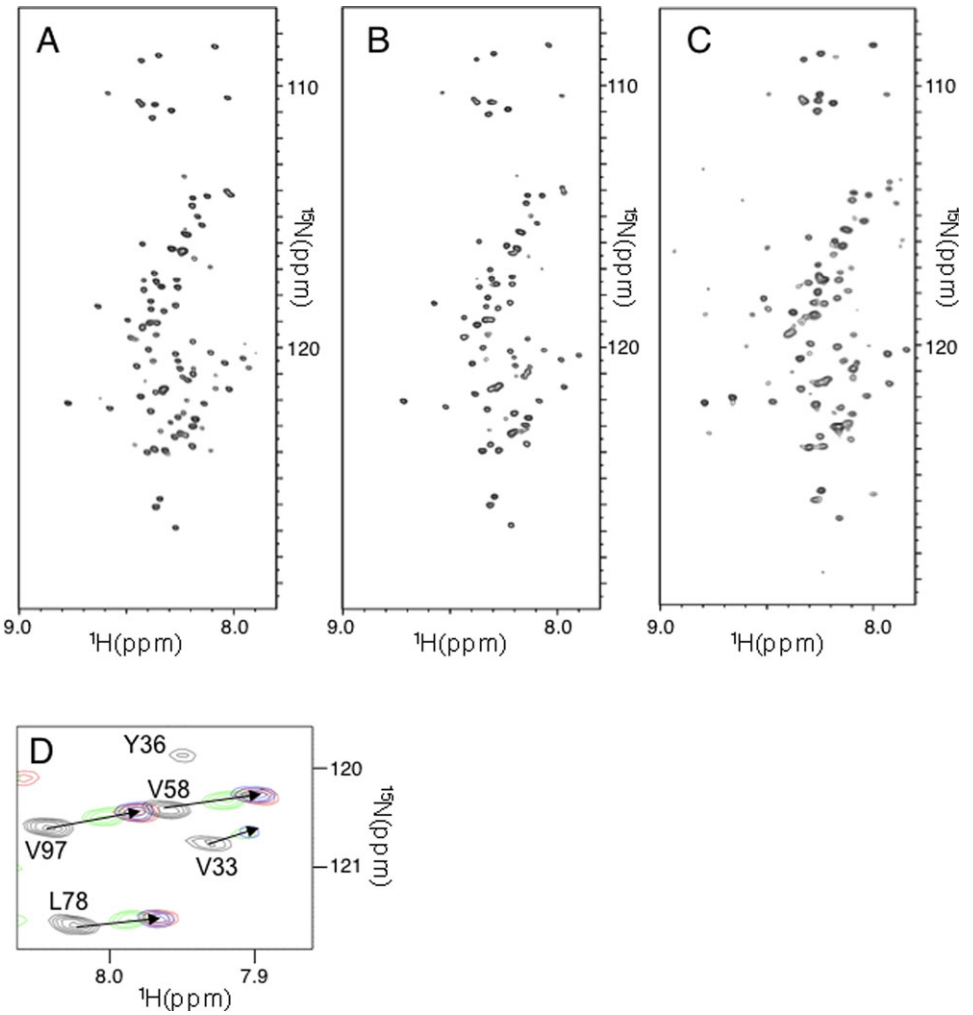

Figure 4. ^{1}H-^{15}N HSQC spectra of the 3Hmut Wil protein in the absence (A) or presence (B) of 0.5*M* sucrose, or (C) 1.0*M* sucrose at pH 2 in 90%H_2O/10%D_2O at 37°C. D: A part of the ^{1}H-^{15}N HSQC spectra in the absence (black) and presence of 0.5*M* glucose (green), sucrose (blue), and trehalose (red) were overlapped. Assignments in the absence of sugar are indicated in the figure.

However, the overall spectra were not very different [Fig. 4(A,B)]. Therefore, the above results indicate that sucrose exhibits almost no direct binding to the polypeptide chain of denatured 3Hmut Wil.

We also examined the chemical shift changes in the absence and presence of 0.5*M* glucose or 0.5*M* trehalose. The overall spectra were not very different from those of 0.5*M* sucrose [Supporting Information Fig. S2(A,B)]. The part of the spectrum in the presence or absence of each sugar is shown in Figure 4(D). The chemical shift changes of each residue in the presence and absence of each sugar are shown in Figure 5. We found that the overall patterns of chemical shift changes for each residue were similar, although the amounts of the individual chemical shift changes were different for each sugar. The averages of the chemical shift changes between the absence and presence of 0.5*M* sucrose, trehalose, and glucose were 0.064, 0.064, and 0.044 ppm, respectively. Interestingly, the effect of all sugars lessened the chemical shift change of the residues Thr24-Ser26, Trp35, Ile48, and Tyr91 (Fig. 5, indicated by arrowheads). The peak intensities of these residues were decreased in the presence of sugar. Additionally, considerable broadening occurred at Tyr36 in the presence of sugars.

However, we found several new dispersed peaks in the presence of more than 1*M* sucrose [Fig. 4(C)]. The peak pattern of the folded 3Hmut Wil at pH 4.5 was similar to the spectra in the presence of 1 or 1.5*M* sucrose [Supporting Information Fig. S3(B–D)], although the peaks broadened. Taken together, the fluorescence and CD results suggested that high concentration of sugar induced the native-like folded protein.

Sugars did not affect the fibril elongation process

In the presence of 1*M* sucrose, the amyloidosis was not proceeded more than 200 h [Fig. 6(A)]. When

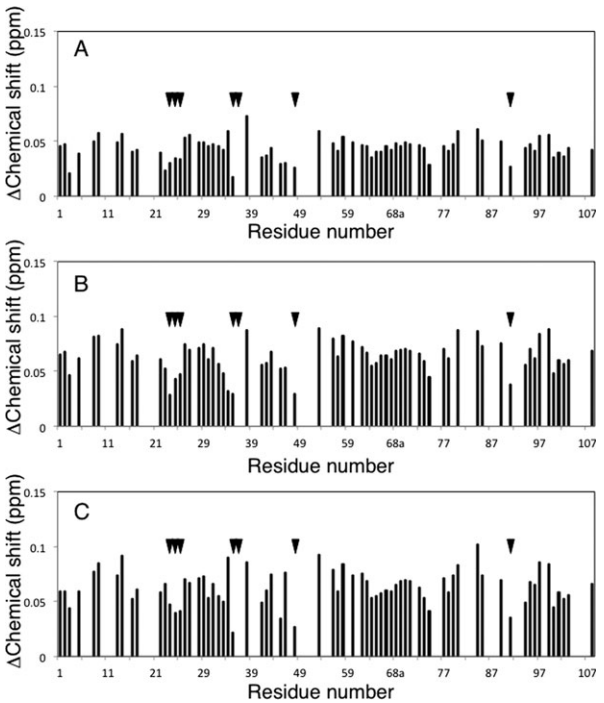

Figure 5. Differences in the chemical shift changes between the absence or presence of 0.5*M* glucose (A), sucrose (B), or trehalose (C), are displayed for the backbone amides as a function of sequence number. All chemical shifts changes in the ^{1}H-^{15}N HSQC spectra were calculated according to the formula $[\Delta\delta(^{1}H)^{2} + (\Delta\delta\ (^{15}N)/7)^{2}]^{1/2}$. The residues, which have small chemical shift changes or signal broadening, were shown by arrow heads.

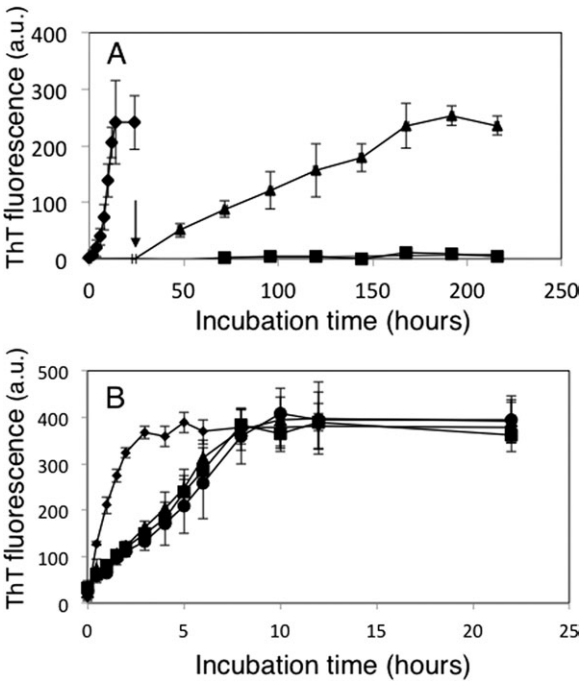

Figure 6. A: After incubation for 24 h in the presence of 1.0*M* sucrose, 1 μL of prepared amyloid seed was added, and then the ThT fluorescence was measured (filled triangles). The arrow indicates the time point of seed addition. As a control, amyloid fibrillation of the 3Hmut Wil protein in the absence (filled diamonds) and presence of 1.0*M* (filled squares) sucrose are also shown. B: Amyloid fibrillation of the 3Hmut Wil protein in the absence (filled diamonds) and presence of 1.25*M* glucose (filled triangles), 0.95*M* sucrose (filled squares), and 0.9*M* trehalose (filled circles), at pH 2 and 37°C. Ten microliters of prepared amyloid seed was added to each solution immediately before incubation.

amyloid seed was added to the sample solution after 24 h, the amyloid fibril was regrown even in the 1*M* sucrose solution [Fig. 6(A)]. Since a part of the protein was unfolded in the 1*M* sucrose solution, as suggested by the fluorescence, CD and NMR results, a seed core introduced the amyloid fibril formation. Therefore, it is suggested that sugar suppressed the amyloid fibril core formation by promoting the folding to native conformation in high sugar concentration solution. However, to examine the possibility that sugar was involved in the fibril elongation process, 3mut Wil was incubated in the presence of 0.9*M* trehalose, 0.95*M* sucrose, or 1.25*M* glucose and the results are shown in Figure 6(B). Each sugar concentration was determined by the efficient half concentration, which we calculated in the above section [Fig. 2(B)], because the thermodynamic equilibrium between the native-like and denatured state remains a constant 1:1. In addition to that condition, to enhance the core formation, the prepared seed was added to each solution immediately before incubation. The resulting amyloidosis formation is shown in Figure 6(B). The fibrillation kinetics was almost identical under all conditions irrespective of the type of sugar. It was suggested that sugar did not affect the amyloid elongation process.

Discussion

In this study, we examined the mechanism of the effects of sugar on amyloid fibril formation using 3Hmut Wil protein. ThT binding suggested that several sugars including sucrose retarded the amyloid fibril formation. Kim *et al*. have demonstrated that the amyloid fibril formation by light chains is predominantly controlled by thermodynamic stability, because sucrose retarded and urea accelerated the amyloid fibril formation of light chains.[14] However, since 3Hmut Wil is almost denatured at pH 2,[18] we examined the effects of the sugars trehalose, glucose, and sucrose on the denatured state of 3Hmut Wil. The fluorescence, CD and NMR results suggested that a high concentration of sugars induced a native-like conformation from the denatured state. These results clearly indicated that sugars stabilized the native state of 3mut Wil by considerably shifting the equilibrium from a denatured state to a native-like state. Thus, we conclude that the stabilization of the native state caused the retardation of amyloid fibril formation. It has been proposed that fibril

formation of the amyloid protein is initiated by protein destabilization.[20–22] Therefore, sugars prevented the destabilization step of the global structure of a protein, resulting in the retardation of amyloid fibril formation.

Sugar is well known to increase protein stability.[9,12] Namely, since sugars have the potential to be excluded from the surrounding proteins, more water molecules make up the shell around the protein surface at high sugar concentrations. It has been considered that this preferential hydration effect is the primary reason for the protein stabilization by sugars. Previous articles[9,12,13,24] have examined the potential for preferential hydration in individual sugars. The effect of the preferential hydration parameter of trehalose was a little greater or similar to that of sucrose, while that of glucose was smaller than that of sucrose, although it should be noted that there were some slight differences in the conditions employed in these articles.[9,12,13,24] Our results showed that the effective order for stabilization of the native-like state of a protein by sugars was trehalose, sucrose, and glucose, and amyloid fibril formation was retarded in the same order. The consistency of the order implied that the primary reason for stabilization of the native conformation of 3Hmut Wil was preferential hydration by sugar. Moreover, Figure 6(B) shows that the type of sugar did not affect the fibril elongation process, suggesting that direct sugar binding to protein could not inhibit the assembly of amyloid fibrils from protein monomers.

As for the effect of sugar on the polypeptide chain at the atomic level, we showed the effect of sugars on the chemical shifts of individual amino acid residues using heteronuclear NMR. Since the spectra patterns of ^{1}H-^{15}N HSQC in the presence and absence of 0.5*M* sugars were not very different, it was evident that the sugar reacted weakly or not at all with 3Hmut Wil in the denatured state [Fig. 4(A,B), Supporting Information Fig. S2]. However, the overall peaks shifted upfield in the presence of a high concentration of sugar. The averages of the NH chemical shifts were different depending on the type of sugar. The average chemical shifts for trehalose and sucrose were both 0.064 ppm, and that for glucose was lower at 0.044 ppm. Interestingly, this order was also consistent with those for stabilization of the native state, retardation of amyloid fibril formation and preferential hydration parameters. It may be that, when the concentration of water molecules surrounding the protein is increased by preferential hydration at a high sugar concentration, a chemical shift in the amide protons occurs because the water concentration affects the exchange between the water protons and amide protons. In fact, most of the chemical shifts of amide protons were upfield-shifted toward the water proton signal in the presence of sugar. However, since we obtained little information about the relationship between the amide proton shift and preferential hydration, further investigations will be needed using the other chemicals involved in preferential hydration.

Moreover, we found that the effect of sugars lessened the chemical shift change of the residues Thr24-Ser26, Trp35, Ile48, and Tyr91. These residues were assigned as the hydrophobic cluster in the denatured state of 3Hmut Wil.[18] The hydrophobic cluster in the denatured state of a protein was first identified by Schwalbe *et al*.[25] When the collapse formation of the hydrophobic cluster forms hydrogen bonds in the polypeptide, it is possible that the chemical shifts of amide protons in the protein are effectively influenced in the manner of temperature coefficients.[26] In this study, the surrounding waters by preferential hydration would be inaccessible to the residues involved in the collapse formation of the hydrophobic cluster in the denatured state of 3Hmut Wil. Thus, the chemical shift changes in a high sugar solution was small, and it might be a simple indicator of a hydrophobic cluster in the denatured state as well as relaxation time.[25] In a series of experiments using hen lysozymes, it was shown that hydrophobic clusters were deeply involved in not only the formation of amyloid fibril formation but also that of protein aggregation.[27–31] Therefore, the simple cluster-finding method might have an advantage for the further investigation of protein folding.

In this study, we demonstrated that sugar stabilized protein even in the denatured state at pH 2, resulting in the retardation of amyloid fibril formation. Their finding was consistent with our previous report that the global structure of the amyloidogenic Wil was less stable than that of the nonamyloidogenic Jto under physiological conditions.[17] Moreover, our results suggested that sugar did not directly interact with protein but indirectly influenced the polypeptide by preferential hydration. These results should be useful in terms of both suppression of amyloidosis and an improved understanding of the mechanisms of protein folding.

Materials and Methods

Materials

^{15}N-ammonium chloride was obtained from Shoko (Tokyo, Japan). ThT was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest quality commercially available.

Preparation of the 3Hmut Wil protein

The 3Hmut Wil protein including ^{15}N-labeled sample was prepared as described in our previous report.[18]

NMR experiments

For 3Hmut Wil, the NMR solution contained 0.1 m*M* protein concentration in HCl at pH 2 in 90% H_2O/10% D_2O. For the preparation of samples containing a high concentration of sugar (sucrose, trehalose, or glucose), the buffer was added to the sample solution in the absence of sugars. NMR experiments were performed at 37°C for 3Hmut Wil using a Varian Inova 600 MHz spectrometer equipped with a triple-resonance pulse-field gradient probe with an activity shielded *z* gradient and a gradient amplifier unit. Signals for 3Hmut Wil were assigned based on the previous reports.[18] The combined index of the chemical shift change of each amide cross-peak was calculated according to the equation, $[\Delta\delta(^1H)^2 + (\Delta\delta(^{15}N)/7)^2]^{1/2}$.

In vitro fibril formation assays

Fibrillation of 100 μ*M* 3Hmut Wil in HCl at pH 2 and 37°C was induced by constant stirring (100 rpm) using a Teflon-coated microstirrer bar (3 × 3 mm) in the absence or presence of 0.5 and 1.0*M* sucrose and the other sugars. Samples were incubated at 37°C in disposable tubes (12 × 75 mm). At various time points, a 5 μL aliquot was taken from each reaction tube and mixed with 995 μL of 20 μ*M* ThT in PBS (10 m*M* Na_2HPO_4 − 10 m*M* NaH_2PO_4 buffer containing 150 m*M* NaCl at pH 7.5). In vitro fibril formation was monitored by the fluorescence of ThT. The excitation wavelength was fixed at 450 nm and the emission was collected at 485 nm.

Preparation of amyloid fibril seeds

One hundred μ*M* 3Hmut Wil was incubated in HCl at pH 2 and 37°C. After the confirmation of amyloid fibril formation, the incubated sample was centrifuged at 15,000 rpm for 60 min. The supernatants were collected and stored at −20°C until analysis.

CD measurements

CD spectra for 3Hmut Wil in the absence or presence of sugar were collected with a J-720 spectropolarimeter (Jasco, Tokyo, Japan) between wavelengths of 200 and 250 nm. 3Hmut Wil was measured at pH 2 in the absence or presence of 0.5*M* sucrose at 37°C. All sample concentrations were 10 μ*M*.

Tryptophan fluorescence measurements

The fluorescence was measured using an FP-6200 fluorescence spectrophotometer (JASCO, Tokyo, Japan). The concentration of 3Hmut Wil was 1 or 10 μ*M* under various conditions. Tryptophan fluorescence was excited at 280 nm, and the emission spectrum was recorded at 300–420 nm.

Acknowledgments

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