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The Effect of Dehydration on the Aggregation Kinetics of Two Amyloid Peptides

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Abstract

It is well known that water plays a crucial role in the folding, dynamics and function of proteins. Here we provide further evidence showing that the aggregation kinetics of peptides also depend strongly on their hydration status. Using reverse micelles as a tool to modulate the accessible number of water molecules and infrared spectroscopy and transmission electron microscopy as means to monitor aggregate formation, we show that the rate of aggregation of two amyloid forming peptides increases significantly under conditions where limited hydration of the peptide molecule is expected to occur. These results are not only in accord with recent computer simulations indicating that the expulsion of interfacial water molecules is a key event in the dimerization/oligomerization of amyloid β (A β) peptides, but also have implications for amyloid formation *in vivo* where molecular crowding is expected to influence the solvation status of proteins.

Keywords

Aggregation; Amyloid Peptide; Dehydration; Infrared; Reverse Micelle

Introduction

Amyloidosis is associated with a variety of human pathological conditions.^{1,2} Thus, recent years have seen a remarkable increase in the number of studies aimed at understanding the molecular mechanism(s) underlying amyloid formation both *in vitro* and *in vivo* using various techniques.^{1–13} While previous studies have shown that various factors, including solvent, temperature, pH, concentration, and sequence, can significantly affect the rate of aggregation and amyloid formation,^{14–22} the precise role of water, the most abundant substance in the living cell, however, is yet to be fully ascertained. Herein, we attempt to assess the effect of dehydration, by varying the available number of water molecules, on the aggregation kinetics of two well-studied peptides, A β _{16–22} (sequence: KLVFFAE) and Sup35_{7–13} (sequence: GNNQQNY), which despite their short chain lengths have been shown to exhibit significant amyloidogenic propensity.^{23,24} In addition, both peptides form dehydrated oligomer/amyloid structures,^{24–28} implying that expulsion of water is an obligatory step along the course of aggregation and fibrillization.

In the current study, modulation of the amount of water accessible to the peptide molecules of interest is achieved by solubilizing them in the water pool of reverse micelles. The reverse micelles were prepared from sodium bis(2-ethylhexyl) sulfosuccinate (AOT), isooctane (IO) and water. Many studies have shown that the number of ‘free’ water molecules confined in the polar core of a specific AOT reverse micelle system is determined by its w_0 value (i.e., [water]/

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[AOT]).²⁹ For example, there are approximately 96, 473, and 4273 free water molecules available to interact with the solute molecules in the core of reverse micelles with w_0 values of 6, 10, and 20, respectively. Thus, the hydration status of an entrapped peptide can be tuned by simply varying the molar ratio of water to surfactant in the reverse micelle.³⁰ In addition, the use of AOT reverse micelles may also prove to be beneficial for the current study because the water confined in the micellar cores has often been proposed to mimic that found in the nanopores of cellular compartments.³¹ Our results show that both A β_{16-22} and Sup35 $_{7-13}$ peptides form soluble aggregates in AOT reverse micelles of low w_0 values at a rate that is much faster than that in bulk water.

Experimental Method

Materials

AOT and isooctane were purchased from Sigma Chemical Co. (St. Louis, MO). AOT was further purified by dissolving it in methanol and stirring the solution overnight in the presence of activated charcoal. Subsequent filtration and removal of methanol by distillation under vacuum yielded the AOT used in the current study. Isooctane was used without further purification. D₂O (D, 99.96%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Peptide synthesis and purification

A β_{16-22} and Sup35 $_{7-13}$ peptides were synthesized using the standard Fmoc-protocol and purified via reverse phase HPLC (Agilent 1100 Series). The identity of the samples was further verified by electrospray-ionization mass spectroscopy. Hydrogen to deuterium exchange as well as removal of residual trifluoroacetic acid from peptide synthesis were accomplished by two rounds of lyophilization against a 0.1 M DCl solution at a peptide concentration of ~50–75 μ M. The deuterated peptide was immediately used in the FTIR experiments.

Sample preparation

The purified AOT was dried under vacuum overnight before use. Then 50, 100, and 300 mM AOT/IO solutions were prepared by dissolving an appropriate mass of AOT in isooctane, which were used to prepare micelle solutions of $w_0 = 6, 10$ and 20, respectively. The resultant solutions were first vortexed for 3 minutes and then sonicated for 10 minutes in a bath sonicator, followed by stirring for one hour.³⁰ The reference solution used in the FTIR experiments with a desired w_0 value was prepared by adding an appropriate amount of D₂O to one of the above AOT/IO solutions. The resultant solution was further stirred for 30 minutes before use. Similarly, the peptide-micelle solution of a desired w_0 value was prepared by directly mixing appropriate amounts of the pre-prepared AOT/IO/D₂O solution and lyophilized peptide solid. This mixture was then incubated under room temperature for 30 minutes with slight vortexing every 10 minutes. Assuming that peptide incorporation does not affect the characteristic micellar distributions, a simple stoichiometric calculation indicates that the peptide to micelle ratio is about 1:5 for all of the AOT reverse micelles used, with the final peptide concentration being ~200 μ M in each case. The peptide/D₂O solution was prepared by directly dissolving lyophilized peptide solid in D₂O (pH* = 2, pH meter reading) and the peptide concentration is ~200 μ M. In all cases, the peptide concentration was determined optically by either the phenylalanine absorbance at 257.4 nm using $\epsilon = 197 \text{ M}^{-1} \text{ cm}^{-1}$ for A β_{16-22} or by the tyrosine absorbance at 276 nm using $\epsilon = 1450 \text{ M}^{-1} \text{ cm}^{-1}$ for Sup35 $_{7-13}$. Because of the small molar absorptivity of phenylalanine and also interference of the peptide backbone absorbance the reported concentrations for A β_{16-22} peptide are expected to have a large uncertainty and are in the range of 100–300 μ M.

FTIR measurements

FTIR spectra were collected on a Nicolet Magna-IR 860 spectrometer using 2 cm^{-1} resolution and at 25°C . A two-compartment sample cell was used in all experiments. For measurements in bulk D_2O , an optical path length of $100\text{ }\mu\text{m}$ was used, whereas for those in AOT reverse micelles, a longer optical path length ($390\text{ }\mu\text{m}$) was used.³⁰ For all the spectra measured in AOT reverse micelles, a background arising from imperfect subtraction of AOT signals was further estimated and subtracted. Because of such baseline correction, all reported spectra should be viewed as only semi-quantitative.

Circular dichroism (CD) measurements

Far-UV CD data were collected on an Aviv 62A DS circular dichroism spectrometer (Aviv Associate, NJ) using a 1 mm sample cuvette. Mean residue ellipticity was calculated using the equation $[\theta] = (\theta_{\text{obs}}/10lc)/N$, where θ_{obs} is the ellipticity in millidegrees, l is the optical path length in cm, c is the concentration of the peptide in M, and N is the number of residues.

Transmission electron microscopy (TEM) measurements

TEM images were recorded using a JEOL JEM-1010 electron microscope operating at 80 kV and equipped with a Hamamatsu ORCA digital camera using AMT Advantage image capture software. Samples used for TEM experiments are identical to those used in the corresponding IR measurements (i.e., $\text{A}\beta_{16-22}$ + AOT reverse micelle solution of $w_0 = 6$). TEM samples were prepared by first depositing an aliquot ($\sim 5\text{ }\mu\text{L}$) of the $\text{A}\beta_{16-22}$ -AOT reverse micelle solution of $w_0 = 6$, which has been incubated for either 24 hours or 9 days, to Formvar coated 300 mesh copper grids. These grids were then negatively stained with 2% uranyl acetate in water. Any excess fluid was blotted off immediately. The grids were then allowed to dry in air for 10 minutes. Grids used for collecting the image of the reference solution (i.e., the AOT reverse micelle solution of $w_0 = 6$) were prepared identically.

Results and Discussion

Aggregate formation was monitored by the amide I' band of the peptides because of its proven sensitivity to conformational changes. As shown (Figure 1a), the amide I' bands of $\text{A}\beta_{16-22}$ and Sup35_{7-13} in bulk D_2O ($\text{pH}^* = 2$), collected 1 hour after the peptide solution was prepared, show typical characteristics of disordered peptides.³² Consistent with this observation, far-UV CD measurements also show that these peptides are predominantly unstructured in solution (Figure 1b). In addition, these spectra (both CD and IR) did not show any appreciable changes even after three days of incubation (data not shown), indicating that when fully hydrated these peptides aggregate rather slowly under the current experimental conditions.

However, incorporation of $\text{A}\beta_{16-22}$ and Sup35_{7-13} in the D_2O pool of AOT reverse micelles of $w_0 = 6$ leads to a drastic enhancement in their aggregation rate. As shown (Figure 2), the amide I' band of $\text{A}\beta_{16-22}$, also collected 1 hour after the peptide sample was prepared, is now characterized by spectral features of soluble peptide aggregates rich in antiparallel β -sheets, 23,25,32–35 namely, the sharp bands centered at ~ 1620 and $\sim 1687\text{ cm}^{-1}$ respectively, indicating that significant peptide aggregation has occurred within the dead time (ca. 1 hour) of the current method. Similarly, the amide I' band of the Sup35_{7-13} peptide (Figure 2), obtained under the same conditions, indicates that it also aggregates at a rate that is too fast to be resolved by the current method. However, the soluble aggregates formed by Sup35_{7-13} are instead rich in parallel β -sheets, as evident from the lack of the characteristic high-frequency component (i.e., the peak around $\sim 1687\text{ cm}^{-1}$) of antiparallel β -sheets in its amide I' band. These results are consistent with several previous studies,^{23–25,27,33–36} showing that the fundamental spectral characteristics of the corresponding aggregation process of these peptides remain unaltered in the presence of AOT reverse micelles. While many factors have been shown to

affect the rate of peptide or protein aggregation (see below),^{14–22} the current results are nonetheless interesting in that they were obtained in an environment wherein only about 96 free water molecules are available per micellar core.³⁷ Thus, it is expected that the hydration status of the entrapped peptide molecule is significantly different from that in bulk water,^{30, 38} leading to changes in its physical and/or chemical properties. A similar idea has also been put forward based on the modulation of water activity in a confined environment.³⁹ Hence, we propose that the observed increase in the rate of peptide aggregation in AOT reverse micelles arises primarily from dehydration or limited hydration of the peptide molecules of interest.

If the above assertion of kinetic enhancement based on the assumption of dehydration is valid, then one would expect the aggregation rate of these peptides to decrease with an increase in the number of free water molecules inside the polar core of the reverse micelles (i.e., an increase in the w_0 value).³⁷ Indeed, the aggregation rate of Sup35_{7–13} in AOT reverse micelles of $w_0 = 10$, as judged by the evolution of its amide I' band as a function of incubation time (Figure 3), becomes slower. These data clearly show that unlike that of $w_0 = 6$, no detectable aggregation has occurred 1 hour after the peptide sample was prepared, with the detectable sign of aggregation starting to develop only after ~6 hours of incubation, as judged by the appearance of the characteristic aggregation band centered at $\sim 1624\text{ cm}^{-1}$. The latter becomes the dominant spectral feature after 24 hours of incubation, indicating that at this time a significant portion of the peptide molecules has assembled into soluble aggregates. Further incubation of this sample for an extended period of time led to the formation of insoluble aggregates, indicative of the dynamic nature of this system.²⁹ As expected, increasing the w_0 value to 20 suppresses the rate of Sup35_{7–13} aggregation to a further extent (Figure 4). It is evident in this case that even after 24 hours of incubation the AOT-peptide sample does not show detectable aggregates. Interestingly, however, the amide I' band of A β _{16–22} in AOT reverse micelles of $w_0 = 10$ (Figure 3) is very similar to that obtained at $w_0 = 6$, indicating that under such conditions aggregation has already taken place to an appreciable extent during the dead time of the current method. On the other hand, the amide I' band of A β _{16–22} in AOT reverse micelles of $w_0 = 20$, also measured after 1 hour of incubation, is dominated by spectral features of disordered peptide monomers (Figure 4), thereby suggesting significant alleviation of peptide aggregation under conditions where the peptide molecules are expected to be extensively hydrated. However, after 24 hours of incubation, an appreciable amount of A β _{16–22} peptide was found to form insoluble aggregates that remained suspended in solution and thus, no further attempt was made to measure its FTIR spectrum under this condition.

Taken together, the above results indeed support the idea that the degree of hydration is an important determinant of the rate of peptide aggregation, and that the actual effect of dehydration on aggregation depends on the sequence. For example, in reverse micelles of $w_0 = 10$, A β _{16–22} aggregates faster than Sup35_{7–13}. Previous studies have shown that the major driving force for the oligomerization of A β _{16–22}, which contains the crucial central hydrophobic cluster⁴⁰ essential for the polymerization of the full length peptide,⁴¹ is hydrophobic interaction,²³ whereas the aggregates formed by the hydrophilic peptide Sup35_{7–13} are primarily stabilized by interstrand backbone-backbone and sidechain-sidechain hydrogen bonds.^{24,26,27,36,42} Therefore, it is not surprising that A β _{16–22} exhibits a faster aggregation rate than Sup35_{7–13} under conditions wherein only limited hydration is possible. This is because under such conditions most of the available water molecules are involved in preferential solvation of the polar or charged groups, leaving the hydrophobic sidechains relatively dehydrated.^{38,43} As a result, the free energy barrier encountered in peptide-peptide association via the mechanism of hydrophobic interaction is expected to be greatly reduced for A β _{16–22}.

Furthermore, it is well established that the rate of peptide aggregation is highly concentration dependent.⁴⁴ Thus, the first concern in the interpretation of the current data is that whether the observed enhancement in the aggregation rate of both peptides is due to an incidental increase in local peptide concentration as a result of confinement in the water pool of the reverse micelles. In order to minimize this possibility, all AOT experiments were carried out under conditions where the ratio of micellar core to peptide was maintained at 5:1. In other words, the percentage of micelles initially containing more than one peptide molecule is fairly small. Thus, the faster aggregation rate of these peptides in AOT reverse micelles, compared to that in bulk water, is unlikely to result from the concentration effect. In addition, previous studies have shown that interactions with lipid membranes play an important role in the aggregation of a number of amyloidogenic peptides.^{45–47} Thus, the second concern is whether the enhanced aggregation rate we observe in the current case arises from interactions of these two peptides with the surfactant-water interface of the reverse micelle. While we cannot entirely rule out this possibility, the overall enhancement is unlikely to have resulted solely from such interactions because the rate of aggregation for both peptides changes as a function of w_0 value. In fact, such interactions in the current case may even slow down aggregation by preventing peptide molecules to diffuse through different water pools, an event necessary for self-association. Based on these arguments and the data acquired at different w_0 values, we therefore attribute the drastically enhanced aggregation rate observed for both A β_{16-22} and Sup35 $_{7-13}$ in AOT reverse micelles primarily to peptide dehydration. Consistent with this picture, several earlier studies^{24,35} have suggested that peptide self-association encounters a substantial desolvation free energy barrier. Of particular interest is a recent MD simulation study wherein water expulsion has been associated with the formation of protofilaments of aggregating A β_{16-22} peptides.⁴⁸ Indeed, dewetting is a common phenomenon observed in the self-association of hydrophobic surfaces and biological molecules.^{49–52} Moreover, it is worth mentioning that the above results also indicate that these reverse micelles must be dynamic in nature,²⁹ allowing the entrapped solute molecules to move from one polar core to another so that peptide-peptide association or aggregation can take place.

While the current study focuses mainly on probing the effects of dehydration on the early stages of the kinetics of peptide aggregation, to confirm that the peptide aggregates formed in AOT reverse micelles can further assemble into larger oligomeric structures we have also performed TEM measurements on the A β_{16-22} -AOT reverse micelle system. As shown (Figure 5), the transmission electron micrograph of negatively stained deposits obtained from A β_{16-22} -AOT reverse micelle solution of $w_0 = 6$, which has been incubated for either 24 hours or 9 days, revealed annular aggregates whereas such structures were distinctly absent from the micrograph obtained from the reference AOT reverse micelle solution (Figure 5c). Such annular-like structures have also been observed in other amyloidogenic systems (e.g., A β peptides, α -synuclein, human hormone calcitonin, immunoglobulin light chain, and ABri peptides).^{53–57} Thus, this result not only substantiates the notion that in AOT reverse micelles of low w_0 value the aggregation rate of A β_{16-22} is enhanced, but it also suggests that AOT reverse micelles may play an important role in controlling the morphology of the aggregates formed in such systems.

The aggregation results obtained in D₂O and AOT reverse micelles of different water contents combined thus provide strong evidence indicating that the degree/extent of hydration plays an important but often understated role in the kinetics of aggregate formation of amyloid peptides, a notion that so far has only been implied in other studies. For example, it has been shown that amyloid fibrils formed from the sequential stacking of β -sheets are primarily anhydrous²⁴ in nature, suggesting that expulsion of water molecules is an obligatory step during aggregate formation and subsequent fibrillization. In particular, a recent molecular dynamics study⁵⁸ on A β_{10-35} has shown that formation of contacts between hydrophobic clusters of aggregating peptides encounters an appreciable free energy barrier arising from the removal of water

molecules solvating the interacting interfaces/domains. Additionally, differences in spatial distribution of solvent (water) molecules around a protein can influence its interaction with other proteins and/or self-association.^{59,60} Moreover, studies involving the diastereomeric assembly of polylysines⁶¹ and the effect of osmolytes⁶² on the morphology of aggregates and the amyloidogenic pathways of the A β peptide also suggest that hydration can indeed be a key factor in controlling the thermodynamics as well as the kinetics of such processes, even though the aggregation process in reverse micelles could be mechanistically quite different from that in aqueous solutions.

Conclusion

In summary, we have studied the aggregation properties of two amyloidogenic peptides, A β _{16–22} and Sup35_{7–13}, in D₂O and AOT reverse micelles of different w_0 values. Our results show that the aggregation rates of these peptides are greatly enhanced when solubilized in the water pool of AOT reverse micelles of $w_0 = 6$ and 10 wherein the number of free water molecules interacting with the peptide molecules are quite limited. Thus, these experimental results provide strong indication that dehydration promotes aggregate formation by reducing the free energy barrier arising from desolvation or removal of water molecules from the interfacial region. In addition, the current results have strong implication for aggregate and amyloid formations *in vivo* (or in the cell) where crowding is expected to reduce the local density of water molecules.

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Figure 1.
The amide I' bands (a) and CD spectra (b) of Aβ₁₆₋₂₂ (blue) and Sup35₇₋₁₃ (red) peptides in D₂O.

Figure 2. The amide I' bands of Aβ₁₆₋₂₂ (blue) and Sup357-13 (red) peptides in AOT reverse micelles of $w_0 = 6$. These data were collected after 1 hour of sample incubation.

Figure 3.

The amide I' bands of Sup35₇₋₁₃ in AOT reverse micelles of $w_0 = 10$ collected after different incubation times, i.e., 1 hour (pink), 6 hours (green), and 24 hours (red). Also shown in blue is the amide I' band ($\times 5$) of A β ₁₆₋₂₂ in AOT reverse micelles of $w_0 = 10$, collected after 1 hour of incubation.

Figure 4.

The amide I' bands of Sup35₇₋₁₃ (red) and Aβ₁₆₋₂₂ (blue) peptides in AOT reverse micelles of $w_0 = 20$. The Sup35₇₋₁₃ spectrum was collected after 24 hours of incubation, while that of Aβ₁₆₋₂₂ was measured after 1 hour of incubation.

Figure 5. TEM images of the $A\beta_{16-22}$ -AOT reverse micelle sample at $w_0 = 6$ after (a) 24 hours incubation and (b) 9 days incubation. Also shown in (c) is the TEM image of the AOT reverse micelle solution of $w_0 = 6$.