Phosphorylation Interferes with Maturation of Amyloid-β Fibrillar Structure in the N Terminus*

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Neurodegeneration is characterized by the ubiquitous presence of modifications in protein deposits. Despite their potential significance in the initiation and progression of neurodegenerative diseases, the effects of posttranslational modifications on the molecular properties of protein aggregates are largely unknown. Here, we study the Alzheimer disease-related amyloid-β (Aβ) peptide and investigate how phosphorylation at serine 8 affects the structure of Aβ aggregates. Serine 8 is shown to be located in a region of high conformational flexibility in monomeric Aβ, which upon phosphorylation undergoes changes in local conformational dynamics. Using hydrogen-deuterium exchange NMR and fluorescence quenching techniques, we demonstrate that Aβ phosphorylation at serine 8 causes structural changes in the N-terminal region of Aβ aggregates in favor of less compact conformations. Structural changes induced by serine 8 phosphorylation can provide a mechanistic link between phosphorylation and other biological events that involve the N-terminal region of Aβ aggregates. Our data therefore support an important role of posttranslational modifications in the structural polymorphism of amyloid aggregates and their modulatory effect on neurodegeneration.

Aggregation of the amyloid-β (Aβ) peptide into oligomers, protofibrils, and amyloid fibrils is the pathological hallmark of Alzheimer disease (AD) (1). Neurotoxic protein aggregation is often characterized by high levels of posttranslational modifications (2, 3). A particularly abundant modification in amyloid deposits is phosphorylation (4). For example, the Tau protein found in intracellular neurofibrillary tangles of AD brains is hyperphosphorylated, and in Lewy bodies in Parkinson disease brains, a large fraction of α-synuclein is phosphorylated at serine 129 (4). In the case of Aβ, it has been shown that phosphorylation at Ser5 is particularly abundant in intraneuronal deposits at very early stages of AD (5), whereas phosphorylation at Ser8 is detected in the later stages of the disease (6).

Several aspects of protein activity are influenced by phosphorylation of neurodegeneration-related proteins (4). It has been shown that phosphorylation of the Parkinson disease-related α-synuclein alters its interactions with other proteins (7) and membrane lipids (8) and plays a role in regulating its subcellular localization (9) and degradation (10–12). In the case of Aβ, phosphorylation at Ser7 attenuates its proteolytic degradation by certain proteases (13). It has also been suggested that the Ser8 phosphorylation level defines the biochemical stage of Aβ aggregate maturation, which is associated with the conversion from preclinical to symptomatic AD (6, 14). Despite its mechanistic importance, little is known on the effect of phosphorylation on the molecular properties of protein aggregates, especially their structure.

Progress over the past decade has provided substantial insight into the structure of Aβ fibrils (15, 16). In most models, Aβ molecules adopt a U-shaped β-strand-turn-β-strand fold. Despite the fact that the N-terminal region of Aβ is mainly unstructured in these fibrillar models, several lines of evidence point to an important role of this region in pathogenic aggregation. In particular, it has been shown that toxic Aβ assembly is enhanced by mutations in this region (A2V, H6R, or D7N) (17, 18) as well as N-terminal truncation with or without pyroglutamate formation (19, 20). Furthermore, antibodies against the N-terminal region inhibit Aβ amyloid formation (21), and the conformational propensity of the N-terminal region controls the partitioning between Aβ oligomers and protofibrils (22). In addition, phosphorylation at Ser8 promotes toxic Aβ aggregation both in vitro and in vivo (23) and increases the stability of Aβ aggregates against pressure- and SDS-induced dissociation into monomers (24).

Because of their putative role in the initiation and spreading of neurodegenerative diseases and their potential as therapeutic targets, there is growing interest in elucidating the effects of posttranslational modifications on the molecular properties of protein aggregates (4). In the present study, we used hydrogen-deuterium exchange coupled with solution-state NMR techniques and demonstrated that phosphorylation of Ser8 favors more exposed conformations in the N-terminal region of Aβ in insoluble aggregates. Our data support an important role of posttranslational modifications in the structural polymorphism of amyloid protein deposits and suggest a modulatory role in the course and severity of neurodegenerative diseases.
**Results**

Monomeric Aβ Remains Disordered after Ser^8^ Phosphorylation—First, we investigated the structural features of non-aggregated Aβ by CD and NMR. The far-UV CD spectra of both non-phosphorylated Aβ1–40 (npAβ) and Ser^8^-phosphorylated Aβ1–40 (pS8Aβ) were indicative of largely disordered conformations as manifested in prominent negative minima at ~199 nm and weak shoulders at ~218 nm (Fig. 1). The one-dimensional proton NMR spectra of the two peptides showed poor resonance dispersion in the amide and methyl regions, further supporting their lack of ordered structure (Fig. 2, A and B). Minor differences between the two spectra were observed in the amide and aromatic regions, including the expected downfield signal that originated from the amide proton of phosphoserine (at ~9.25 ppm) and two other peaks from the backbone amide proton of Tyr^10^ and the side chain ε1 proton of His^6^, both in the vicinity of the phosphorylated residue (Fig. 2A). In pulsed field gradient NMR experiments, the two peptides showed identical decay curves of NMR signal intensity, indicating that they had the same apparent diffusion coefficient of 8.27 × 10^{-7} cm²·s⁻¹ at 5 °C and thus the same assembly state (Fig. 2C). A calculated hydrodynamic radius of ~1.6 nm demonstrated that the NMR-visible states of the two peptides were mainly monomeric.

Serine 8 Undergoes Extensive Conformational Dynamics in Monomeric Aβ—NMR chemical shifts are sensitive probes of local conformational dynamics in polypeptide chains. The random coil index (25) calculated from backbone chemical shifts (HN, HA, N, CA, and CO) of npAβ reveals that serine 8 is located in a region of high conformational flexibility in the N-terminal region of Aβ that extends down to residue Glu^11^ (Fig. 3). Interestingly, Ser^26^, the second potential site of Aβ phosphorylation, occurs in a region of high mobility too, although the mobility level of these two regions is quantitatively different. It has been shown that phosphorylation of Ser^26^ results in a significant loss of conformational plasticity in Aβ that is otherwise essential for the progression of Aβ aggregation toward fibrils (26). It is therefore important to see how Ser^8^ phosphorylation may influence the local conformational dynamics in Aβ.

Phosphorylation at Ser^8^ Alters Local Conformational Dynamics of Aβ—The HN and HA chemical shifts obtained by two-dimensional homonuclear experiments showed that the backbone conformation of npAβ and pS8Aβ differs mainly around the phosphorylation site. The largest chemical shift

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**FIGURE 1.** Both wild-type and Ser^8^-phosphorylated Aβ are largely disordered in the monomeric state. Far-UV CD spectra of freshly dissolved (“monomeric”) states of both npAβ and pS8Aβ show spectral features that are characteristic for random coil conformation. mdeg, millidegrees.

**FIGURE 2.** Aβ peptide is in an unstructured monomeric state independent of the phosphorylation state at Ser^8^. A and B, one-dimensional ^1^H NMR spectra of npAβ (black) and pS8Aβ (gray) in the amide/aromatic (A) and aliphatic/methyl (B) regions, respectively. The low chemical shift dispersion of amide and methyl proton resonances is in agreement with the unstructured nature of the two peptides. Marked (*) peaks originate from the amide proton of phosphorylated Ser^8^, the amide proton of Tyr^10^, and the ε1 proton of His^6^, both in the vicinity of the phosphorylated residue (Fig. 2A). In pulsed field gradient NMR experiments, the two peptides showed identical decay curves of NMR signal intensity, indicating that they had the same apparent diffusion coefficient of 8.27 × 10^{-7} cm²·s⁻¹ at 5 °C and thus the same assembly state.
deviations were observed at Ser⁸ and Asp⁷ followed by residues 4–16 (Fig. 4A). The amide proton of His⁶ was not detectable in npA/H9252, probably due to severe exchange broadening, but with pS8A/H9252, a strong His⁶ HA-HN correlation peak was observed. The signs of HA shift changes were opposite for residues preceding and following Ser⁸: Phe⁴, Asp⁷, and Ser⁸ rose in HA shift, whereas residues 9–12 fell (Fig. 4A, inset). This suggests a differential influence of phosphorylation on these residues: the conformation of the N-terminal residues gets more extended, whereas succeeding residues tend to become less extended. The latter is in line with enhanced HN(i)-HN(i+1) NOE peaks of residues Gly⁹–Glu¹¹ in pS8A/H9252 (Fig. 4, B and C). The side chain resonances of Asp⁷ and Tyr¹⁰ provided further support for the differential impact of Ser⁸ phosphorylation on the structural dynamics downstream and upstream of the phosphorylation site. The two HB resonances of Asp⁷, unresolved in npA/H9252, became well separated following phosphorylation, whereas the two well resolved HB resonances of Tyr¹⁰ in npA/H9252 lost their dispersion upon phosphorylation (Fig. 5). Notably, a significant increase in the intensity of sequential NOE peaks between the amide protons of residues Asp²³–Gly²⁸ and Gly³⁰–Ala³¹ was also observed, suggesting that a long range conformational change may occur after Ser⁸ phosphorylation. Together, our data show that monomeric Aβ preserves its disordered structure in the Ser⁸-phosphorylated state with structural changes mainly induced around the site of phosphorylation.

**Phosphorylation at Ser⁸ Decreases Compaction of Aβ Aggregates in the N-terminal Region**—Next, the effect of Ser⁸ phosphorylation on the structure of aggregated Aβ was investigated. First, a control experiment confirmed that pS8A/H9252 possessed a higher rate and amount of thioflavin T-reactive aggregation than npA/H9252, in line with previous reports (data not shown) (23). Then we used hydrogen-deuterium exchange coupled to solution-state NMR to provide high resolution information on the backbone structure of Aβ aggregates dependent on Ser⁸ phosphorylation. After 7 days of aggregation (37 °C with gentle stirring), which led to formation of amyloid fibrillar aggregates, the

**FIGURE 3.** Serine 8 is located in a region of high conformational flexibility in the N-terminal region of Aβ. The random coil index (RCI) and random coil index-based squared order parameter (S²), derived from the backbone chemical shifts of Aβ, are shown. Serine 8, marked by an asterisk, along with residues Gly⁹–Glu¹¹, exhibit higher mobility than the flanking residues Arg⁵–Asp⁷ and Val¹²–His¹⁴.

**FIGURE 4.** Effect of Ser⁸ phosphorylation on the structure of monomeric Aβ. A, combined HA and HN chemical shift perturbation (CSP) indicates conformational changes around the phosphorylation site. In the inset, the HA chemical shift perturbations are shown. B, superposition of two-dimensional ¹H-¹H NOESY spectra of npA/H9252 (red) and pS8A/H9252 (green) showing some of the sequential HN-HN NOE peaks. C, intensity of HN-HN NOE peaks shown in B after calibration with respect to the NOE peak between Gln¹⁵ side chain proton. Higher intensities of sequential HN-HN peaks for Gly³⁰–Glu¹¹ support a phosphorylation-induced decrease of extended conformation for residues C-terminal to the phosphorylation site. Error bars represent S.D. and were estimated on the basis of the signal-to-noise ratio in the spectra.
insoluble Aβ aggregates were spun down by ultracentrifugation and then resuspended in pure D2O in a way that unprotected protons could be exchanged with deuterons. Then, after a second run of ultracentrifugation and following dissociation of the pellet with 5% dichloroacetic acid, 95% DMSO, we obtained two-dimensional 1H,1H TOCSY spectra and evaluated the intensity of cross-peaks between backbone amide protons and non-exchangeable aliphatic protons. For residues 15–40, the ratio of cross-peak intensity between pS8Aβ and npAβ was 0.54 ± 0.03 (Fig. 6A). Because a similar ratio of ~0.55 was observed for the intensity of non-exchangeable methyl resonances, the lower cross-peak intensity of residues 15–40 is attributed to a lower amount of insoluble aggregates formed by pS8Aβ when compared with npAβ. The prominent drop observed in the intensity ratio of the N-terminal residues indicates a higher solvent exposure of this region in pS8Aβ aggregates. In one-dimensional CLEANEX NMR experiments, monomeric pS8Aβ shows a smaller collective water-amide proton exchange rate than npAβ, excluding the possibility that the intensity profile of the hydrogen-deuterium exchange NMR data (shown in A) arises because of a phosphorylation-induced increase in the intrinsic exchange rates. Error bars represent S.D. and were estimated on the basis of the signal-to-noise ratio of the spectra.
rate constants of 26.0 ± 2.5 and 20.5 ± 2.9 s⁻¹ were obtained for npAβ and pS8Aβ, respectively. Because the intrinsic exchange rate in pS8Aβ is not larger than in npAβ, the possibility that the enhanced efficiency of hydrogen-deuterium exchange in pS8Aβ arises because of its higher intrinsic exchange rate is excluded. Our data thus demonstrate that the peptide backbone in the N-terminal region is more solvent-exposed in insoluble pS8Aβ aggregates than in aggregates of npAβ.

To further investigate the solvent exposure of the N-terminal region of Aβ aggregates, we performed dynamic quenching measurements in which the fluorescence emission of Tyr10 was probed in the presence of the neutral quencher acrylamide. When the lifetime of fluorescence excited states does not significantly vary, the quenching constant (the Stern-Volmer constant (KSV)), which is obtained through the dependence of fluorescence intensities on quencher concentration, reflects how much the fluorophore is exposed to solvent. For monomeric npAβ and pS8Aβ, similar quenching constants of 6.30 ± 0.26 and 6.18 ± 0.17 M⁻¹ were obtained. In contrast, KSV was significantly higher in pS8Aβ (3.98 ± 0.08 M⁻¹) than in npAβ aggregates (3.30 ± 0.06 M⁻¹) (Fig. 7). The higher KSV of pS8Aβ aggregates provides further support for the lower compaction of the N-terminal region of phosphorylated Aβ aggregates.

Phosphorylated Aβ Fibrils Exhibit Distinct Sedimentation Behavior—Phosphorylation at Ser8 enhances the oligomeric and fibrillar aggregation of Aβ (23). The fibrils generated by npAβ and pS8Aβ are similar in morphology, but they show significant differences in the stability against SDS- and pressure-induced dissociation (24). To further investigate the effects of Ser8 phosphorylation on the properties of Aβ aggregates, 5-day-aggregated Aβ samples were briefly centrifuged (16,000 × g for 30 min), and the pellets and supernatants were examined separately. NMR measurements of the dissociated pellets showed that the concentration of pS8Aβ in the pelleted aggregates was smaller than in the pellet obtained from the npAβ sample (Fig. 8) in agreement with higher CD intensities of pS8Aβ in the supernatant (Fig. 9). EM examination further demonstrated that the pS8Aβ supernatant but not the npAβ supernatant was rich in fibrils (Fig. 10). In addition, the fibrils observed in the supernatant of pS8Aβ exhibited detailed features on their surface that were not present in the few fibrils that were found in the npAβ supernatant (Fig. 10). The data suggest that Ser8 phosphorylation alters the surface properties of Aβ fibrils in a manner that reduces their higher order assembly and increases the number of dispersed Aβ fibrils.

Discussion
Aβ aggregation proceeds through several steps in which Aβ forms different conformational and assembly states. Although a U-shaped strand-turn-strand conformation of Aβ is a structural motif commonly preserved along the aggregation pathway (27, 28), a high level of conformational rearrangement underlying the interconversion of Aβ aggregates is essential for progression of Aβ aggregation (29). One striking example of the role of conformational plasticity in Aβ aggregation is observed in the turn region around Ser26 where rigidification of this region by Ser26 phosphorylation is connected to the prevention of fibrillar Aβ aggregates (26). Similarly, maturation of Aβ fibrillar aggregates in later stages of aggregation involves extension of the β-sheet structure toward the N terminus (30). Moreover, the N-terminal region of Aβ fibrils manifests significant structural polymorphism with various degrees of solvent protection due to variations in experimental conditions (31). Our data demonstrate that the conformational dynamics of Ser8 and its adjacent residues are significantly affected by phosphorylation. In agreement with the crucial importance of local flexibility for structural remodeling of Aβ aggregates, our hydrogen-
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Despite the higher solvent accessibility of pS8A, the stability of Aβ aggregates is rather different from that of npAβ aggregates. The data reported in the current study demonstrate that phosphorylation at Ser8 not only influences the stability but also the structure of Aβ aggregates. Although an increase in the stability of pS8Aβ aggregates against pressure-induced dissociation, despite the higher solvent accessibility of pS8Aβ aggregates, seems at first sight counterintuitive, it should be noted that the pressure stability and solvent accessibility methods monitor two different aspects of Aβ aggregate structure. For example, in the npAβ aggregates, the N-terminal region of Aβ is rather protected from the solvent as evidenced by hydrogen-deuterium exchange and fluorescence quenching data (Figs. 6 and 7). At the same time, this does not exclude the presence of water-excluded cavities in this region in the structure of npAβ aggregates, and the lower pressure stability of npAβ aggregates (24) suggests that indeed this might be the case. In line with this model, the solvent-exposed N-terminal region of pS8Aβ aggregates would contain fewer water-excluded cavities, resulting in a higher resistance of pS8Aβ aggregates against pressure-induced monomer dissociation. In addition, we showed that the volume change upon monomer release from pS8Aβ aggregates is around 3 times smaller than that of npAβ aggregates (8 versus 25 ml/mol), suggesting that the pS8Aβ aggregates have higher compressibility without undergoing monomer release. According to molecular dynamics simulations (24), the increased number of intramolecular electrostatic interactions in pS8Aβ aggregates and their susceptibility to pressure-induced disruption contribute to the higher compressibility of pS8Aβ aggregates without Aβ monomer release. The combined data show that phosphorylation at Ser8 modulates both the stability and structure of Aβ aggregates.

Despite the largely disordered structure of Aβ in aqueous solution, several short segments of the Aβ sequence exhibit non-random conformational preferences (32). Of special interest in this study is the segment Ser8–Val12, which adopts a transient turnlike structure (32, 33) and thereby partially brings the

**FIGURE 8.** A smaller amount of pS8Aβ than npAβ is incorporated into insoluble Aβ aggregates as indicated by one-dimensional proton NMR spectra of npAβ (black) and pS8Aβ (gray). After 5 days of aggregation, the Aβ samples were centrifuged (16,000 × g for 30 min), and the pellets were dissociated into monomers by 5% dichloroacetic acid, 95% DMSO and examined by NMR. The signal intensity ratio in the methyl region (between 1 and 0.5 ppm) was ~0.8 for pS8Aβ

**FIGURE 9.** The aggregated pS8Aβ contains higher amounts of soluble Aβ assembles than npAβ. After 5 days of aggregation, CD spectra of the whole aggregated samples (solid lines) and supernatant fractions (dotted lines; 16,000 × g for 30 min) were recorded. Stronger CD intensities were observed in the supernatant of aggregated pS8Aβ than in that of npAβ. mdeg, millidegrees.

**FIGURE 10.** pS8Aβ fibrils exhibit distinct surface properties. After 5 days of aggregation, EM examination of supernatant fractions (16,000 × g for 30 min) revealed many more Aβ fibrils in the pS8Aβ sample than in the npAβ sample in which several pS8Aβ fibrils showed detailed features on their outer surface (examples are indicated by arrows).

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N-terminal region of Aβ close to the functionally important central hydrophobic cluster of Aβ (residues Leu17–Phe20) (34, 35). In addition, residues Phe20–Val24 tend to form a helical turn (32), which is in line with the helical propensity of this segment observed in SDS micelle environments and non-aqueous helix-inducing solvents (36–38). Helical propensity of residues His13–Asp23 has been suggested to be crucial for the early aggregation of Aβ and its interaction with membranes (39). Local perturbation of Aβ conformation by Ser8 phosphorylation, as demonstrated by our data, can therefore be associated with subtle long range conformational alterations, further influencing its aggregation behavior. The observation that the fine modulation of Aβ conformation by Ser8 phosphorylation influences its aggregation is of potential wider interest, especially because Aβ aggregation in vivo usually occurs in various environments such as cellular membrane interfaces where interaction with membrane lipids influences Aβ conformation (40, 41).

The N-terminal region of Aβ is host for a wide range of events such as phosphorylation (23), metal binding (28, 42), tyrosine nitration (43), and truncation (19, 20). These events may modulate the role of Aβ in oxidative damage (44) and induction of inflammation (45) and alter the efficiency of prion-like self-propagation (24) and their interaction with biological targets such as cellular lipid membranes (46). Based on the observation that phosphorylation of Ser8 increases the N-terminal exposure of Aβ aggregates, we hypothesize that phosphorylation might contribute to a mechanism of cross-talk between different N-terminal modifications of Aβ aggregates and thus influence the biochemical maturation of Aβ aggregates (14) and progressive course of AD. This hypothesis remains to be tested by additional experiments probing different N-terminal modifications dependent on Ser8 phosphorylation.

In conclusion, phosphorylation of Aβ at Ser8 changes the structure of the N-terminal region of Aβ aggregates in favor of more solvent-exposed conformations. Our data provide experimental support at the molecular level for the potential role of posttranslational modifications in structural polymorphism of amyloids.

**Experimental Procedures**

*Materials—*Synthetic npAβ and pS8Aβ were obtained from Peptide Specialty Laboratory (Germany); both had a purity of more than 95% and were used without further purification. The 15N, 13C-labeled Aβ1–40 (non-phosphorylated) was purchased from AlexaTech (Sweden); it had a purity above 95% and was used without further purification. For monomerization, the Aβ powders were dissolved in 20 mM NaOH at 2 mg/ml peptide concentration, and after 1 min of sonication and 30 min of shaking in the cold room (4 °C), they were split into 50-μl aliquots, flash frozen in liquid nitrogen, and stored at −80 °C until use.

*Transmission Electron Microscopy—*For EM examination, aggregated samples of npAβ and pS8Aβ were deposited onto carbon-coated copper mesh grids and negatively stained with 2% (w/v) uranyl acetate. Excess stain was washed away, and the sample grids were allowed to air-dry. Subsequently, the samples were examined using a Philips CM 120 BioTwin transmission electron microscope (Philips Inc., Eindhoven, The Netherlands).

*Far-UV CD Spectroscopy—*After a two-step solubilization, first in 1,1,1,3,3,3-hexafluoro-2-propanol and then in 100 mM NaOH, synthetic npAβ and pS8Aβ peptide variants were dissolved in 20 mM sodium phosphate (pH adjusted to 7.40) at a concentration of 0.3 mg/ml. CD spectra were recorded on a Chirascan spectropolarimeter using a cuvette with 1-mm path length at 10 °C. The CD spectra were recorded from 260 to 190 nm at 0.5-nm intervals with an acquisition time per data point of 8 s. Temperature control with an accuracy of ±0.5 °C was achieved with a heating/cooling accessory using a Peltier element.

*Dynamic Fluorescence Quenching Experiment—*Tyr10 fluorescence emission spectra of monomeric and aggregated npAβ and pS8Aβ samples (50 μM in 50 mM sodium phosphate, 50 mM NaCl, pH 7.4) were measured in the presence of specified concentrations of the neutral quencher acrylamide. The aggregated Aβ samples were incubated in aggregation-prone conditions (37 °C with gentle stirring) for 72 h. The excitation wavelength was 279 nm, and emission spectra were recorded between 292 and 350 nm. The maximum emission intensity obtained through interpolation was used for Stern–Volmer analysis. KSV was obtained from the following equation.

\[
\frac{I_0}{T} = 1 + K_{SV}[Q]
\]

in which \(I_0\) is the maximal emission intensity obtained in the absence of quencher and \([Q]\) is the molar concentration of the quencher.

*Two-dimensional Homonuclear NMR Experiments—*NMR samples contained 0.4 mg/ml Aβ in 20 mM sodium phosphate buffer, pH 7.2, and measurements were performed at 5 °C. Chemical shift referencing at this temperature was made with respect to external 4,4-dimethyl-4-silapentane-1-sulfonic acid (0.0 ppm). All NMR spectra were processed and analyzed with NMRPipe (47) and Sparky (50). Two-dimensional 1H, 1H TOCSY and NOESY spectra were acquired on a Bruker (Germany) Avance 800-MHz spectrometer equipped with a cryogenic probe. The time domain data contained 2,048 and 600 complex data points in t2 and t1, respectively. MLEV17 was used for mixing in the TOCSY experiment with a total duration of 60 ms. The mixing time in the NOESY experiment was 200 ms. Water signals were suppressed through a WATERGATE element. 1H resonance assignments were made on the basis of the two-dimensional 1H, 1H TOCSY and NOESY spectra. The combined HN and HA chemical shift perturbation (CSP) induced by Ser8 phosphorylation was evaluated using the following equation.

\[
\text{CSP} = \sqrt{(\Delta \text{HN})^2 + (\Delta \text{HA})^2}
\]

*Pulsed Field Gradient NMR—*Pulsed field gradient NMR experiments were performed on a Bruker AMX 600-MHz spectrometer equipped with a triple resonance cryogenic probe. The sample contained dioxane as an internal hydrodynamic radius standard and viscosity probe. The 1H spectra were collected as a function of gradient amplitude using the stimulated echo sequence with bipolar gradient pulses with gradient strengths increasing from 0.674 to 32.030 G/cm (after correction for the sine shape of the gradient pulses) in a linear manner. The gradient distance (Δ) was 200 ms, and the total
gradient length ($\delta$) was 4 ms. Peaks in the aliphatic region of the $^1$H spectra were picked, and the apparent translational diffusion coefficients were calculated after fitting the intensity versus gradient strength data to a corresponding diffusion equation.

Two-dimensional and Three-dimensional Heteronuclear NMR Experiments—NMR samples containing 0.4 mg/ml $^{15}$N,$^{13}$C-labeled Aβ (20 mm sodium phosphate, pH 7.2) were measured at 5 °C on a 700-MHz Bruker spectrometer with cryogenic probe. Chemical shift referencing was made with respect to an internal 4,4-dimethyl-4-silapentane-1-sulfonic acid standard (0.0 ppm). The $^1$H,$^{15}$N heteronuclear single quantum correlation, $^1$H,$^{13}$C heteronuclear single quantum correlation, HNCA, and HNCO experiments were used for backbone assignment. The residue-specific random coil index calculated through the random coil index web server. Relative extent of hydrogen-deuterium exchange in npA samples (50 μM) in PBS were incubated in aggregation-prone conditions (37 °C with gentle stirring) for 7 days. Subsequently, aggregated Aβ samples were ultracentrifuged (100,000 × g at 25 °C) for 4 h, and the pellet was dried using strips of filter paper, resuspended in D$_2$O with gentle vortexing, and incubated at room temperature for 1 h to allow for forward exchange from D$_2$O to exchangeable amide protons. After D$_2$O treatment, insoluble Aβ aggregates were again collected by ultracentrifugation (100,000 × g at 25 °C) for 4 h and carefully dried to remove residual D$_2$O. The collected Aβ aggregates were then dissolved in 250 μl of 5% dichloroacetic acid, 95% DMSO mixture and immediately transferred to an NMR tube to start data acquisition on an 800-MHz Bruker NMR spectrometer. One-dimensional $^1$H, two-dimensional $^1$H, $^1$H TOCSY, and NOESY spectra were obtained. Mixing times of 60 ms for TOCSY and 200 and 300 ms for NOESY experiments were used. Peak assignments were made through a standard homonuclear sequential assignment strategy and further checked with the assignments reported previously (48). The relative extent of hydrogen-deuterium exchange in npAβ and pS8Aβ aggregates was evaluated through comparing the TOCSY cross-peak intensities between the two peptides.

Water-Amide Proton Exchange Rates—Intrinsic water-amide proton rates in monomeric npAβ and pS8Aβ samples (75 μM; buffered with 20 mM sodium phosphate, pH 7.4) were measured through one-dimensional CLEANEX-PM experiments on a 700-MHz Bruker spectrometer at 15 °C. In this experiment, a selective water excitation pulse was followed by a mixing time ($\tau_m$) of durations 5, 10, 15, 25, 50, 100, 200, and 500 ms during which chemical exchange between water and amide protons takes place. The global intensity of amide protons as a function of mixing time was fitted to the following equation.

$$V / V_0 = \frac{k \cdot \exp(-R_{1B} \cdot \tau_m) - \exp[-(R_{1A} + k) \cdot \tau_m]}{(R_{1A} + k - R_{1B})}$$

(Eq. 3)

where $V_0$ is the intensity in a control experiment, $k$ is the normalized rate constant related to the forward exchange rate constant between water and amide protons, and $R_{1A}$ and $R_{1B}$ are apparent longitudinal relaxation rates for protein and water (49).

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