Early Divergence in Misfolding Pathways of Amyloid-Beta Peptides

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The amyloid cascade hypothesis proposes that amyloid-beta (Aβ) aggregation is the initial triggering event in Alzheimer’s disease. Here, we utilize NMR spectroscopy and monitor the structural dynamics of two variants of Aβ, Aβ40 and Aβ42, as a function of temperature. Despite having identical amino acid sequence except for the two additional C-terminal residues, Aβ42 has higher aggregation propensity than Aβ40. As revealed by the NMR data on dynamics, including backbone chemical shifts, intra-methyl cross-correlated relaxation rates and glycine-based singlet-states, the C-terminal region of Aβ, especially the G33-L34-M35 segment, plays a particular role in the early steps of temperature-induced Aβ aggregation. In Aβ42, the distinct dynamical behaviour of C-terminal residues at higher temperatures is accompanied with marked changes in the backbone dynamics of residues V24-K28. The distinctive role of the C-terminal region of Aβ42 in the initiation of aggregation defines a target for the rational design of Aβ42 aggregation inhibitors.

Amyloid-beta peptides (Aβ) are the main constituents of senile plaques in the brains of Alzheimer’s disease (AD) patients.[1, 2] Several lines of evidence suggest a crucial role for Aβ aggregation as the initial triggering event in AD pathology.[1] Consequently, Aβ aggregation is widely considered as an ideal target for anti-AD drug development.

The two common variants of Aβ are Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42), which are identical in the amino acid sequence except for the two additional C-terminal residues in Aβ42 (Figure 1A).[2] Despite being similarly unstructured in the monomeric state,[3] Aβ42 is more aggregation prone and neurotoxic than Aβ40.[4] Early studies of Aβ aggregation suggest that the C-terminus of Aβ plays a key role in amyloidogenesis and that the oligomeric assembly of Aβ40 and Aβ42 proceeds through distinct pathways.[4, 5] The Aβ42 was shown to be more rigid than Aβ40 at the C-terminus,[6] probably due to the transient formation of a β-sheet structure in Aβ42.[7] In recent years, several structural models of Aβ40 and Aβ42 fibrils have been published, providing clear evidence for their differences in the fibril state.[8–11] In addition, structural models of the intermediate Aβ aggregates implicate that Aβ42 oligomers are structurally distinct in the C-terminal region.[12]

The rational design of an Aβ aggregation inhibitor ideally requires a mechanistic understanding of the early stages of aggregation before any toxic Aβ species is formed. Solution-state NMR spectroscopy allows monitoring early events during Aβ aggregation at atomic resolution. Here, we utilize this technique to probe the backbone and sidechain dynamics of...

Figure 1. A) Amino-acid sequence of Aβ1-40(42), showing the sequence distribution of glycine (purple squares) and methyl-containing (orange squares) residues. B) Temperature-dependence of backbone dynamics in Aβ40 and Aβ42. Residue-specific squared order parameters (S²) of Aβ40 and Aβ42 in dependence of temperature, based on the Random Coil Index (RCI) values derived from backbone chemical shifts, are shown. Note the differences between Aβ40 and Aβ42, in particular from residue V24 towards the C-terminus. See Tables S1 and S2 for residue-specific chemical shifts, esp. with regard to residues with missing assignments.
Aβ40 and Aβ42 and exploiting them as local proxies of aggregation-related events (Figure 1A), we provide evidence for the early divergence of the misfolding pathways of Aβ40 and Aβ42.

NMR chemical shifts are sensitive probes of conformational dynamics of proteins at pico-to-microseconds. To monitor the backbone dynamics of Aβ in dependence of temperature, we measured the backbone (HN, HA, N, CA, CO) chemical shifts of Aβ40 and Aβ42 at three temperatures of 278, 288 and 298 K (SI, Tables S1 and S2). We then followed the random coil index (RCI)-based order parameters (S2) approach of ref. 14 which in intrinsically disordered proteins (IDPs) represents the conformational heterogeneity of their ensembles and therefore enables “qualitative” monitoring of temperature effects on Aβ’s backbone dynamics. As shown in Figure 1B, the increase in temperature altered the structural dynamics of Aβ40 and Aβ42 in two different ways: in Aβ40, increasing the temperature from 278 to 288 and then 298 K had little impact on the backbone mobility of residues proximal to G29, while the backbone mobility of residues A30 towards the C-terminus was clearly enhanced. In Aβ42, up to residue D23, the temperature dependence of mobility was almost the same as that of Aβ40. However, sharp differences were observed from residue V24 onwards; residues V24-K28 became more rigid at higher temperatures, residues G29 and L34-G38 retained their mobility, and only residues A30-G33 and to lesser extent V39-I41 got more mobile at higher temperatures. The distinctive dynamical behavior of the C-terminal region of Aβ42 is in qualitative agreement with the 15N relaxation-based reports of Aβ’s ps-ns dynamics, 15 although a quantitative comparison of the 15N relaxation- and RCI-based S2 is not possible.

Next, to investigate the temperature-dependent changes in the side-chain dynamics of Aβ40 and Aβ42, the 13C,1H HSQC spectra were measured for the methyl (CH3) groups of alanine, valine, leucine, isoleucine and methionine residues (SI, Figure S1). As suggested by the structural models of Aβ40 and Aβ42 fibrils,18-11 these hydrophobic residues play significant roles in the stability of Aβ aggregates. Hydrophobic interactions are strongly temperature-dependent and their destabilization at very low or high temperatures promotes dissociation of amyloid fibrils.15 The temperature-induced perturbations in chemical shifts were overall similar for Aβ40 and Aβ42, however, the C-terminal residues, I41 and A42, of Aβ42 showed a relatively large chemical shift change (Figure 2). The 13C and 1H chemical shifts did not show any significant Aβ concentration dependence (SI, Figure S2), suggesting that their temperature variation reflected Aβ’s conformational dynamics predominantly in the monomeric state. In both Aβ40 and Aβ42, the methyl groups exhibited large variation in temperature dependence of their peak intensity (SI, Figures S3 and S4). Interestingly, in both Aβ40 and Aβ42, the most prominent intensity reduction was observed for the methyl group of M35. Less prominent albeit considerable intensity loss was observed for the methyl groups of L34, V40 and V39 in Aβ40 (SI, Figure S3), and those of A42, V36/V39, L34, L17 and V18 in Aβ42 (SI, Figure S4). In Aβ42, all the methyl groups showed an intensity drop at 302 K, as a consequence of aggregation-induced monomer loss.

To further investigate the effect of temperature on methyl dynamics in Aβ40 and Aβ42, the cross-correlated relaxation (CCR, Γ) rates between three 13C,1H dipolar couplings of the methyl groups were measured through constant-time 1H-coupled 13C,1H HSQC spectra from the intensities of different peaks in the quartet, as described in Ref. 16 (Figure 3). The methyl CCR rates report the reorientational mobility of the three-fold symmetry axis of the methyl groups.16,17 In Aβ40, the CCR rates of most of the methyl groups decreased by temperature, in accord with the temperature-dependent decrease in viscosity/temperature (η/Τ) ratios and its resultant enhancement in side-chain mobility (SI, Table S2). However, the methyl groups of L17, L34, M35 and V40 showed an unexpected increase in the CCR rates at 310 K, indicating their significantly lower mobility at this temperature (Figure 4A). In comparison, in Aβ42, the methyl groups of L17 obeyed the general trend of mobility enhancement upon temperature increase up to 298 K, but the methyl groups of L34, V40 and A42 followed an opposite trend and became relatively rigid at 298 K (Figure 4B and SI, Table S3).

Aβ contains six glycine residues, G9, G25, G29, G33, G37 and G38. To utilize glycines as local probes of structural dynamics of Aβ during aggregation, we monitored glycines of Aβ40 and Aβ42 through a singlet-filtered NMR method.18 Singlet-states are effective spin-0 states formed in homonuclear spin-1/2 pairs and can be detected only indirectly.19,20 The singlet-states have been previously utilized for monitoring protein conformational and dynamical changes during unfolding.21,22 Glycines have a

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Figure 2. Combined 13C and 1H chemical shift perturbation (CSP) of the methyl groups of Aβ40 (A) and Aβ42 (B) upon temperature rise, evaluated with respect to the chemical shifts obtained at 278 K. Note that the C-terminal methyl groups of Aβ42 (of I41 and A42) exhibit a relatively large CSP in dependence of temperature.
pair of HA spins, which in deuterated solvents become largely isolated from all other protons. Since the HA pairs of the six glycines of $\alpha\beta$ experience different coupling regimes (depending on the ratio between their chemical shift difference, $\Delta\omega$, and the $^2J$ scalar coupling between them), they could be monitored selectively through the gc-M2S sequence (Figure 5 and SI, Figure S5). The singlet-filtered approach is particularly advantageous in $\alpha\beta$ where the peaks of glycine residues in proton-based homonuclear NMR spectra are not well resolved.

In $\alpha$40, the G25 and to a lower extent G29, G37, G38 and G9 residues showed an initial increase in the intensity upon heating, followed by the intensity loss at higher temperatures (Figure 6A). The G33 residue however obeyed an opposite trend: its intensity decreased up to 288 K, then increased by further heating up to 310 K. The distinction between G33 and other glycine residues became more pronounced in $\alpha$42: upon heating to 293 K, the G33 residue showed an intensity gain by a factor of $\sim 2$, while the other glycine residues kept (G25) or lost (G29, G38, G9 and G37) their intensity (Figure 6B). Notably, in $\alpha$40 and especially in $\alpha$42 the intensity gain of G33 were accompanied by temperature-induced $\alpha\beta$ monomer loss due to aggregation.

Subsequently, we measured the singlet-state ($T_s$) and spin-lattice relaxation times ($T_1$) of $\alpha\beta$'s glycines at different temperatures. There are three distinct dynamical regimes in peptide-based singlet-states: fast, intermediate, and slow. In “fast” and “slow” regimes, the $T_s$ and $T_1$ change in the same direction when the rotational correlation time ($\tau_c$) is altered, while in the “intermediate” regime, they change in the opposite directions. Consequently, the $T_s/T_1$ ratio exhibits its largest sensitivity to dynamical changes in the intermediate regime. Due to the relatively low sensitivity and long duration of the singlet-state NMR experiments, these relaxation measurements were performed only for the less aggregation-prone $\alpha$40. As shown in SI, Figures S6 and S7, all glycine residues of $\alpha$40 except G33 showed slight temperature-dependent increase in $T_s$ and decrease in $T_1$ at temperatures above 281 K, which considering their $\tau_c$ being greater than $1/\omega_H \sim 230$ ps, suggests their relative mobilization in the intermediate regime. Unlike them, the G33 residue seems to have undergone a relative rigidification in the same temperature range. When the $T_s/T_1$ ratio was plotted against the temperature, the distinct behavior of the G33 residue became more evident (Figure 6 and SI, Figure S8). Overall, the singlet-state intensity (and
relaxation) data point to a particular role of G33 in temperature-induced initiation of Aβ aggregation.

To investigate whether the temperature-induced alterations in the conformational dynamics of Aβ are due to intramolecular changes in Aβ monomers or alternatively caused by the formation of Aβ oligomers in rapid exchange with NMR-visible monomers, we measured the hydrodynamic radii ($R_h$) of Aβ40 and Aβ42 through NMR diffusion experiments. At 278 K, the $R_h$ of Aβ40 and Aβ42 were 2.0 ± 0.1 and 2.1 ± 0.1 nm, respectively, consistent with the predominantly monomeric state of NMR-visible Aβ peptides. Upon temperature increase to 288 and 298 K, the $R_h$ of Aβ40 remained unchanged, while the $R_h$ of Aβ42 showed slight reduction to 2.0 ± 0.2 and 1.8 ± 0.2 nm, respectively. The slight reduction of the $R_h$ of Aβ42 at 298 K occurred despite significant (~75%) monomer loss due to aggregation, indicating that the exchange between Aβ42 monomers and oligomers is slow in the diffusion timescale. It is notable that our diffusion data does not exclude the weak interaction between Aβ monomers and stable high-molecular-weight Aβ aggregates, as shown previously. However, in the present study, we have avoided the experimental conditions, e.g. high Aβ concentrations, which induce the formation of protofibrils or other large aggregates. Overall, in line with the lack of concentration dependence of NMR chemical shifts (SI, Figure S2), our data suggest that the temperature-induced conformational changes of Aβ are predominantly intramolecular.

Aβ molecules contain small percentage of β-sheet structure already in the monomeric state, in particular at the C-terminus. Our results demonstrate the distinctive behavior of residues G33, L34 and M35 of both Aβ40 and Aβ42 in response to a temperature rise. The G33 residue is part of the GxxGxxGxxxG motif of Aβ (G25-G29-G33-G37), which may be involved in transmembrane oligomerization of Aβ by forming a glycine zipper. Previous studies have shown that the G33 substitution with alanine or isoleucine diverts Aβ aggregation toward less-toxic Aβ oligomers. Furthermore, the F19-L34 contact is formed at an early stage of Aβ40 aggregation, and
the L34 V mutation of Aβ promotes Aβ aggregation. Besides, the role of M35 residue in Aβ40 oligomerization has been detected via 19F NMR. Our data are in accord with the previous reports and furthermore suggest that the alterations in the conformational dynamics of the G33-L34-M35 segment constitute an "early" step of Aβ aggregation. Notably, this region is distinct from the reported "hot spots" of Aβ involved in seeded Aβ aggregation, nevertheless the observed oscillation in the peak intensity profile of Aβ's C-terminal residues during seeded aggregation is in agreement with the proposed role of the C-terminal region during early Aβ aggregation. Furthermore, these residues of Aβ may play a role in the micelle-like peptide oligomerization, as suggested in an arguably key step in the aggregation of Aβ and other amyloid peptides, and their cross-seeded aggregation.

In both variants of Aβ, the C-terminal residues (V40 in Aβ40 and A42 in Aβ42) appear to be involved in the early steps of aggregation. However, unlike Aβ40, the distinct temperature-dependence of side-chain dynamics in the C-terminal region of Aβ42 is accompanied by changes in the backbone dynamics of V24-K28 and relative rigidity of M35-G38 (Figure 1B). Besides, previous studies have shown that S26 phosphorylation or phosphonimetic mutation (S26D) induces the formation of a local salt bridge with K28 side chain and increases the mobility of C-terminal residues distal to K28. This non-native salt bridge was proposed to underlie the observed anti-aggregation effect of S26 phosphorylation.

Inspired by these data, we hypothesize that elevated temperature brings the middle and C-terminal regions of Aβ closer to each other and promotes the formation of a salt bridge between K28 side chain and the C-terminal carboxyl group. As shown in another protein system, the formation of this salt bridge can be favored at higher temperatures because of the entropic gain originating from desolvation effects. The formation of this long-range salt bridge leads to the relative compaction of Aβ42 monomers, consistent with the slight reduction of the Rg of Aβ42 at high temperatures. This salt bridge has been observed in recent structural models of Aβ42 fibrils, and our data proposes that it may form during the "early" steps of the misfolding of Aβ42. It is however notable that the salt-bridge disrupting K28E mutation induces only small differences in the chemical shifts of A30-G37 of Aβ42 at 278 K, indicating that the speculated salt bridge is not present at low temperatures. Further studies are required to test the proposed hypothesis.

Glycine residues are believed to play important roles in controlling protein aggregation and liquid-liquid phase separation. Proteins undergoing neurodegeneration-related aggregation, such as Aβ and tau protein, α-synuclein and dipeptide repeat (DPR) proteins contain large numbers of glycines frequently located in the key regions of the protein sequence. In AD, several mutations introduce glycine into Aβ sequence, which can alter the structure and dynamics of Aβ fibrils. In addition, glycine residues of Aβ are potential players in peptide-lipid membrane interactions. The use of glycine-based singlet-states as exemplified in this study allows site-specific monitoring of conformational changes during aggregation without the need for uniform or selective isotope labeling of proteins. Unlike amide protons, the use of glycine HAS is not compromised by rapid amide-water exchange rates in disordered proteins. Besides, new insights into the mechanism of peptide aggregation or peptide-membrane interaction can be obtained through dynamical information encoded in the singlet-state relaxation times.

In summary, we have demonstrated that the C-terminal region of Aβ plays a distinctive role during the earliest steps of temperature-induced misfolding and aggregation. Our data highlights the plasticity of the aggregation mechanism in Aβ peptides and defines the C-terminal region as a potential target in the rational design of Aβ42 aggregation inhibitors.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] D. J. Selkoe, J. Hardy, EMBO Mol. Med. 2016, 8, 595–608.
[2] R. Sengoku, Neuropathology 2020, 40, 22–29.
[3] R. Riek, P. Guntert, H. Dobeli, B. Wipf, K. Wuthrich, Eur. J. Biochem. 2001, 268, 5930–5936.
[4] J. T. Jarrett, E. P. Berger, P. T. Lansbury Jr., Ann. N. Y. Acad. Sci. 1993, 695, 144–148.
[5] G. Bitan, M. D. Kirkitadze, A. Lomakina, S. S. Vollers, G. B. Benedek, D. B. Teplow, Proc. Natl. Acad. Sci. USA 2003, 100, 330–335.
[6] Y. L. Yan, C. Y. Wang, J. Mol. Biol. 2006, 364, 853–862.
[7] T. Kakeshpour, V. Ramanujam, C. A. Barnes, Y. Shen, J. Ying, A. Bax, Biophys. Chem. 2020, 270, 106531.
[8] J. X. Lu, W. Ong, W. M. Yau, C. D. Schwiersets, S. C. Meredith, R. Tycko, Cell 2013, 154, 1257–1268.
[9] L. Gremer, D. Scholzel, C. Schenk, E. Reinartz, J. Labahn, R. B. G. Ravelli, M. T. Colvin, R. Silvers, Q. Z. Ni, T. V. Can, I. Sergeyev, M. Rosay, K. A. Donovan, B. Michael, J. Wall, S. Linse, R. G. Griffin, J. Am. Chem. Soc. 2016, 138, 9663–9674.
[10] M. A. Walti, F. Ravotti, H. Arau, C. G. Glase, J. S. Wall, A. Bockmann, P. Guntert, B. H. Meier, R. Riek, Proc. Natl. Acad. Sci. USA 2016, 113, E4976–4984.
[11] S. Parthasarathy, M. Inoue, Y. Xia, Y. Matsumura, Y. Nabeshima, M. Hoshi, Y. Ishii, J. Am. Chem. Soc. 2015, 137, 6480–6483.
[12] D. S. Wishart, Prog. Nucl. Magn. Reson. Spectrosc. 2011, 58, 62–87.
[13] M. V. Berjanskii, D. S. Wishart, J. Am. Chem. Soc. 2005, 127, 14970–14971.
[14] H. Y. Kim, M. K. Cho, D. Riedel, C. O. Fernandez, M. Zweckstetter, Angew. Chem. Int. Ed. 2008, 47, 5046–5048; Angew. Chem. 2008, 120, 5124–5126.
[15] T. M. Sabo, D. Bakhitani, K. F. Walter, R. L. McFeeters, K. Giller, S. Becker, C. Griesinger, D. Lee, Protein Sci. 2012, 21, 562–570.
[16] Y. Yan, J. Liu, S. A. McCallum, D. Yang, C. Wang, Biochem. Biophys. Res. Commun. 2007, 362, 410–414.
