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Isomerization of the Asp7 Residue Results in Zinc-Induced Oligomerization of Alzheimer's Disease Amyloid β(1–16) Peptide

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Alzheimer's disease (AD)—a fatal neurodegenerative disorder that primarily affects the elderly—is pathophysiologically characterized by the extracellular deposition of a 40/42-amino-acid-long protein, referred to as amyloid-β peptide (Aβ), in the brains of AD victims.[1] Although the molecular mechanism of AD onset is unknown, the transformation of Aβ from its native monomer conformation via soluble dimers and higher oligomers into insoluble fibrillar β-sheet aggregates, which finally accumulate into the amyloid plaques, is believed to be a key event in AD pathogenesis.[2] One plausible hypothesis suggests that the amyloid neuropathology of AD depends on zinc ions released during neurotransmission, and so it is assumed that binding of zinc to Aβ might play an important role in initiating pathogenic amyloid deposition,[3] as well as some additional still unidentified proteinaceous factors.[4]

The Aβ molecules isolated from AD brain lesions have numerous endogenous post-translational modifications (PTMs),[5] which should profoundly affect both the Aβ conformation and its oligomeric state and make up a pool of potential pathogenic agents in AD. The most abundant PTM of Aβ is isomerization of the Asp7 residue; this results in the formation of an L-*iso*Asp7 isoform (isoaspartate). This nonenzymatic modification occurs spontaneously in polypeptides through an intramolecular rearrangement of aspartate or asparagine residues and is generally regarded as a degradation reaction that occurs in vivo during tissue ageing.[6] In the case of isomerized Aβ (*iso*Aβ) it is still unclear whether the isoaspartyl residues are the cause or the result of the pathological accumulation of Aβ.[7] Nevertheless, recent in vitro experimental evidence indicates that *iso*Aβ might potentially be involved in the onset of AD.[8]

To investigate the role of the Asp7 isomerization in zinc-induced oligomerization of Aβ we have studied the thermodynamics of zinc binding and the oligomeric states of two synthetic model peptides that correspond to region 1–16 in Aβ and in *iso*Aβ: $A\beta_{16}$ and *iso*$A\beta_{16}$, respectively. Earlier, this region was identified as the zinc-binding domain of Aβ,[9–11] which binds Zn^{2+} with 1:1 stoichiometry and a 6 μM dissociation constant.[12] Both $A\beta_{16}$ and its complex with Zn^{2+} were found to be monomeric under physiological conditions for at least six months over a wide concentration range,[9,11] and so were used as monomer reference standards throughout this work. The *iso*$A\beta_{16}$ was also shown to possess zinc-binding ability;[11] however, the properties of the Zn^{2+}–*iso*$A\beta_{16}$ complex have not been studied previously.

To compare Zn^{2+} binding to $A\beta_{16}$ and to *iso*$A\beta_{16}$ (in 50 mM Tris buffer at pH 7.3), isothermal titration calorimetry (ITC) was used. The thermodynamic data demonstrate that $A\beta_{16}$ binds one zinc ion with an association constant of $1.7(\pm0.4)\times10^4\ M^{-1}$ (Figure 1), which corresponds to previously published data.[12]

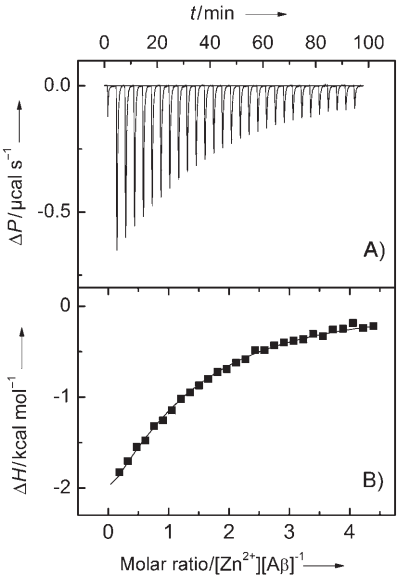

Figure 1. Typical ITC curves of Zn^{2+} interaction with $A\beta_{16}$ at 25 °C in Tris buffer (pH 7.3, 50 mM): A) titration of $A\beta_{16}$ with Zn^{2+}; B) binding isotherm derived from A).

Zinc binding to $A\beta_{16}$ is both enthalpy-driven and entropy-favorable ($\Delta H = -3.8 \pm 0.2$ kcal mol^{-1}; $T\Delta S = 2.0 \pm 0.1$ kcal mol^{-1}). The thermodynamic parameters determined for the $A\beta_{16}$ peptide acetylated at the N terminus and amidated at the C terminus correspond, within experimental error, to recently published data for the $A\beta_{16}$ peptide with unprotected termini.[13] Asp7 isomerization does not noticeably affect either the associ-

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ation constant ($K_a = 1.3(\pm 0.4) \times 10^4\ M^{-1}$) or the thermodynamic profile ($\Delta H = -4.8 \pm 0.2$ kcal mol^{-1}; $T\Delta S = 0.8 \pm 0.1$ kcal mol^{-1}) of zinc binding to *iso*Aβ_{16}. The positive entropy could be explained in terms of burial of hydrophobic surfaces and/or the release of water molecules from the Zn^{2+} ion upon its binding to peptides. Since the contribution of zinc desolvatation to the binding entropy—as well as the effect of the reduction of the number of particles from two to one—is the same for both peptides, it appears that the difference in the Zn-binding entropy values of the peptides mostly depends on conformational changes. The differences in thermodynamic values thus suggest that Asp7 isomerization slightly changes the mechanism of zinc-complex formation with *iso*Aβ_{16} relative to that for Aβ_{16}. These observations agree with earlier conclusions based on NMR spectroscopy data.[11]

The oligomeric states of zinc-free and zinc-bound Aβ_{16} and *iso*Aβ_{16} were studied by electrospray ionization mass spectrometry (ESI-MS), which allowed noncovalently bound multimeric peptide species to be detected.[14] All samples were prepared in Tris buffer (pH 7.3, 5–10 mM) containing the peptide of interest (0.01–1 mM) and $ZnCl_2$ (0–10 mM). The mass spectrum of zinc-free Aβ_{16} shows intense signals of ions that correspond to multiprotonated and sodiated Aβ_{16} monomers (Figure 2).

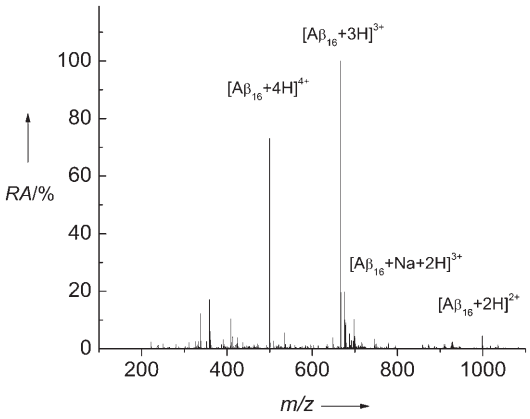

Figure 2. The 200–1000 *m/z* range fragment of a positive ion mass spectrum obtained for zinc-free Aβ_{16} peptide at a concentration of 10 μM in Tris buffer (pH 7.3, 10 mM). Major signals at *m/z* 499.99, 666.31, and 998.97, which correspond to multiprotonated monomeric molecules of Aβ_{16} that have charge states of 4^+, 3^+, and 2^+, respectively, are indicated, together with the doubly protonated and sodiated ions of Aβ_{16} (with *m/z* 673.65).

The ions' identities were then verified in collision-induced dissociation (CID) MS^2 experiments. The mass spectrum of zinc-free *iso*Aβ_{16} demonstrated signals identical to those of Aβ_{16} (data not shown). The CID mass spectrum of the doubly protonated *iso*Aβ_{16} ions (data not shown) revealed the "$b_6 + H_2O$" ion, which was absent in the corresponding MS^2 spectrum of Aβ_{16}. This ion serves as a characteristic feature of the presence of *iso*Asp7 in amyloid-β peptides.[15]

Altogether, the data have shown that in the absence of Zn^{2+} both peptides exist exclusively in monomeric form. Addition of zinc ions to the same samples, however, resulted in strong differentiation between the oligomeric states of the peptides, as evidenced by ESI-MS. The mass spectrum of zinc-bound Aβ_{16} (data not shown) demonstrated no changes relative to that of zinc-free Aβ_{16}. In contrast, in the mass spectrum of the zinc-bound *iso*Aβ_{16} sample an additional isotopic pattern in the 1330.9–1332.3 *m/z* interval was registered (Figures 3 and 4).

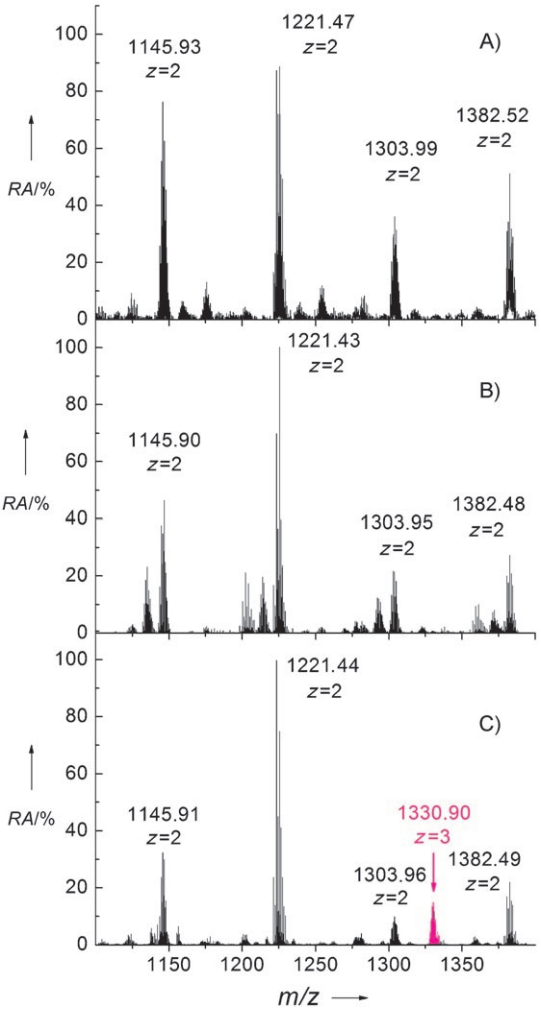

Figure 3. The 1100–1400 *m/z* fragments of the positive ion mass spectra obtained for: A) Tris buffer (pH 7.3, 10 mM) in the presence of $ZnCl_2$ (0.1 mM), B) zinc-bound Aβ_{16}, and C) zinc-bound *iso*Aβ_{16}, both of which were dissolved in the Tris buffer. The four major signals (corresponding to the ions with charge state $z = +2$) in each spectrum are indicated by their monoisotopic *m/z* values. The peaks' amplitudes were about 0.5–3% of that corresponding to the $[A\beta_{16}+3H]^{3+}$ ion signal shown in Figure 2. Each peak from these fragments was subjected to CID fragmentation, and according to these experiments (data not shown) none contained any Aβ_{16} isoform except for the peak with monoisotopic *m/z* 1330.90 (in magenta, panel C), the isotopic pattern of which is shown in Figure 4. This peak was assigned to a triply protonated noncovalent *iso*Aβ_{16} homodimer.

This pattern was attributed to the triply protonated noncovalent homodimer formed by *iso*Aβ_{16}. MS^2 and MS^3 experiments confirmed the *iso*Aβ_{16} homodimer identification (Figure 5).

No higher oligomers or zinc-bound dimers of *iso*Aβ_{16} were observed, possibly due to the instability of such species under

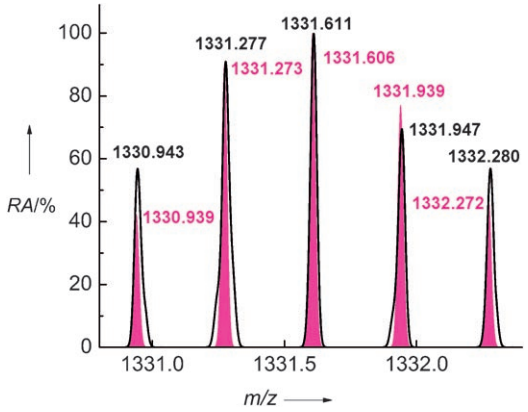

Figure 4. The observed (black) and calculated (magenta) MS isotopic patterns of triply protonated noncovalent *iso*Aβ_{16} homodimer. ESI-MS measurements of *iso*Aβ_{16} (10 μM) were performed in Tris buffer (pH 7.3, 10 mM) in the presence of $ZnCl_2$ (0.1 mM).

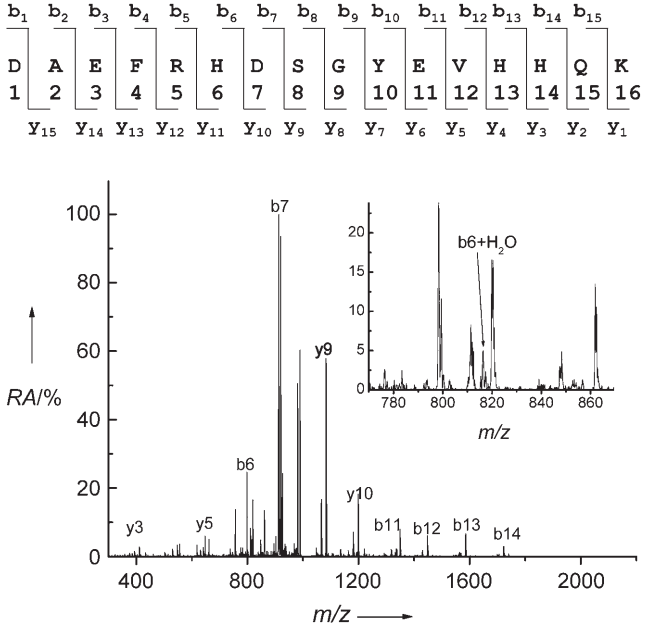

Figure 5. The MS^3 spectrum of the *iso*Aβ_{16} homodimer. The precursor ion was the doubly protonated peptide (*m/z* 998.95). Some of the b and y series product ions that corresponded to fragments of *iso*Aβ_{16} (see scheme, top panel) are marked. The inset shows expansion of the 780–880 *m/z* range to demonstrate the "$b_6 + H_2O$" ion, which is the characteristic marker of *iso*Asp7. Attribution of all observed fragment ions is shown in Table S1 in the Supporting Information.

our experimental conditions. The ESI-MS data presented here therefore unambiguously indicate that *iso*Aβ_{16} forms oligomers in the presence of zinc ions. In contrast, normal Aβ_{16} showed no signs of oligomerization under any of the experimental conditions examined.

Earlier it was demonstrated that Asp7 isomerization does not affect the biophysical and biological properties of the full-length Aβ; however, its behavior was studied in the absence of zinc ions,[16] whereas it has been shown that zinc plays an important role in pathological Aβ oligomerization.[3] In the work presented here, the synergetic effect of both zinc ions and Asp7 isomerization on Aβ oligomerization has been studied with ESI-MS by using the zinc-binding domain of Aβ as a model. It was found that isomerization of Asp7 results in zinc-induced oligomerization of this domain under such conditions. On the basis of these results it is reasonable to suggest that this modification will also cause the full-length Aβ to form oligomers in the presence of zinc ions. Since excessive formation of Aβ oligomers is assumed to trigger AD,[2] our results support the protein-ageing hypothesis of AD, which suggests that *iso*Aβ is a causative agent in AD rather than a product of the pathology progression.[7] In the context of this hypothesis our results show that any endo- or exogenous factor that favors isoaspartate formation at Aβ residue 7 will presumably lead to subsequent accumulation of zinc-bound *iso*Aβ in the brain, and will potentially be involved in Alzheimer's disease. Ageing of Aβ in biological tissues could be one such factor. Another consideration could be substitution of Asp7 by Asn in Aβ, since asparagine is known to be much more susceptible than aspartate to spontaneous conversion into isoaspartate.[6] This factor could explain why the recently discovered intra-Aβ mutation Asp7Asn (the Tottori-Japan mutation)[17] is linked to early-onset familial AD.

In summary, in this work it has been shown that Asp7 isomerization—a standard post-translational modification of Aβ species accumulated in AD brain lesions[5]—gives rise to a new function of the Aβ zinc-binding domain: namely, its ability to oligomerize upon interactions with zinc ions. In the context of the already known molecular features of AD, these in vitro results suggest a need for further in vivo analysis of biological effects of isomerized Aβ in AD pathogenesis.

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