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Up-and-down topological mode of amyloid β-peptide lying on hydrophilic/hydrophobic interface of ganglioside clusters

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Abstract Growing evidence has indicated that GM1 ganglioside specifically interacts with Amyloid β-peptide (Aβ) and thereby promotes Alzheimer's disease-associated Aβ assembly. To characterize the conformation of Aβ bound to the ganglioside, we performed 920 MHz ultra-high field NMR analyses using isotopically labeled Aβ(1–40) in association with GM1 and lyso-GM1 micelles. Our NMR data revealed that (1) Aβ(1–40) forms discontinuous α-helices at the segments His^{14}-Val^{24} and Ile^{31}-Val^{36} upon binding to the gangliosidic micelles, leaving the remaining regions disordered, and (2) Aβ(1–40) lies on hydrophobic/hydrophilic interface of the ganglioside cluster exhibiting an up-and-down topological mode in which the two α-helices and the C-terminal dipeptide segment are in contact with the hydrophobic interior, whereas the remaining regions are exposed to the aqueous environment. These findings suggest that the ganglioside clusters serve as a unique platform for binding coupled with conformational transition of Aβ molecules, rendering their spatial rearrangements restricted to promote specific intermolecular interactions.

Keywords Amyloid β-protein · Ganglioside · NMR · Alzheimer's disease

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Abbreviations

Aβ	Amyloid β-peptide
AD	Alzheimer's disease
CD	Circular dichroism
HSQC	Heteronuclear single-quantum correlation
NMR	Nuclear magnetic resonance
PG	Phosphatidylglycerol
TROSY	Transverse relaxation-optimized spectroscopy

Introduction

Alzheimer's disease (AD) is associated with the progressive accumulation of amyloid deposits in the brain and is pathologically diagnosed through the formation of the extracellular senile plaques and intracellular neurofibrillary tangles [1]. The major component of plaques is a 4-kDa peptide known as amyloid β-peptide (Aβ), consisting of 40 or 42 amino acid residues with a high propensity for aggregation to form cross-β-fibrils [2, 3]. While the conversion of soluble, nontoxic, monomeric Aβ to its insoluble, toxic, aggregated form has been considered to be a crucial step in AD [1], the soluble Aβ oligomers have recently been proposed to be the primary toxic Aβ species in AD [4]. In the case of familial AD, the expression of responsible genes likely results in the enhancement of Aβ generation and/or assembly; however, no evidence has so far been provided to indicate an increased level of Aβ production in sporadic AD [1, 5]. Thus, it remains to be elucidated how the Aβ assembly is accelerated in the brain in age-dependent and region-specific manners.

Yanagisawa *et al.* have previously identified a unique Aβ species in cerebral cortices from AD patients, that is tightly associated with GM1 ganglioside, a glycosphingolipid abundant in neuronal membranes [6]. A series of *in vitro* studies have indicated that the GM1-bound Aβ exhibits an extremely high potential to facilitate Aβ assembly [7]. Thus, this Aβ species has been proposed to act as a seed for Aβ fibrillogenesis in the brain [8]. Furthermore, an enhanced formation of a GM1-induced toxic soluble Aβ(1–40) assembly has been reported in a hereditary variant-type Aβ termed *Arctic* [9]. It is also noteworthy that Aβ(1–40) forms toxic fibrils upon specific interaction with GM1 micelles [10]. Hence, the structural characterization of the GM1-interacting Aβ species is crucial for understanding the molecular mechanisms underlying the onset and the development of AD.

On the basis of the circular dichroism (CD) data, Kakio *et al.* have demonstrated that Aβ undergoes a conformational transition from an α-helix-rich structure to a β-sheet-rich structure when Aβ density increases on GM1-containing liposomes [11]. Their CD data along with fluorescence spectroscopic data have also indicated that GM1 micelles provide a binding platform for Aβ, closely mimicking the GM1-containing raft-like membranes. A recent NMR study has shown that small but significant chemical shift perturbations were induced for the amino acid residues localized within the N-terminal region of Aβ upon titration of GM1 micelles under Aβ-excess conditions [12]. However, no detailed structural study has so far been reported on the Aβ molecules tightly bound to GM1 clusters because the molecular size of their complex has been considered to exceed the size limitation of conventional high-resolution NMR analyses.

In the previous studies, we have demonstrated that 920 MHz ultra-high field NMR spectroscopic techniques provide improved spectral resolution and sensitivity, which are extremely advantageous in the structural analyses of biological macromolecules [13, 14]. In view of this situation, we herein characterize the conformation of Aβ(1–40) bound to the micelles of GM1 and lyso-GM1 using stable-isotope-assisted ultra-high field NMR spectroscopy. We successfully applied these techniques to the structural analyses of ^{2}H-, ^{13}C- and ^{15}N-labeled Aβ(1–40) and thereby obtained detailed information about its conformation in association with the glycolipid micelles.

Materials and methods

Materials

Powdered bovine brain GM1 ganglioside and lyso-GM1 were purchased from Sigma-Aldrich and from Takara Bio Inc., respectively. GM1-pentasaccharide, Galβ1→3-GalNAcβ1→4(Neu5Acα2→3)Galβ1→4Glc, was from IsoSepAB. Choresterol, L-α-phosphatidylglycerol-DL-glycerol sodium salt (PG) from egg yolk lecithin and sphingomyelin (SM) from bovine brain were purchased from Sigma-Aldrich.

Construction of plasmids

The expression vector encoding Aβ(1–40) was constructed as described in the literature [15] with slight modifications. The DNA fragment encoding Aβ(1–40) was amplified by the PCR from cDNA of full-length human Amyloid Precursor Protein as a template with the following primers: 5''-GTCTACTCTTCATCTTGTCTTAAGACTTC GTGGTGGTTAAC-3'' and 5''-GTTAACCACCACGAAG TCTTAAGACAAGATGAAGAGTAGAC-3''. The *AflII* site was introduced into a DNA encoding ubiquitin (Ub) using the site-directed mutagenesis method with the following primers: 5''-TTTCTTAAGACTTCGTGGTGG TGATGCAGAATTCCGACAT-3'' and 5''-ATG

GTGGGCGGTGTTGTCTAACTCGAGTTT-3′′. The DNA fragment corresponding to Ub-fused Aβ(1–40) was cloned into pET28a(+) plasmid vector (Novagen) with an N-terminal hexa-histidine tag moiety.

Expression and purification of Aβ(1–40)

The expression and purification of recombinant Aβ(1–40) were performed as described in the literature with slight modifications [15]. For the production of isotopically labeled Aβ(1–40) protein, cell were grown in M9 minimal media containing [^{15}N]NH_4Cl (1 g/L), [U-$^{13}C_6$] glucose (2 g/L) and/or ^2H_2O. Aβ(1–40) was dissolved at an approximate concentration of 2 mM in 0.1% (*v*/*v*) ammonia solution then collected and stored in aliquots at −80°C until use.

Preparation of micelles and vesicles

GM1 and lyso-GM1 was dissolved in methanol. The solvent was removed by evaporation in a rotary evaporator. The residual ganglioside was suspended at a concentration of 12 mM in 10 mM potassium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and 0.05 mM NaN_3, and then vortex-mixed. A suspension of PG vesicles was prepared in a similar manner. Micelle sizes were determined by dynamic light scattering using a DynaPro Titan (Wyatt technology). Large unilameller vesicles composed of GM1/choresterol/SM (40:30:30) were prepared according to the literature [16], except that we used the buffer described above.

Dot blot analysis

Mouse monoclonal antibodies 6E10 (COVANCE), 4G8 (COVANCE), and 1A10 (IBL), which are directed against the amino acid residues 1–10, 17–24, and 35–40, respectively, of human Aβ(1–40), were used for the dot blot analysis. Aβ(1–40) (0.2 mM), GM1 (6 mM) and their mixture with a 1:30 molar ratio of Aβ(1–40) and GM1 were incubated at 4°C for 1 hr and blotted onto nitrocellulose membranes (GE Osmonics) as described elsewhere [8]. The blots were reacted with 6E10 (1:10,000), 4G8 (1:5,000) or 1A10 (1:500), and subsequently with horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Tec). The bound-enzyme activities were visualized with an enhanced chemiluminescence system (GE Healthcare).

CD measurements

Aβ(1–40) was dissolved at a concentration of 0.05 mM in 10 mM potassium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and 0.05 mM NaN_3. CD spectra were measured at 37°C on a Jasco J-725 apparatus, using a 1.0-mm path length quartz cell. Eight scans were averaged for each sample. The averaged blank spectra were subtracted.

NMR measurements

NMR spectral measurements were made on a JEOL EC-920 spectrometer employing GORIN application [17] as well as a Bruker DMX-500 spectrometer equipped with a cryogenic probe. The probe temperature was set to 37°C. Isotopically labeled Aβ(1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer containing 0.5 mM EDTA, 0.05 mM NaN_3 and 10% (*v*/*v*) ^2H_2O in the presence or absence of 6 mM GM1 or lyso-GM1. For ^{1}H-^{15}N TROSY measurements [18], the spectra were recorded using ^{2}H- and ^{15}N-labeled Aβ(1–40) at a ^{1}H observation frequency of 920 MHz with 96 (t_1) x 1024 (t_2) complex points and 128 scans per t_1 increment. The spectral width was 2,760 Hz for the ^{15}N dimension and 11,040 Hz for the ^{1}H dimension. The ^{1}H, ^{13}C, and ^{15}N resonances of the backbone were assigned using a standard set of double- and triple-resonance experiments [19]. NMR spectra were processed and analyzed with the program nmrPipe/Sparky. ^{1}H chemical shifts were referenced to external 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS), while ^{13}C and ^{15}N chemical shifts were indirectly referenced to DSS by using the absolute frequency ratios. Secondary structural elements were identified based on the backbone chemical shifts by using the program TALOS [20]. For the saturation transfer experiments [21, 22], ^{1}H-^{15}N TROSY spectra were recorded using ^{2}H- and ^{15}N-labeled Aβ(1–40) peptide with on-resonance irradiation at 1.3 ppm (the acyl chains of lyso-GM1 or GM1) and at 4.69 ppm (H_2O) along with off-resonance irradiation at 40.0 ppm by continuous wave technique with an irradiation duration of 2.5 s. The strength of the irradiation field was adjusted to 24 Hz and 63 Hz for the saturation of water and the acyl chains, respectively, in the experiments by observing Aβ(1–40) bound to GM1 micelles, while a saturating field of 64 Hz was used for observation of lyso-GM1-bound Aβ(1–40).

Results and discussion

Overall structure of GM1-bound Aβ

For structural characterization of Aβ(1–40) bound to GM1 micelles, we first examined its reactivity with the monoclonal antibodies directed against the different parts of the Aβ(1–40) molecules. The dot blot analysis showed that the reactivity to 4G8 and 1A10 were significantly compromised in the presence of the GM1 micelles, while 6E10 was

bound to Aβ(1–40) in a GM1-independent manner (Fig. 1). These data suggest that the N-terminal region (Ala^{1} - Tyr^{10}) of Aβ(1–40) is exposed, whilst the middle (Leu^{17}-Val^{24}) and the C-terminal (Met^{35}-Val^{40}) regions are buried in the GM1 micelles.

The CD data indicated that Aβ(1–40) adopts an α-helical structure upon binding to the GM1 micelles whereas neither the PG vesicles nor the carbohydrate moiety released from GM1 induced such conformational change (Fig. 2). The inability of Aβ(1–40) to interact with PG and with the isolated pentasaccharide has been consistent with what was previously reported [12, 23, 24]. Intriguingly, the smaller lyso-GM1 micelles also interacted with Aβ(1–40) and thereby induced its α-helix formation as the larger GM1 micelles and the GM1-containing liposome did (Fig. 2 and Supplementary Fig. 1). These data suggest that the specific carbohydrate moieties clustered on a hydrophilic/hydrophobic interface are prerequisites for the interaction of Aβ(1–40) and its consequent conformational alteration.

Interaction mode of Aβ with ganglioside clusters

To provide a more detailed understanding of the interaction mode of Aβ and gangliosides, we attempted to carry out high resolution NMR analyses. Dynamic light scattering data showed that lyso-GM1 and GM1 form large micelles with approximate molecular masses of 60 kDa and 140 kDa, respectively, which hamper conventional NMR analyses due to severe line-broadening resulting from slower molecular tumbling. To cope with this difficulty, we performed ultra-high field NMR analyses of isotopically labeled Aβ(1–40) bound to the micelles. ^{1}H-^{15}N TROSY spectral data indicated that lyso-GM1 and GM1 micelles cause chemical shift changes for most of the Aβ(1–40) residues in similar fashions (Fig. 3 and Supplementary Fig. 2). The torsion angle prediction based on the backbone chemical shifts of Aβ(1–40) (Supplementary Table 1) revealed that the segments His^{14} - Val^{24} and Ile^{31}-Val^{36} form two discontinuous α-helices flanked by the N- and C-terminal random-coil regions upon binding to lyso-GM1 micelles (Fig. 4a). The α-helical content calculated from the CD data (ca. 23%) was significantly lower than what is expected from the NMR-based estimation. Discrepancies in α-helical contents between CD and NMR measurements have been widely recognized because far-ultraviolet CD of peptides are generally influenced by helix fraying and non-ideal helix geometry and by the presence of aromatic residues in a helical segment resulting in underestimation of the helical content [25]. It has been reported that the two α-helices are formed in the almost corresponding regions of Aβ(1–40) molecules in organic solvents-containing media as well as in SDS micelles [26–29].

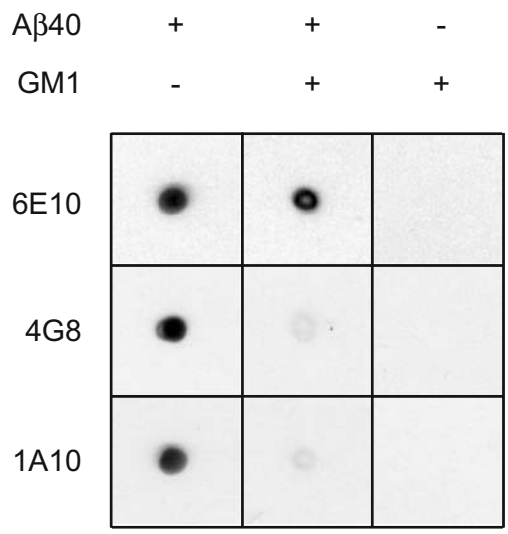

Fig. 1 Dot blot of Aβ(1–40) incubated in the presence and absence of GM1 micelles. Aβ(1–40), GM1 and a mixture of Aβ(1–40) and GM1 at a 1:30 molar ratio were incubated and blotted onto nitrocellulose membranes. The blot was reacted with monoclonal antibodies 6E10, 4G8, and 1A10, which are directed against the amino acid residues 1–10, 17–24, and 35–40, respectively

Deuteration of Aβ(1–40) also prompted us to determine its position with respect to the gangliosidic micelles by TROSY-based saturation transfer experiments. In the present study, the selective irradiation of the complex with a frequency corresponding to the proton resonances of the lyso-GM1 acyl chains (CH_2) primarily affects the micelles exclusively, because there exists no aliphatic protons in the deuterated Aβ(1–40) molecules. Upon the irradiation, the effective saturation resulted as caused by the proton resonances originating from the hydrophobic interior of the micelles, due to spin diffusion, and was further transferred to the amide groups of Aβ(1–40) in contact with the hydrophobic environment through the cross-saturation phenomena [21]. A comparison of the peak intensities of the amide groups of Aβ(1–40) bound to the micelles on the ^{1}H-^{15}N TROSY spectra with on-resonance and off-resonance irradiation allow us to identify the micelle-buried segments, *i.e.* Val^{12}-Gly^{25}, Ile^{31}-Val^{36}, and Val^{39}-Val^{40}. On the other hand, the selective saturation of H_2O resonance resulted in the attenuation of the amide peaks originating from the regions Glu^{3}-Tyr^{10} and Ser^{26}-Ala^{30} segments, and the Gly^{37}-Gly^{38} dipeptide, indicating that these segments were exposed to the aqueous environment and therefore undergo rapid hydrogen exchange. On the basis of these obviously complementary profiles of the effects caused by the CH_2 and H_2O irradiation, we conclude that the Aβ(1–40) molecules in association with the lyso-GM1 micelles exhibit an up-and-down topological mode in which the two α-helices and the C-terminal Val^{39}-Val^{40} dipeptide contact with the hydrophobic interior,

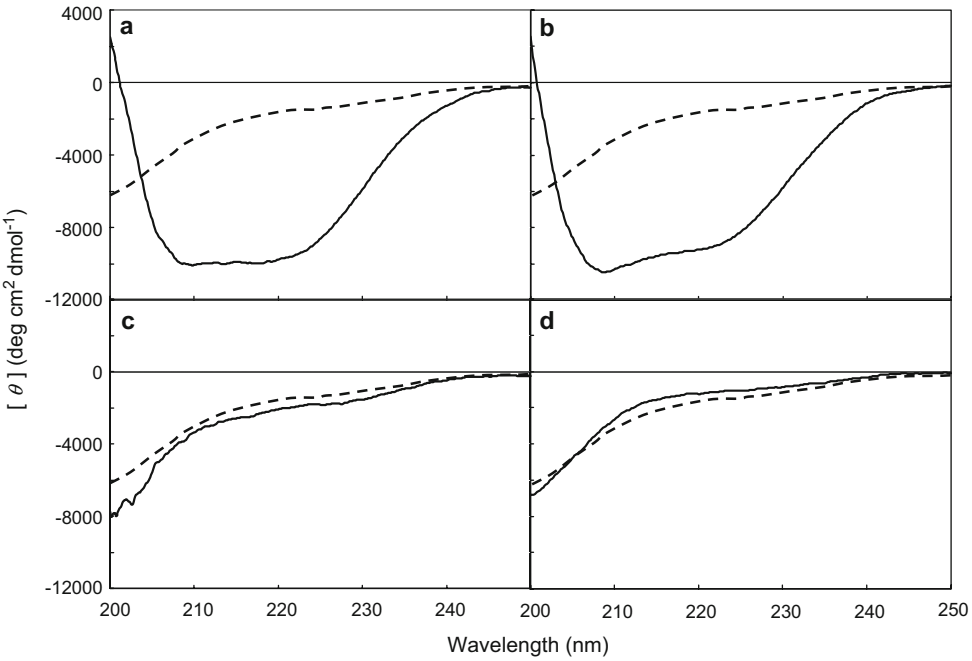

Fig. 2 CD spectra of 50 μM Aβ(1–40) were recorded in the absence (*dashed line*) and presence of GM1 (1.5 mM) **a**; lyso-GM1 (1.5 mM) **b**; the carbohydrate moiety alone of GM1 (1.5 mM) **c**; and PG (1.5 mM) **d**

whilst the N-terminal segment, the linker connecting the two helices, and the penultimate Gly^{37}-Gly^{38} dipeptide are exposed to water-accessible environment (Fig. 4b). Aβ(1–40) bound to GM1 micelles exhibited similar saturation transfer profiles with that of lyso-GM1-bound Aβ(1–40) although the peaks originating from the segment spanning residues His^{13}-Phe^{20} were not detectable due to severe broadening and the solvent exposure of the Gly^{37}-Gly^{38} dipeptide was less pronounced (Supplementary Fig. 3). The dot blot data are consistent with these saturation transfer data.

Williamson *et al*. have characterized the interactions of Aβ(1–40) with GM1 and asialo-GM1 micelles by inspection of their NMR titration data [12]. They have reported that the amino acid residues localized to the N-terminal half of Aβ(1–40) exhibit progressive chemical shift changes, although subtle, depending on their sialic acid moieties, while those in the C-terminal half show little or no chemical shift changes rather loss of peak intensity in sialic acid-independent manners. On the basis of the direction of the observed chemical shift change of the amide groups, they concluded that binding to GM1 micelles causes a confor-

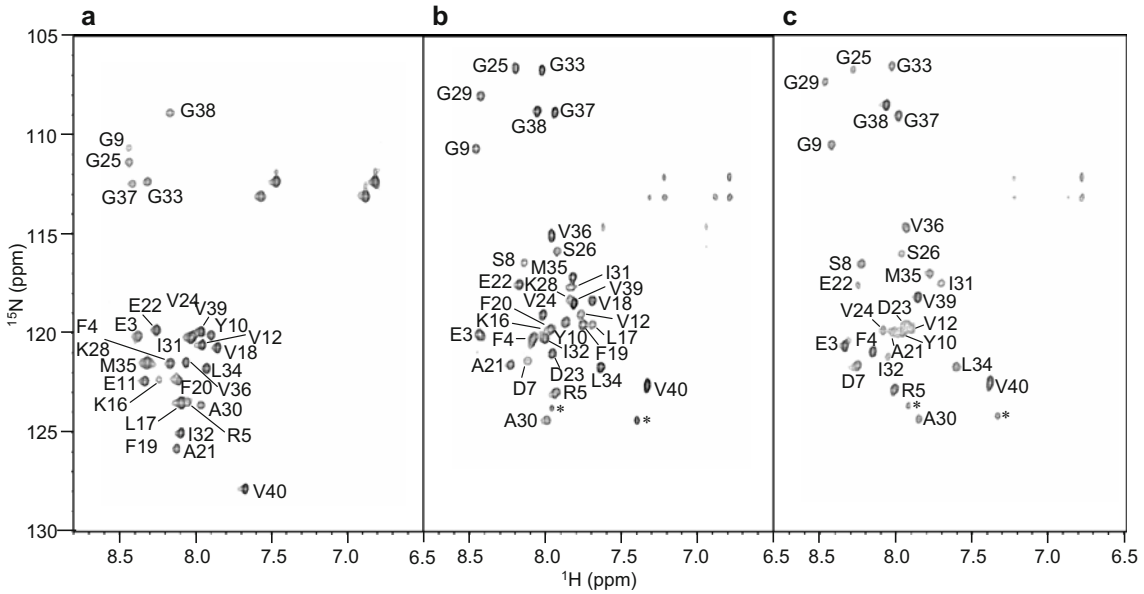

Fig. 3 21.6 T ^{1}H-^{15}N shift correlation spectra of isotopically labeled Aβ(1–40) in the absence (**a**) and presence of lyso-GM1 (**b**) or GM1 (**c**) micelles. The HSQC (**a**) and TROSY (**b** and **c**) spectra are presented. *Asterisk* indicates the peak originating from the gangliosidic micelles

mational transition from random coil to α-helix in the N-terminal region, leaving the C-terminal region unstructured. This apparent discrepancy from our findings can be explained based on the fact that many of the resonances affected upon titration under the Aβ(1–40)-excess condition did not move directly towards the peak position corresponding to the fully micelle-bound state shown herein (Supplementary Fig. 2). This means that the progressive, subtle chemical shift change under the Aβ(1–40) excess condition occur from a binding process of Aβ(1–40) to form a weak complex with GM1 micelles in fast exchange with the free state, as recently observed in coupled folding and binding of the phosphorylated kinase inducible activation domain of the transcription factor CREB onto its target protein [30]. Inspection of all these data suggests that Aβ(1–40) forms a weak encounter complex with the gangliosidic micelles through the interaction between its N-terminal region and the sialic acid moieties and subsequently forms α-helices in its C-terminal region, which are stabilized presumably by the hydrophobic interactions with the acyl chains of the glycolipid molecules.

Using NMR and paramagnetic probes, Gräslund and coworkers have shown that Aβ(1–40) exhibits a similar positioning in SDS micelles with the exposed N-terminal segment and the buried α-helices [27]. Noteworthy, they have demonstrated that the C-terminal segment of Aβ(1–40) in the SDS micelles is exposed to the Mn^{2+} ions added in the solution and exhibits higher mobility [27, 29]. In

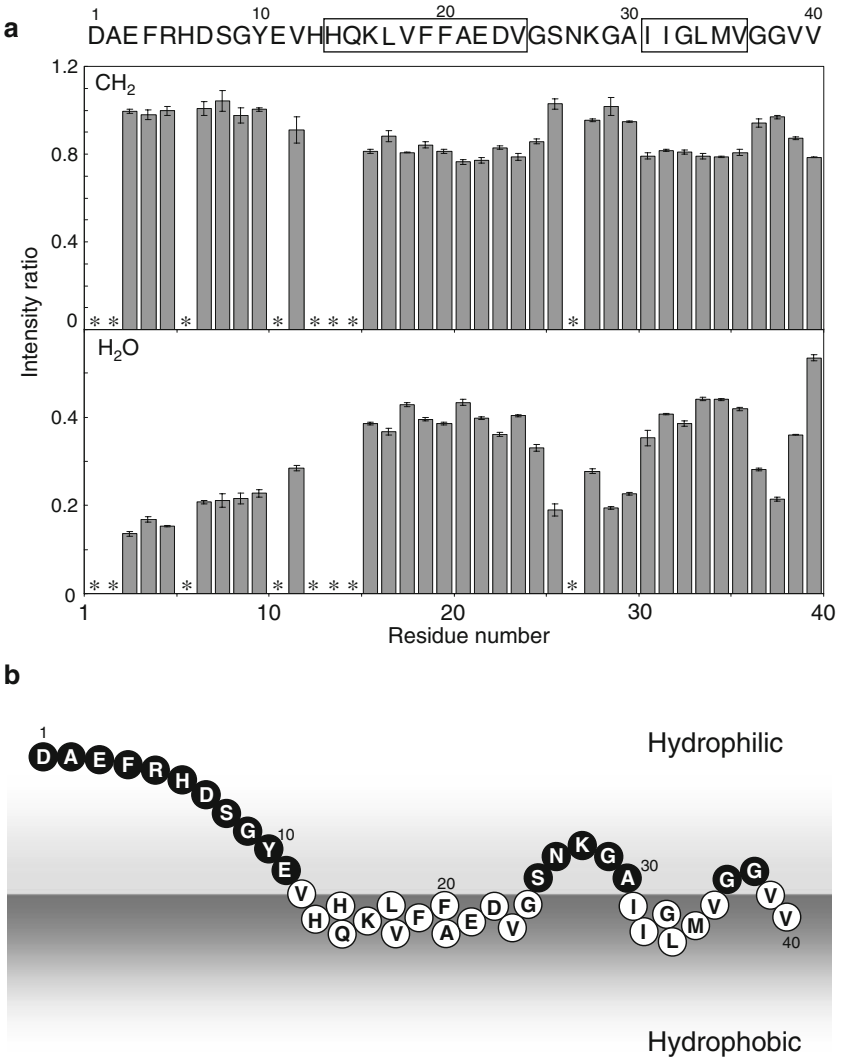

Fig. 4 **a** Plots of the intensity ratios of the backbone amide peaks of Aβ(1–40) with on-resonance and off-resonance irradiation of the acyl (CH_2) groups of lyso-GM1 (*upper*) and H_2O (*lower*). The primary structure of Aβ(1–40) peptide with *boxes* indicating the α-helical regions is represented at the top of the plots. *Asterisk* indicates the amino acid residue that did not exhibit observable peak in the spectrum due to severe broadening. Intensity ratio are the mean±S.D. of three independent experiments. **b** The topological model of Aβ(1–40) lying on ganglioside clusters based on the present NMR data. The amino acid residues exposed to the hydrophilic and hydrophobic milieus are represented by *closed and open circles with single-letter codes*

contrast, the present saturation transfer data of Aβ(1–40) in the lyso-GM1 micelles clearly indicate that the C-terminal Val^{39}-Val^{40} dipeptide is inserted into the hydrophobic interior, while the preceding Gly^{37}-Gly^{38} dipeptide is exposed to the solvent. Indeed, deletion of the C-terminal valine residue of Aβ(1–40) resulted in an impaired binding to GM1 micelles (M. Utsumi *et al.* unpublished data). Conversely, it is possible that the additional hydrophobic Ile-Ala dipeptide at the C-termini of Aβ(1–42) serves as an anchor enhancing interaction with the GM1 clusters. Mandal and Pettegrew have reported that asialo-GM1 embedded in SDS micelles affected conformation and dynamic of the C-terminal segment of Aβ(1–40) by a different fashion of interaction with the GM1 micelles used in the present study [31]. All these data suggest that the interaction mode of the C-terminal segment of Aβ(1–40) is influenced by the existence, abundance and structures of the carbohydrate moieties displayed on micelles.

Accumulating evidence, including the present data, indicates that the GM1 clusters in lipid bilayers provide a unique platform for binding, conformational transition, and subsequent interaction of Aβ molecules [7]. It is easily conceivable that the up-and-down topological mode restricts the spatial rearrangements of Aβ molecules on the hydrophilic/hydrophobic interface and thereby promotes their specific intermolecular interactions. The next important issue is the structural characterization of putative Aβ-Aβ interactions on the ganglioside clusters. Application of the recently sophisticated NMR techniques [30, 32] would be a valuable approach for observation of the transient interactions of Aβ molecules on various ganglioside clusters. The structural information provided in the present study would offer fundamental insights into the molecular mechanisms of the Aβ action in the pathogenesis of AD and the opportunity for designing drugs that target the Aβ molecules bound to the ganglioside clusters in the brain.

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