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Interaction Models of Substrate Peptides and β-Secretase Studied by NMR Spectroscopy and Molecular Dynamics Simulation

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The formation of β-amyloid peptide (Aβ) is initiated from cleavage of amyloid precursor protein (APP) by a family of protease, α-, β-, and γ-secretase. Sub W, a substrate peptide, consists of 10 amino acids, which are adjacent to the β-cleavage site of wild-type APP, and Sub M is Swedish mutant with double mutations on the left side of the β-cleavage site of APP. Sub W is a normal product of the metabolism of APP in the secretary pathway. Sub M is known to increase the efficiency of β-secretase activity, resulting in a more specific binding model compared to Sub W. Three-dimensional structures of Sub W and Sub M were studied by CD and NMR spectroscopy in water solution. On the basis of these structures, interaction models of β-secretase and substrate peptides were determined by molecular dynamics simulation. Four hydrogen bonds and one water-mediated interaction were formed in the docking models. In particular, the hydrogen bonding network of Sub M-BACE formed spread over the broad region of the active site of β-secretase (P5-P3′), and the side chain of P2-Asn formed a hydrogen bond specifically with the side chain of Arg235. These are more favorable to the cleavage of Sub M by β-secretase than Sub W. The two substrate peptides showed different tendency to bind to β-secretase and this information may useful for drug development to treat and prevent Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is characterized by the progressive formation of insoluble amyloid plaques and neurofibrillary tangles in the brain (Cras et al., 1991; Kagan et al., 2004; Li et al., 2004; Tang et al., 2005). Amyloid precursor protein (APP) is a single transmembrane protein with a 590 to 680 amino acid long extracellular amino terminal domain and an approximately 55 amino acid cytoplasmic tail that contains intracellular trafficking signals. Amyloid β-peptide (Aβ) is the major component of the plaques; the misfolding of Aβ leads to its dysfunctions and fibrillization, the latter of which is associated with a cascade of neuropathogenetic events that produce the cognitive and behavioral decline hallmarks of AD (Buxbaum et al., 1990; Xu et al., 2005). The formation of Aβ is initiated from the cleavage of APP by a family of protease, α-, β-, and γ-secretase (Numan et al., 2000). Because β-secretase initiates the cleavage of APP, β-secretase is the most important drug target for AD than γ-secretase (Haass et al., 2004; Numan et al., 2000; Roggo et al., 2002).

β-secretase, also known as β-site of APP cleaving enzyme (BACE) or memapsin-2, is one of the aspartic protease and transmembrane proteins, contains two active site aspartate residues in its extracellular protein domain, and may function as a dimer (Citron et al., 2004; Schmechel et al., 2004; Vassar et al., 1999). The three-dimensional structure of human β-secretase has been determined by crystallography (Hong et al., 2000; Patel et al., 2004).

Aβ is a normal product of the metabolism of APP in the secretary pathway. A number of missense mutations in APP have been implicated in forms of early-onset familial AD (Esler et al., 2001). All these mutations are at or near one of the canonical cleavage sites of APP. Mutations in APP linked to autosomal dominant inheritance of AD have been found to alter the production of Aβ. β-Secretase cleavage is highly sequence specific. The Swedish double mutation of the proximal to the N-terminus of Aβ (K670N and M671L) found in the "Swedish" familial AD mutation, which causes early-onset AD, is immediately adjacent to the β-cleavage site and increases the efficiency of β-secretase activity, resulting in more total Aβ (Haass et al., 1995).

We focused on a 10 amino acid region of both wild-type APP (Sub W) and APP that contains Swedish mutations on the left side of the β-cleavage site (Sub M) to determine the interaction model with BACE. The cleavage site of β-secretase was located in the center of the substrate peptides (Na et al., 2007). Schematic models of Sub W and Sub M are presented in Fig. 1. According to the standard protease nomenclature, these residues are labeled P5-P4-P3-P2-P1-P1′-P2′-P3′-P4′-P5′, and the actual cleavage site is located between the P1 and P1′ regions (Sauder et al., 2000).

The specificity constant (k_{cat}/K_m) of Sub M is known to be 60-fold higher than that of Sub W, which is consistent with the increased Aβ production and the early onset of Swedish familial

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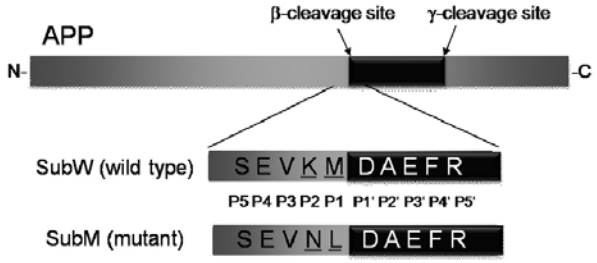

Fig. 1. The structure of the amyloid precursor protein

AD (Roggo et al., 2002; Toulokhonova et al., 2003). Sub M in transfected cells increases the production of Aβ by approximately 5-fold, suggesting a causal link between altered APP processing and the development of AD (Martin et al., 1995).

In this study, we determined the three-dimensional structures of two substrate peptides, Sub W and Sub M, by NMR spectroscopy and proposed binding models of peptides and BACE studied by molecular dynamics simulation.

MATERIALS AND METHODS

Preparation of substrate peptides

Sub W and Sub M were synthesized with purity of more than 95% by A&Pep Co., Inc. (Korea). The purity and identity of the peptides were verified by high-performance liquid chromatography (HPLC) and mass spectrometry.

Circular dichroism analysis

Circular dichroism (CD) experiments were performed using a J-820 spectropolarimeter (Jasco, Japan) with a 1-mm path length cell. The CD spectra of the peptides at 100 μM were recorded at 283 K, 298 K, 308 K, and 318 K at 0.1 nm intervals from 190 to 250 nm. To investigate the conformational changes induced by water, a solution of a defined composition was added to the peptides. For each spectrum, the data from 10 scans were averaged and smoothed using J-820. Data were expressed as the mean residue ellipticity [θ] in deg•cm^2•dmol^{-1}.

Nuclear magnetic resonance experiments

Peptides were dissolved at 1.0 mM in 0.45 ml 90% H_2O/10% D_2O to investigate the free conformation of Sub W and Sub M. Phase-sensitive, two-dimensional experiments, including double-quantum-filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY), were performed by time-proportional phase incrementation (Bax and Davis, 1985a; 1985b; Citron et al., 2004; Derome et al., 1990; Marion et al., 1983). For TOCSY and NOESY experiments, 450 to 512 transients with 2K complex data points were collected for each increment, with a relaxation delay of 1.2 s between the successive transients, and the data along the t1 dimension were zero-filled to 1 K before two-dimensional Fourier transformation. TOCSY experiments were performed using 50- and 70-ms MLEV-17 spin-lock mixing pulses. Mixing times of 150 and 250 ms were used for NOESY experiments. For DQF-COSY experiments, 512 transients with 4K complex data points were collected for each increment, and the data along the t1 dimension were zero-filled to 4K before the two-dimensional Fourier transformation. The $^3J_{HN\alpha}$ coupling constants were measured from the DQF-COSY spectra with a spectral width of 4194.631 Hz and a digital resolution of 1.02 Hz/point. Chemical shifts are expressed relative to the DSS signal at 0 ppm. All the NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer in Konkuk University and KBSI. NMR spectra were processed with NMRPipe (Delaglio et al., 1995) and visualized with Sparky (Goddard et al., 2001).

Structure calculations of substrate peptides

Distance constraints were extracted from the NOESY spectra with mixing times of 150 and 250 ms. The volumes of the nuclear Overhauser effects (nOes) between the two beta protons of the Phe residue were used as references. All other volumes were converted into distances by assuming a $1/r^6$ distance dependence. All the NOE intensities were divided into three classes depending on their distance ranges: strong, 1.8 to 2.7 Å; medium, 1.8 to 3.5 Å; and weak, 1.8 to 5.0 Å (Clore et al., 1989; 1994). Structure calculations were carried out using X-PLOR version 3.851 with the topology and parameter sets topallhdg.pro and parallhdg.pro, respectively. Standard pseudoatom corrections were applied to the non-stereospecifically assigned restraints (Wüthrich et al., 1983), and an additional 0.5 Å was added to the upper bounds for NOEs involving methyl protons (Clore et al., 1987). A hybrid distance geometry-dynamical simulated annealing protocol was employed to generate the structures (Kuszewski et al., 1992; Nilges et al., 1988). The target function minimized during the simulated annealing comprises only quadratic harmonic potential terms for covalent geometry, square-well quadratic potentials for the experimental distance, torsion angle restraints, and a quartic van der Waals repulsion term for the nonbonded contacts. The target function did not contain hydrogen bonding, electrostatic, or 6-12 Lennard-Jones empirical potential energy terms. A total of 50 structures were generated, and of these, 20 structures with the lowest energies were selected for further analysis.

Docking and molecular dynamics study of β-secretase and peptides

We performed molecular dynamics (MD) simulations of Sub W and Sub M with BACE. Information available from the known crystal structure of BACE was used to determine the putative ligand-binding pocket of Sub W and Sub M (Hong et al., 2000; Hu et al., 2006; Na et al., 2007). The starting structures of the substrate peptides were obtained by NMR spectroscopy, as mentioned in the previous section. Two peptides were placed at the binding region, and the ligand-receptor complex was subjected to energy minimization. An automated docking study was performed for these models using AutoDock (Goodsell et al., 1996). In order to determine the precise conformation of the ligand–receptor complex, MD simulation was performed at a constant temperature of 298 K for 2 ns. An explicit solvent model TIP3P water was used, and the solvent model was constructed with a 15 Å water cap from the center of mass of BACE. The Discover module of InsightII, which is a software package of Accelrys (Accelrys, USA), was used for MD simulation. The consistent-valence forcefield (CVFF) was used for energy minimization and MD simulations. These simulations were performed in Linux environment.

RESULTS AND DISCUSSION

Structures of substrate peptides

CD spectra in Fig. 2 show that Sub W has a random structure and Sub M has a turn structure in an aqueous solution. We tried structural studies of amyloid peptides in various buffer conditions. Aqueous solution gave the best resolution in NMR spectra for the structural determination. Therefore, all NMR experiments were performed in aqueous solution. To confirm

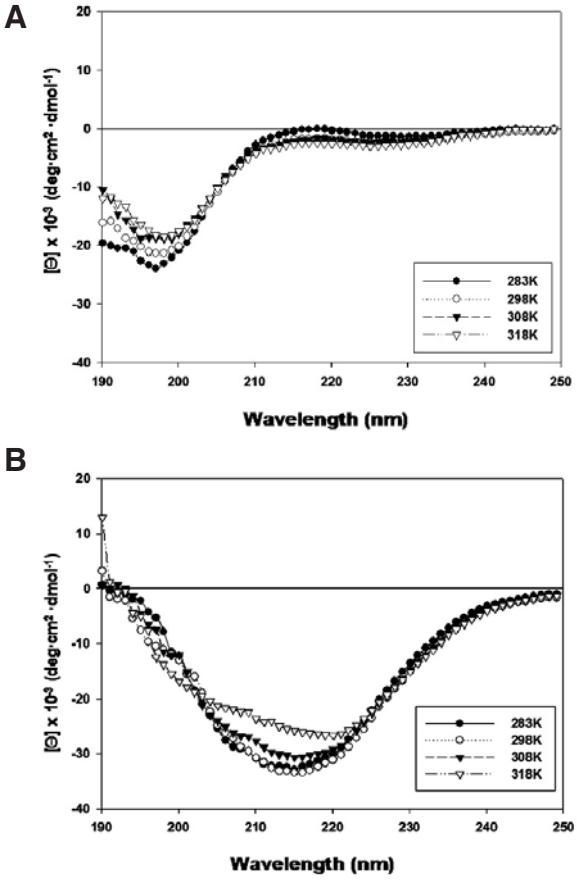

Fig. 2. The CD spectra of Sub W and Sub M in an aqueous solution. (A) Sub W and (B) Sub M.

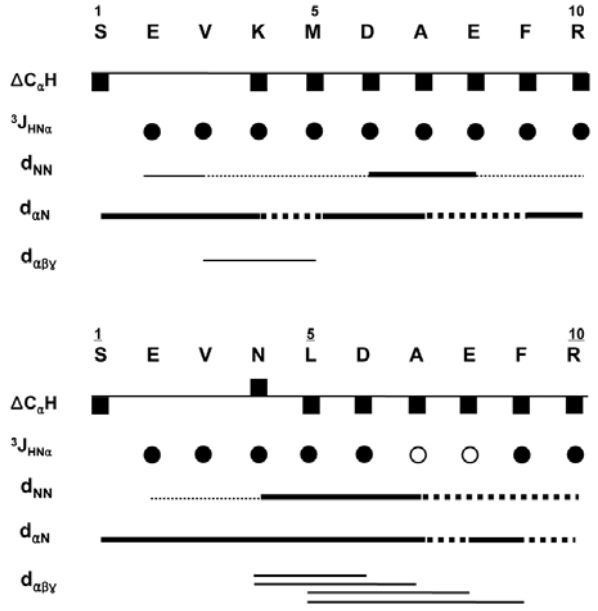

Fig. 3. NOE connectivity, $J_{HN\alpha}$ coupling constant (●, $J_{HN\alpha}$< 6 Hz), and CαH chemical shift index for (A) Sub W and (B) Sub M in an aqueous solution. Line thickness for the NOEs reflects the intensity of the NOE connectivities.

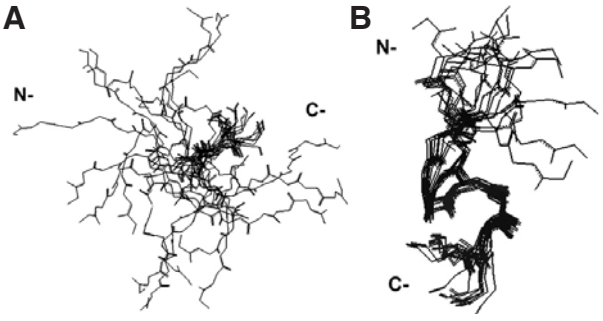

Fig. 4. The superposition of the 20 lowest energy structures calculated from the NMR data. (A) Sub W and (B) Sub M in an aqueous solution.

the three-dimensional structure of these two peptides, we made sequence-specific resonance assignments using mainly the DQF-COSY, TOCSY, and NOESY data (Han et al., 2008; Wüthrich et al., 1986).

As shown in Fig. 3, for Sub W, only one medium-range NOEs (|i-j| = 2) was observed between P3-Val and P1-Met. For Sub M, 11 nonsequential medium-range NOEs such as P2-Asn/P1′-Asp, P2-Asn/P2′-Ala, P1-Leu/P3′-Glu, and P1-Leu/P4′-Phe were observed. The observed values of the $^3J_{HN\alpha}$ coupling constants for the turn region of all the peptides were generally from 7 Hz to 9 Hz.

To calculate the tertiary structures of Sub W and Sub M, we used experimental restraints such as distance restraints and torsion angle restraints. For Sub W, 8 sequential restraints and 1 medium-range NOEs were used. For Sub M, 24 sequential restraints and 11 medium-range restraints were used. Torsion angle restraints were used for all residues.

We analyzed 20 output structures with the lowest energy for each peptide. Figure 4 shows the superposition of the 20 lowest energy structures of Sub W (A) and Sub M (B) over the backbone atoms in H_2O. When we superimposed the 20 lowest energy structures of Sub W (from P2′-Ala to P5′-Arg) and Sub M (from P1-Leu to P3′-Glu) over the backbone atoms, their root-mean-squared deviations (RMSDs) from the mean structures were 0.927 ± 0.23 Å and 0.163 ± 0.04 Å for the backbone atoms (N, Cα, C′, O), and 2.111 ± 0.21 Å and 0.748 ± 0.15 Å for all heavy atoms, respectively. According to the Procheck analysis, Sub M has β-turn structures (type IV) from P2-Asn to P2′-Ala, from P1-Leu to P3′-Glu, from P1′-Asp to P4′-Phe, and from P2′-Ala to P5′-Arg (Laskowski et al., 1993). These results imply that Sub W has a random coil structure, while Sub M has more stable β-turn structure.

Docking and MD study

To appreciate the interaction between BACE and Sub W and between BACE and Sub M, the initial docking models of BACE with peptides are determined by automated docking and MD simulation. The three-dimensional conformation of the complex structure is described in Fig. 5A. During simulation, these peptides were highly mobile with an RMSD of 1.7 (Sub W) and 2.1 Å (Sub M) from the initial structure. The backbone structure of Sub W retained a random coil conformation during the simulation, while several turn structures (from P2 to P5′) of Sub M were extended to a random structure.

The active site of BACE consists of both hydrophilic and small

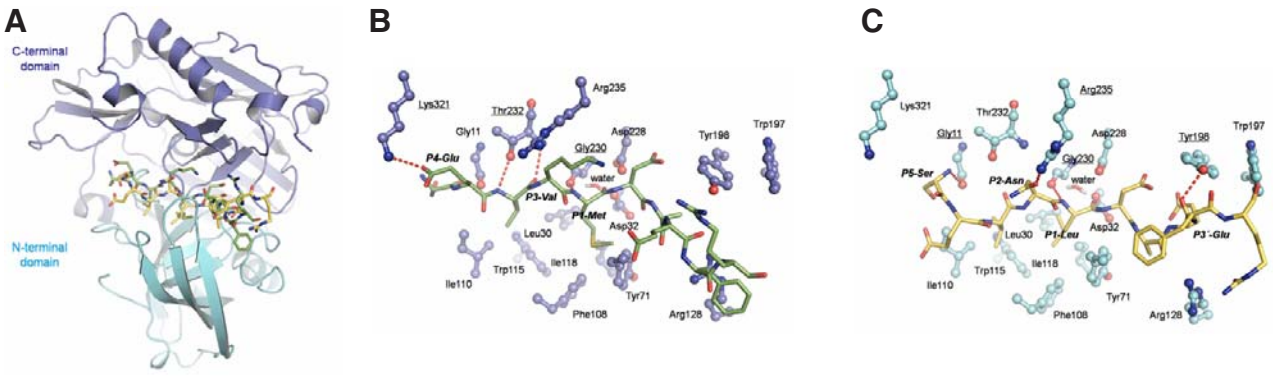

Fig. 5. Docking model of two substrate peptides with BACE. (A) Superimposed model of two docking models; (B) Interaction Model of Sub W and BACE; and (C) Interaction Model of Sub M and BACE. The vicinity enzyme residues of docked peptides are also listed. Enzyme residues (shown as ball and stick) that participated in hydrogen bonding interactions with the peptide substrates are underlined.

hydrophobic pockets. Analysis of the interactions between substrates and enzyme based on docking results showed that the 2 substrate peptides had several hydrogen bonding interactions with BACE specifically.

Interactions between the substrate peptides and BACE

As mentioned, the active site of BACE consists of both hydrophilic and small hydrophobic pockets. Hydrophobic interactions between the two peptides and BACE at the active site are important for the interaction of the substrate and BACE (Turner et al., 2005). P3-Val of the two peptides participated in hydrophobic interaction with Ile30, Ile110, and Trp115 of BACE. Another phobic site is the P1 region, including Met of Sub W and Leu of Sub M. The P1 region formed a hydrophobic interaction with one aliphatic residue, Ile118, and 3 aromatic hydrophobic residues, Tyr71, Phe108, and Trp115.

Four hydrogen bonds and one water-mediated interaction were formed between BACE and peptides, and these are represented in Table 1 and Figs. 5B and 5C. Hydrogen bonding interactions between Sub W and BACE are localized on the P5-P1 region. The side chain oxygen of P4-Glu of Sub W formed a hydrogen bond with the side chain of Lys321. The backbone of P3-Val and P1-Met of Sub W participated in interactions with Thr232 and Gly230. The P3-Val of Sub W formed two tight hydrogen bonds with Thr232, and the backbone of P1-Met formed a hydrogen bond with the backbone carbonyl oxygen of Gly203.

Hydrogen bonding network between Sub M and BACE is spread from P5 to P3′, and the hydrogen binding distance between the atoms in Sub M are closer than that in Sub W. Backbone NH of P5-Ser and P1-Leu formed hydrogen bonds with the backbone carbonyl oxygen of Gly11 and Gly230, respectively, and P3′-Glu formed a hydrogen bond with side chain OH of Tyr198. In particular, in the docking model of Sub M-BACE, P2-Asn participated in hydrogen bonding interaction with Arg235 of BACE. Arg235 is known to be important in controlling the catalytic activity of BACE, which is located on the active site near the 2 catalytic aspartic acid residues, Asp32 and Asp228 (Rajamani et al., 2004; Toulokhonova et al., 2003). The P2-Lys of Sub W is replaced with Asn in Sub M. A hydrogen bond with Arg235 would vanish, and the positive charge would interact unfavorably with the side chain of Arg235 (Grüninger-Leitch et al., 2002). Among several aspartic proteases such as Cathepsin D and E, Pepsin, Napsin A, Rennin, BACE-2, and BACE, Sub M is known to show substrate activity only for BACE and BACE-2. Substrate specificity of these seven aspartic proteases is determined by the P2-P1 amino acid of the substrate (Gregor et al., 2006). Five proteases have a substitution of Arg235 (in case of BACE) with other amino acids such as Thr, Ser, or Val, which are among the non-positive charged amino acids; thus, the hydrogen bond between Sub M and Arg235 only subsists on binding BACE and BACE-2. Therefore, the hydrogen bonding interaction of Sub M with Arg235 might be specific and critical for the binding of the substrate of BACE and we confirmed this by MD simulation.

Table 1. List of hydrogen bonds between the two substrate peptides and BACE

Substrate	Residue of substrate	Atom	Residue of enzyme	Atom	Distance (Å)
Sub W	P4-Glu	OE1	Lys321	NZ	3.1
	P3-Val	N	Thr232	OG1	3.2
	P3-Val	O	Thr232	N	3.1
	P1-Met	N	Gly230	O	3.2
Sub M	P5-Ser	N	Gly11	O	3.2
	P2-Asn	OD1	Arg11	NH1	2.6
	P1-Leu	N	Gly230	O	2.8
	P3′-Glu	O	Tyr198	O	2.9

Sub M binds tightly tighter to BACE than Sub W and the dock energy of Sub M-BACE is lower than Sub W-BACE. This result may be an effect of spread hydrogen bonding interactions from P5 to P3′ region and the shorter hydrogen binding distance between the atoms of the Sub M-BACE complex. This could explain why the affinity of Sub M to BACE is much higher than that of Sub W.

Water-mediated interaction formed between Asp32 and APPs is well known (Brik et al., 2003; Suguna et al., 1987). A water molecule is placed between two catalytic Asp residues in aspartic protease and plays a role in the transfer of a proton from the enzyme to the substrate. According to our docking models, Asp32 activates the water molecule by abstracting a proton, and the water molecule can then attack the carbonyl carbon of the P1 residue of the APP's scissile peptide bond (Brik et al., 2003; Suguna et al., 1987). The water molecule of the Sub W-BACE model moved inside the active site approximately 1.0 Å compared with the Sub M-BACE model, and the distance between water and Sub M and that between water and Sub W are 1.7 and 2.6 Å, respectively. Conformation of the

Asp32-water-Sub M is more profitable than that of the Sub W model.

It is probable that spread hydrogen bonding interaction and forming a hydrogen bond between P2-Asn and Arg235 contributed to the cleavage of Sub M by the action of the two aspartic acid residues providing spatial advantage, and this advantage may increasingly contribute to the production of Aβ.

CONCLUSION

In this study, we determined three-dimensional structures of 10 amino acids substrate peptides, Sub W and Sub M, surrounding the β-cleavage site of APP by NMR spectroscopy in an aqueous solution. Sub W had a random coil structure, while Sub M has β-turn structures from P2-Asn to P2′-Ala, from P1-Leu to P3′-Asp, from P1′-Ala to P4′-Asp, and from P2′-Ala to P5′-Arg. We confirmed these features by CD data.

MD simulation was performed on two substrate peptides and BACE on the basis of the NMR structure of peptides. For the docking model of Sub W with BACE, all four hydrogen bonds were included, but it is probable that only four hydrogen bonds related P4-Glu, P3-Val, and P1-Met with Lys321, Thr322, and Gly230 and contributed to the enzyme-substrate binding. The hydrogen bonding network between Sub M and BACE was different from that of the Sub W model. In particular, the hydrogen bond that formed with the substrate P2-Asn side chain and the side chain of Arg235 of enzymes had the most important effect on the binding activity of Sub M as a substrate of BACE. This hydrogen bond vanished in the Sub W-BACE model. A water-mediated interaction was formed between Asp-32 and P1 residue of the 2 peptides. The water molecule of the Sub W and BACE model moved inside the active site at approximately 1.0 Å compared with the Sub M-BACE model. Conformation of Asp32-water-Sub M is more profitable than the Sub W model for cleavage by BACE.

In this study, we demonstrated docking models of two substrate peptides and BACE by NMR and MD simulations. The sequential difference between Sub W and Sub M caused discrepancy in the three-dimensional structures of the two substrate peptides and the biological mechanism that is related to cleavage by BACE. These differences were defined by the structure of substrate peptides and the formation of hydrogen bonds with enzyme. Therefore, understanding the substrate specificity of BACE is necessary for appreciation of its role in normal situations and in initiation of Alzheimer's disease. We demonstrated that the docking models presented here explain the specificity of β-secretase for the cleavage site in APPs that produces Aβ associated with Alzheimer's disease.

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