A pH-dependent conformational transition of A β peptide and physicochemical properties of the conformers in the glial cell

Yoichi MATSUNAGA^{*1}, Nobuhiro SAITO^{*}, Akihiro FUJII[†], Junichi YOKOTANI[†], Tadakazu TAKAKURA[†], Tomoaki NISHIMURA[‡], Hiroyuki ESAKI[‡] and Tatsuo YAMADA^{*}

^{*}Fifth Department of Internal Medicine, School of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0133, Japan, [†]Discovery Laboratories, Toyama Chemical Co. Ltd., Toyama, Japan, and [‡]Nippon Laser & Electronics Laboratory, Nagoya, Japan

In the present study we identified the epitopes of antibodies against amyloid β -(1–42)-peptide (A β 1–42): 4G8 reacted with peptides corresponding to residues 17–21, 6F/3D reacted with peptides corresponding to residues 9–14, and anti 5-10 reacted with peptides corresponding to residues 5–10. The study also yielded some insight into the A β 1–42 structures resulting from differences in pH. An ELISA study using monoclonal antibodies showed that pH-dependent conformational changes occur in the 6F/3D and 4G8 epitopes modified at pH 4.6, but not in the sequences recognized by anti 1-7 and anti 5-10. This was unique to A β 1–40 and A β 1–42 and did not occur with A β 1–16 or A β 17–42. The reactivity profile of 4G8 was not affected by blockage of histidine residues of pH-modified A β 1–40 and A β 1–42 with diethyl pyrocarbonate; however, the mutant [Gln¹¹]A β 1–40 abrogated the unique pH-dependence towards 4G8 observed with A β 1–40. These findings suggest that these

epitopes are cryptic at pH 4.6, and that Glu¹¹ is responsible for the changes. We suggest that the abnormal folding of 6F/3D epitope affected by pH masked the 4G8 epitope. A study of the binding of metal ions to A β 1–42 suggested that Cu²⁺ and Zn²⁺ induced a conformational transition around the 6F/3D region at pH 7.4, but did not affect the region when it was modified at pH 4.6. However, Fe²⁺ had no effect, irrespective of pH. A β modified at pH 4.6 appeared to be relatively resistant to proteinase K compared with A β s modified at pH 7.4, and the former might be preferentially internalized and accumulated in a human glial cell. Our findings suggest the importance of micro-environmental changes, such as pH, in the early stage of formation of A β aggregates in the glial cell.

Key words: Alzheimer's disease, anti-(amyloid β peptide) antibodies, epitope map, metal ions, protease resistance.

INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease and prion disease share the characteristic feature of accumulation of insoluble protein in the brain [1]. The pathological hallmark of Alzheimer's disease is the formation of insoluble deposits of amyloid β protein (A β) [2], which is mainly composed of A β 1–40 (A β 40) and A β 1–42 (A β 42) [3,4]. These peptides are derived by proteolytic cleavage of the transmembrane region of the 110–135 kDa amyloid precursor protein [5,6], probably in acidic vesicles such as lysosomes or endosomes [7,8]. However, the subsequent mechanisms by which soluble A β peptides are converted into insoluble forms remain unclear. Insoluble proteins in human tissue have the structure of a molten globule [9], and are characterized by β -sheet-rich conformational intermediates [10], stability under denaturing conditions and protease resistance [11]. Recently, a number of techniques, including CD [12], Fourier-transform IR spectroscopy [13], NMR [14], X-ray diffraction [15] and electron microscopy [16] have been employed to determine the secondary structure, conformation and morphology of A β , and much knowledge on the structural transition to the insoluble protein has been acquired.

The native conformation of A β is modulated by environmental factors that include pH, metal ions, temperature, peptide concentration and ionic strength, resulting in β -sheet-rich conformational intermediates [17]. We focused on the conformational

transition induced by pH and metal ions in the present study. The formation of A β aggregates at low pH has been reported [18], and electrostatic changes in aspartic acid and histidine residues in amyloidogenic proteins seem to be crucial for amyloidogenesis [19]. However, little is known about the influence of pH shifts on the folding of A β and which amino acid residues are directly affected by pH. The monoclonal antibodies anti 5-10 [20], 6F/3D [21] and 4G8 [22] have been used for A β 42 in the study of Alzheimer's disease, but there is no map of their epitopes. We performed epitope mapping for these monoclonal antibodies, and an ELISA study using the antibodies provided the critical pH value for A β folding and determined the amino acid residue responsive to pH.

It has been reported that metal ions promote the aggregation of A β *in vitro* and that they participate in the deposition of Alzheimer's senile plaque. Copper, iron and zinc significantly increase within amyloid plaques [23], and chemical modification and mutation studies have provided some information about the metal-binding site, which contains three histidine residues at positions 6, 13 and 14 of A β [24–26]. We investigated the effect of the bioessential metal ions Cu²⁺, Zn²⁺ and Fe²⁺ on A β 42 under two different pH conditions, pH 4.6 and 7.4, to determine the possible participation of these metal ions in the conformational transition in the A β peptide and their pH-dependence.

Furthermore, we characterized the different physicochemical properties of the pH-modified A β conformers in the human glial

Abbreviations used: A β , amyloid β peptide; DEPC, diethyl pyrocarbonate; PK, proteinase K; Fmoc, fluorenylmethoxycarbonyl; MBHA, 4-methylbenzhydrylamine.

¹ To whom correspondence should be addressed (e-mail yoichima@fukuoka-u.ac.jp).

cell. The amyloid proteins share resistance to the proteolytic enzymes that prevent accumulation of excessive or aged proteins in tissues [27,28]. The mechanism and factors involved seem to be polymerization, fibril formation and aggregation of the proteins, all of which are based on conformational changes such as the adoption of β -sheet structures [29]. We demonstrated intracellular accumulation of the A β conformer modified at pH 4.6 in glial cells. Here we suggest that the different physicochemical properties of the pH-modified A β peptides are based on the different susceptibilities of the conformers to proteinase K (PK). We suppose that pH-modified A β in an acidic state provides a possible structural intermediate in the early steps of A β aggregation.

EXPERIMENTAL

Materials

A β peptides

A β 1–42, A β 1–16, A β 17–42, A β 1–40 and [Gln¹¹]A β 1–40, in which Glu¹¹ is replaced by glutamine, were purchased from Anaspec (San José, CA, U.S.A.). Each A β derivative was dissolved at a concentration of 1 mg/ml in water, and the A β 17–42 peptide was dissolved in a minimal amount of DMSO before dilution with water.

Antibodies

4G8 (monoclonal) was purchased from Signet Pathology Systems Inc. (Dedham, MA, U.S.A.), 6F/3D (monoclonal) was from DAKO (Glostrup, Denmark), anti 5-10 (monoclonal) were from QCB (Camarillo, CA) (note: the product analysis sheet from QCB calls anti 5-10 'anti 1-16' because the immunogen was synthetic A β 1–16; however, our present results showed that its epitope was only A β 5–10). Anti 1-7 (polyclonal) [30] from QCB was produced against a chemically synthesized peptide A β 1–7 and the antiserum was negatively absorbed with the peptide corresponding to the region of both –5 to +7 and –2 to +10 of A β . As a secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG from Promega (Madison, WI, U.S.A.) and goat anti-rabbit IgG from Jackson ImmunoResearch (West Grove, PA, U.S.A.) was used for the monoclonal and polyclonal antibody respectively.

Chemicals

Rink Amide MBHA (4-methylbenzhydrylamine) resin and all the protected amino acids were purchased from NovaBiochem (Läufelfingen, Switzerland). BSA, *p*-nitrophenyl phosphate and diethyl pyrocarbonate (DEPC) were from Sigma (St Louis, MO, U.S.A.). Metals, including CuCl₂, ZnCl₂ and FeCl₂, were from Aldrich Chemicals (Milwaukee, WI, U.S.A.). PK and PMSF were from Boehringer Mannheim (Germany). Na₂HPO₄, citric acid, Tris and Tween-20 were from Sigma-Aldrich (Tokyo, Japan).

Methods

Synthesis of peptides

A series of peptides representing residues 1–32 of A β 42 that were eight residues in length and overlapped by seven residues at the N-terminus were synthesized manually using solid-phase peptide chemistry with fluoren-9-ylmethoxycarbonyl (Fmoc)-protected amino acids on Rink Amide MBHA resin. All reactions were performed at room temperature. Coupling with Fmoc-amino-

acid-protected groups was performed in the presence of benzotriazol-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate, 1-hydroxybenzotriazole hydrate and *N,N*-diisopropylethylamine in *N,N*-dimethylacetamide for 1 h, and the Fmoc-protected group was removed by treatment with 20 % piperidine in dimethylformamide for 20 min. The protected peptide-resin was treated with trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5, by vol.) for 2 h to cleave the peptide from the resin and remove the remaining side-chain protecting groups. The exhausted resin was filtered, the filtrate was mixed with diethyl ether and the precipitate was isolated by centrifugation. The precipitate was purified by reverse-phase HPLC before freeze-drying. HPLC was performed with a Hitachi (Tokyo, Japan) L-7100 chromatograph apparatus equipped with a L-7400 UV detector (peak detection at 230 nm) using ODS-A columns (YMC-Pack; YMC Co., Kyoto, Japan), 250 mm (length) $\times$ 20 mm (diameter) for preparation and 150 mm (length) $\times$ 4.6 mm (diameter) for analysis. Linear gradient elution was carried out with 0.1 % trifluoroacetic acid/acetonitrile: 5:95–50:50 (v/v) at 8.0 ml/min for isolation, and 5:95–60:40 (v/v) at 1.0 ml/min for analysis. The peptides were dissolved in water as indicated and used as samples for epitope mapping against anti-A β antibodies. Some residues of amino acids 16–23, amino acids 17–24, amino acids 18–25 and amino acids 25–32 were dissolved in a minimal amount of DMSO before dilution with water.

Epitope mapping using synthetic peptides in ELISA

Epitope mapping was performed in 96-well Immulon 2 MICROELISA microtiter plates (Dynatech, Alexandria, VA, U.S.A.). All assays were carried out in triplicate. Each well was coated for 24 h at 4 °C with 50 μ l of a 4 μ g/ml suspension of the synthetic peptides. The immunoreactivity of antibodies (1 μ g/ml), 4G8, 6F/3D and anti 5-10 against synthetic peptides was determined using a standard ELISA system as described below.

Initial pH modification of A β peptides

Samples of A β 42, A β 40, [Gln¹¹]A β 40, A β 1–16 and A β 17–42 were incubated at 4 μ g/ml for 24 h at 4 °C in Eppendorf tubes with selected buffers in the range pH 3.0–8.0 in 0.2 pH unit steps. The buffer used in pH modifications was 20 mM citric acid adjusted with Na₂HPO₄.

ELISA assay

The assay was performed by coating each well of the microtiter plates for 24 h at 4 °C with 50 μ l of a 4 μ g/ml suspension of pH-modified A β peptides. After the removal of excess sample, the wells were first incubated with Tris-buffered saline (TBS; 20 mM Tris/34 mM NaCl, pH 7.4), containing 3 % BSA, pH 7.4, for 2 h at 37 °C, washed with TBST (TBS with 0.1 % Tween-20, pH 7.4) buffer and incubated for a further 2 h at 37 °C with 50 μ l of 1 μ g/ml antibody, either 4G8, 6F/3D, anti 5-10 or anti 1-7 in TBS containing 1 % BSA, pH 7.4. After incubation, the wells were washed with TBST, and incubated for an additional 1 h at 37 °C with 50 μ l of a 1:5000 dilution of alkaline phosphatase-conjugated secondary antibody as described under 'Materials' above. After washing with TBST, bound antibodies were detected by the addition of *p*-nitrophenyl phosphate, as measured after 30 min at 405 nm using a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). All washing steps were performed six times using a microplate autowasher, Model EL404 (BioTek Instruments, Winooski, VT, U.S.A.).

Figure 1 Epitope map of antibodies against A β 1–42 peptide

Epitopes of each monoclonal antibody, anti 5-10, 6F/3D and 4G8, were determined as described in the Experimental section. Anti 1-7 was a polyclonal antibody against a chemically synthesized peptide, A β 1–7.

Blockage of histidine residues of A β 42 and A β 40

A β 40/42 peptides (1.0 mg/ml) were incubated with 5 mM DEPC in 25 mM phosphate buffer, pH 7.4, for 30 min at 4 °C [31], and the formation of *N*-carbethoxyhistidine residues was followed by the characteristic increase in A_{240} [32]. The sample was dialysed to remove excess DEPC, and the samples were prepared as histidine-modified A β 40 [A β 40(His*)] and A β 42 [A β 42(His*)] for the ELISA assay.

Incubation of A β 42 with metals

A β 42 (4 μ g/ml) was incubated in buffers at different pH values (4.6 and 7.4) for 24 h at 4 °C with no metal, Cu²⁺ (20 μ M), Zn²⁺ (20 μ M) or Fe²⁺ (20 μ M). Reaction mixtures were coated on the plate for another 24 h at 4 °C, and standard ELISA assays were performed to determine the influence of the metal ions on the conformational changes around the 1–7, 6F/3D and 4G8 regions at different pH values.

Samples of pH-modified A β 42 from the plate surface

The 24-well flat-bottomed multiplates (Sumitomo Bakelite, Tokyo, Japan) were coated with 150 μ l of pH-modified A β 42 (4 μ g/ml) at either pH 4.6 or 7.4 for 24 h at 4 °C. After removal of the excess sample, wells were washed twice with PBS with Mg²⁺ and Ca²⁺, and the peptides coating the plate surfaces were collected with a cell scraper (Asahi Techno Glass. Co. Ltd., Tokyo, Japan) in 1 ml of PBS and used as pH-modified A β 42 to study PK sensitivity.

Cell line

A human glial cell line, KG-1-C [33], was obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (complete medium) at 37 °C in a humidified atmosphere containing 5% CO₂.

Preparation of cell pellet and supernatant

The cells were cultured in 24-well flat-bottom multiplates coated with or without pH-modified A β 42 peptides. The wells were pre-coated with 150 μ l of a 4 μ g/ml suspension of A β 42 modified at either pH 4.6 or 7.4 for 24 h at 4 °C. After removal of excess sample, wells were washed twice with PBS, and 1 ml of cell suspension, at a density of 2×10^4 cells/ml in the complete medium, was plated into the wells and incubated for 5 days at 37 °C in a humidified atmosphere containing 5% CO₂. After 5

days culture, the supernatant was collected, and the cells were collected with a cell scraper in 1 ml of PBS. After being washed twice, the cells were re-adjusted to a concentration of 2×10^4 cells/ml in PBS and used as a cell pellet. The viability of the cells was over 95% as judged by the Trypan Blue-dye-exclusion test performed throughout the experiments.

Dot-blot procedure

The cell-blotting method performed was similar to the procedure previously reported [34,35] with modifications. We used PVDF membrane (0.45 μ m pore size; Millipore Corp., Bedford, MA, U.S.A.) in place of nitrocellulose. The cell pellet and supernatant were spotted on to the membrane using the dot-blot apparatus. Samples (200 μ l) of cell suspension (2×10^4 cells/ml) in PBS or the supernatant were applied to the DP-48 Dot Plate (Advantec, Tokyo, Japan) and blotted on to methanol-immersed PVDF membrane by absorption with a vacuum pump. The membrane was then removed, rinsed in TBS, and digested with PK as indicated. In the cell-free culture with A β 42 peptides, samples of pH-modified A β 42 peptides from the plate surface were also spotted on to the membrane using the same procedure as that described above.

PK digestion and Western blotting

Each membrane was incubated with 5 ml of PK solution at concentrations of 0, 5, 20 and 200 μ g/ml in TBS, pH 7.4, for 1, 3, 5 or 6 h at 37 °C with constant shaking. After removal of the PK solution, the reaction was stopped by washing with TBST, followed by incubation with 2 mM PMSF at room temperature for 30 min and the membrane rinsed twice with TBST. After blocking the membrane with 3% non-fat milk for 4 h, it was allowed to react with primary antibody 4G8 (1:10000 dilution in TBS) for 4 h, followed by the secondary antibody with peroxidase-linked anti-mouse IgG (1:5000 dilution in TBS) for another 1 h at 37 °C. After three washes with TBST, the spots on the membranes were detected with enhanced chemiluminescence (ECL[®]; Amersham Pharmacia Biotech., Uppsala, Sweden) according to manufacturer's instructions.

RESULTS

Identification of epitopes

We performed an ELISA study to determine the reactivity of monoclonal antibodies 4G8, 6F/3D and anti 5-10 towards a series of synthetic peptides representing residues 1–32 of A β 42. In all, 25 peptides that were eight residues in length and that

Figure 2 Reactivity patterns of antibodies differ after pH-modification of the A β 1–42 peptide

The pH-modified A β 1–42 peptides (4 μ g/ml) at various pH values were tested for antibody [e.g. anti 1–7 ($\square$), anti 5–10 ($\circ$), 6F/3D ($\bullet$) and 4G8 ($\blacksquare$)] binding at a concentration of 1 μ g/ml on the standard ELISA system. Absorbance was measured at 405 nm. Results are expressed as means $\pm$ S.E.M. ($n = 6$).

Figure 3 Reactivity patterns of antibodies towards the mixture of A β 1–16 and A β 17–42 peptides after pH modification are unchanged

The mixture of pH-modified peptides of A β 1–16 (4 μ g/ml) and A β 17–42 (4 μ g/ml) at various pH values were tested for the antibodies [e.g. 6F/3D ($\bullet$) and 4G8 ($\circ$)] binding at a concentration of 1 μ g/ml on the standard ELISA system. Results are expressed as means $\pm$ S.E.M. ($n = 6$).

overlapped by seven residues at the N terminus were tested, and we identified the binding epitopes recognized by these antibodies. 4G8 reacted with peptides corresponding to residues 17–21, 6F/3D reacted with peptides corresponding to residues 9–14, anti 5–10 reacted with peptides corresponding to residues 5–10 (Figure 1).

Reactivity patterns of antibodies differ after pH modification of A β 42 peptides

We employed an ELISA system to determine the reactivity of antibodies towards A β 42 that had been incubated at various pH values; the object was to discover whether the pH caused any structural modification. The anti 1–7 and anti 5–10 antibodies

Figure 4 Reactivity patterns of 4G8 towards $A\beta$ 42 after histidine blockage and $[Gln^{11}]A\beta$ 1–40

(a) The $A\beta$ 1–42 peptide was incubated with DEPC and, after the confirmation of formation of *N*-carboxyhistidine residues as described in the Experimental section, it was used as histidine-modified $A\beta$ 1–42 (■): $A\beta$ 1–42(*His). The samples ($4 \mu\text{g/ml}$) were subsequently incubated at various pH values, and they were tested for 4G8 ($1 \mu\text{g/ml}$) binding on the standard ELISA system. The pH-modified $A\beta$ 1–42 without histidine blockage (□) was also tested as a control. Results are expressed as means $\pm$ S.E.M. ($n = 6$). (b) The $A\beta$ 1–40 (○), $[Gln^{11}]A\beta$ 1–40 (□) and histidine-blocked $A\beta$ 1–40 (●); $A\beta$ 1–40(*His) at $4 \mu\text{g/ml}$ was subsequently incubated at various pH values and tested for 4G8 ($1 \mu\text{g/ml}$) binding on the standard ELISA system. Results are expressed as means $\pm$ S.E.M. ($n = 6$).

showed very similar high levels of reactivity towards $A\beta$ 42 incubated at various pH values, the reactivity remaining constant over the entire pH range. In contrast, the 6F/3D antibody showed decreasing reactivity from 1.7 to 0.9 absorbance units as the pH was lowered from 8.0 to 4.6, followed by a dramatic increase from 0.9 to 1.8 absorbance units as the pH was lowered

from 4.6 to 3.0. Interestingly, reactivity of the 4G8 antibody showed a profile similar to that shown by antibody 6F/3D, with the lowest signal at pH 4.6 (Figure 2). The reactivity of antibodies towards a mixture of the shorter peptides of $A\beta$ 1–16 and $A\beta$ 17–42 was also tested to investigate aggregate formation as a function of pH. However, the signal was almost constant over the pH

Figure 5 Metal binding to A β 1–42

A β 1–42 (4 μ g/ml) was incubated in buffers of differing pH (4.6 or 7.4) for 24 h at 4 °C with no metal (white bars), Cu²⁺ (20 μ M) (stippled bars), Zn²⁺ (20 μ M) (hatched bars) or Fe²⁺ (20 μ M) (black bars). The samples were tested towards antibodies binding: (a) anti 1–7; (b) 6F/3D; (c) 4G8 on standard ELISA assay. Results are expressed as means $\pm$ S.E.M. (n = 6). The significances of the differences were determined by Student's *t* test. *: *P* < 0.0001 compared with no metal.

range from pH 8.0 to 3.0, although the signal decreased slightly as the pH was lowered (Figure 3). Thus, the 6F/3D and 4G8 regions in the A β 42 peptide revealed a unique pH-dependence.

Conformational changes induced by pH shifts are preserved in the ELISA assay

The standard ELISA assay was carried out at pH 7.4. We were concerned that, after the protein had bound to the ELISA plate, any conformational modifications induced in the A β peptide by incubation in buffers at other pH values might be affected by the subsequent shift to pH 7.4. We prepared pH 3.0-, 4.6- and 7.4-incubated A β 42 peptides, bound them to the ELISA plate, changed the pH to 7.4 and measured the absorbance as a function of time at pH 7.4. The signal levels from the 6F/3D antibody against A β 42 originally incubated at pH 3.0, 4.6 or 7.4 were not affected by incubation at pH 7.4 for times ranging from the minimum measurable (a few minutes) to 2 h (results not shown). Thus immobilization of pH-modified A β 42 on the ELISA plate preserved any conformational change that had been induced by the initial change in the pH, or, alternatively, any refolding induced by a change from pH 4.6 to 7.4 was very slow.

Identification of the amino acid residue of A β peptide responsible for the pH modifications

We modified the amino acids in the 6F/3D epitope of A β 40/42 and monitored the signals against 4G8 to investigate the effect of pH on the A β folding by modification in the 6F/3D region. The reactivity of 4G8 with A β 40 and A β 40(His*)/42(His*) showed a pH-dependence similar to that with A β 42, except that the lowest signal with A β 40(His*)/42(His*) was shifted to pH 4.2 (Figures 4a and 4b). As expected, 6F/3D with A β 40(His*)/42(His*) and [Gln¹¹]A β 40 showed very low signals (below 0.3 absorbance units) because the amino acids in the 6F/3D region had been modified (results not shown). By contrast, 4G8 showed high signals with [Gln¹¹]A β 40 over the entire pH range, with no dramatic changes in *A*₄₀₅ at about pH 4.6 as observed with A β 40 (Figure 4b).

Metal ions affect the conformational changes of A β 42 at neutral pH

Cu²⁺ and Zn²⁺ caused a conformational change around the 6F/3D region of A β 42 when incubated at pH 7.4, with about a 40% decrease in absorbance compared with that with no metal at the same pH (*P* < 0.0001). However, there was no such effect when A β 42 was incubated at pH 4.6 (Figure 5b). The 1–7 and 4G8 regions of A β 42 after incubation at pH 7.4 or 4.6 showed no significant decrease in absorbance, indicating that these regions were not affected by the metal ions (Figures 5a and 5c), and Fe²⁺ did not appear to affect any of these regions of pH-modified A β 42 (Figures 5a–5c).

Different susceptibilities of pH-modified A β 42 to PK

We measured the susceptibility of A β conformers at pH 4.6 and 7.4 to PK digestion to confirm the different characteristics of the conformers. The protein remaining on the membrane after PK digestion is shown in Figure 6(a). The density of the spots was analysed by NIH (National Institutes of Health) imaging software, and the data were expressed as average pixel density after subtracting the mean background pixel density in Figure 6(b). The susceptibility depends on PK concentration and incubation time in both pH-modified conformers. The conformer at pH 7.4 was sensitive to a 1 h incubation with 5 μ g/ml PK and was completely digested, whereas the conformer at pH 4.6 was relatively resistant to the same concentration of PK for 3 h and there was still some undigested material after 6 h, and it showed resistance to PK at 20 μ g/ml for 1 h and there was still some undigested material after 3 h. The conformer at pH 4.6 still

Figure 6 Different susceptibility of pH-modified $A\beta_{42}$ peptides to PK

(a) The $A\beta_{42}$ (200 μ l) modified either at pH 7.4 (A) or 4.6 (B) remaining on the plate surface was spotted on to PVDF membranes and exposed to PK at different concentrations (0, 5, 20 and 200 μ g/ml) for the indicated incubation times (1, 3, 5 and 6 h) at 37 °C. The PK-resistant protein on the membrane was detected with 4G8 (1:10 000 dilution). Visualization was by the ECL[®] system. The experiment was repeated three times. (b) Quantification of PK-resistant $A\beta$ on PVDF membranes by computerized densitometry. Measurements of average pixel density in the spots were made by subtracting the mean background pixel density measured in the areas of the PVDF membrane surrounding the spots. The open symbols refer to $A\beta_{42}$ modified at pH 7.4 and the closed solid symbols refer to that modified at pH 4.6. PK concentrations (μ g/ml): 0 (◇, ◆), 5 (□, ■), 20 (△, ▲) and 200 (○, ●).

Figure 7 Accumulation of $A\beta$ aggregate in glial cells

The KG-1-C cells (2×10^4 cells/ml) were cultured on the pre-coated plates with either (A) pH 7.4- or (B) pH 4.6-modified $A\beta_{1-42}$ peptide (4 μ g/ml) and the cells were cultured without $A\beta_{1-42}$ peptides as a control (N). The cells were harvested on day 5 of culture and re-adjusted to a concentration of 2×10^4 cells/ml in PBS. The cell pellet in PBS suspension (200 μ l) and supernatant (200 μ l) were spotted on to PVDF membranes and treated with different concentrations of PK (0, 5, 20 and 200 μ g/ml) for 90 min at 37 °C. The signals from remaining proteins on the membrane were detected with 4G8 antibody (1:10 000 dilution) using the ECL[®] system. The experiment was performed three times.

remained at 200 μ g/ml PK after 1 h incubation, although both conformers were completely digested after 3 h at that proteinase concentration. Thus the conformer at pH 4.6 showed a relative resistance to PK.

pH-modified $A\beta_{42}$ peptide accumulates in glial cells

To identify the physiological features of pH-modified $A\beta_{42}$ peptides in KG-1-C cells, we cultured them with the conformers for 5 days and studied the internalization of the conformers into the cells and the PK-sensitivity of proteins in the cells. The signals from cell pellets with $A\beta$ at pH 4.6 were stronger than those with $A\beta$ at pH 7.4 at PK concentrations of 0 and 20 μ g/ml, and the contrast at 20 μ g/ml of PK was noteworthy. However, in the supernatant, the signal from $A\beta$ at pH 7.4 was stronger than that at pH 4.6 (Figure 7). The pixel density of $A\beta$ modified at pH 7.4 was 5834 in the supernatant and 3474 in the cell pellet, indicating that 63 % of the peptide was detected in the supernatant. The density of $A\beta$ modified at pH 4.6 was 1758 in the supernatant and 4902 in the cell pellet, indicating that 70 % of the peptide was in the cell pellet (Table 1). Moreover, the cells

Table 1 Quantification of A β aggregate intermediates in the glial cell by computerized densitometry

Average densities of the pixels in Figure 7 were analysed with NIH imaging software. The values in parentheses indicate the percentage of pixels compared with that without PK digestion. Abbreviations used: Ap and Bp, KG-1-C cells with A β modified at pH 7.4 (Ap) and at pH 4.6 (Bp); As and Bs, supernatant at pH 7.4 (As) and at pH 4.6 (Bs).

PK(μ g/ml)	Pixel density			
	Ap	As	Bp	Bs
0	3474 (100)	5834 (100)	4902 (100)	1758 (100)
5	948 (28)	1700 (29)	3759 (77)	412 (23)
20	649 (19)	1027 (18)	2035 (42)	645 (37)
200	221 (6)	81 (1)	151 (3)	83 (5)

with pH 4.6-modified A β showed a remarkable difference in PK-sensitivity compared with that with pH 7.4-modified A β . The amount of protein remaining after PK digestion is shown as a percentage in Table 1. The level of A β conformer at pH 4.6 in the cell pellet was particularly high, being 77 and 42% at 5 and 20 μ g/ml PK respectively. However, the percentages in the supernatant were 23% and 37% at the same concentrations of PK. The percentages in the cell pellet and the supernatant with A β conformer at pH 7.4 were about 30 and 20% at 5 and 20 μ g/ml PK. All the samples were almost completely digested at 200 μ g/ml PK, and the percentage was below 6% (Table 1). Thus the A β conformer at pH 4.6 was preferentially internalized into the cell, and accumulated A β showed a relative resistance to PK, whereas the A β conformer at pH 7.4 remained in the medium and was sensitive to PK.

DISCUSSION

In the present study we investigated the possibility that slightly different environmental conditions, such as pH modification, can determine the propensity of the peptide to adopt different conformational states. The hypothesis that amyloid formation involves acid-mediated conformational changes in A β peptides, mimicking the pH of a lysosome, has been proposed, and the optimum pH range for the formation of A β aggregates has been reported to be pH 5–6 [17,18,36]. However, this is not very informative, because the conformational transition induced by pH is essentially global. We identified the sequences in the A β 40/42 peptide that are affected by pH and determined the critical pH value for the change in reactivity. It was found to be pH 4.6, which is comparable with lysosomal pH, and the pH-dependence of the reactivity of 6F/3D exactly mirrored that of 4G8, indicating that those two epitopes might participate in pH modification (Figure 2). We speculate that abnormal folding of the 6F/3D epitope induced by the pH shift masked the 4G8 epitope. A mixture of the shorter peptides, A β 1–16 and A β 17–42, was very different from A β 42 in its tendency to form aggregate intermediates as a function of pH (Figure 3), suggesting a different character of the 6F/3D and 4G8 epitopes in shorter fragments compared with that in full-length A β 42. These findings support the characteristic property of the full-length A β 42 to form aggregate intermediates, which might be a very early step to form A β aggregates. We analysed the pH-modified A β binding to the plate using surface plasmon resonance and confirmed that the pH-dependence is not simply a plate-coating phenomenon of A β (results not shown). We also confirmed that the antibody reactivity was not affected by pH changes after

immobilization, showing retention of conformation after binding to the plate surface.

A recent NMR study of the solution structures of micelle-bound A β 40/42 showed that the three-dimensional structure of A β 42 consists of an extended chain (Asp¹–Gly⁹), two α -helices (Tyr¹⁰–Val²⁴ and Lys²⁸–Ala⁴²), and a loop region (Gly²⁵–Ser²⁶–Asp²⁷), with the most stable regions being Gln¹⁵–Val²⁴ and Lys²⁸–Val³⁶ [37]. We speculate that the folding of the first α -helix of A β 10–24, containing the 6F/3D and 4G8 epitopes, might be affected by pH, thereby exposing or hiding the epitopes. Thus any conformational variability in this region is of considerable interest. The amino acids sensitive to a pH shift most likely to cause structural transition in proteins are the positively charged group of histidine, arginine and lysine and the negatively charged group of glutamic acid and aspartic acid. A β 42 contains charged amino acids in a limited part of the N-terminal 1–28 region that corresponds to the extracellular domain, but none in the 29–42 region, which corresponds to the transmembrane domain [6]. Because the reactivity profiles of anti 1–7 and anti 5–10 antibodies, which should cover the sequence amino acid 1–10, were not affected by pH (Figures 1 and 2), we focused on the acidic group, Glu¹¹, Glu²² and Asp²³, and the positive group, His¹³ and His¹⁴ and Lys¹⁶ and Lys²⁸. It is reported that the substitution of Glu²² for Gln found in Dutch-type amyloidosis cerebrovascular haemorrhagic disease [38] yields an A β analogue with an increased ability to form amyloid fibrils [39]. We examined the possibility that histidine at residues 13 and 14 might be responsible for the changes, because the pK_a of histidine should be about 6, and it could be completely protonated at pH 4.6. However, histidine residues appear to contribute little to the conformational changes, because blockage of His⁶, His¹³ and His¹⁴ of A β 40/42 did not basically affect the reactivity profile. However, any change occurring at pH values around 4.6 probably arises from the titration of an aspartic acid or glutamic acid residue. The substitution of a mutant (Gln¹¹ instead of Glu¹¹) peptide in A β 40 abrogated the unique pH-dependent profile of A β 40 against 4G8. We suggest that the Glu¹¹ is one of the amino acid residues responsible for the change, and that the pH-dependent structure in A β 42 is caused by a weak association of the Glu¹¹ with either of the positively charged amino acids Lys¹⁶ or Lys²⁸.

Several studies have suggested the participation of bioessential metals ions, including Cu²⁺, Zn²⁺ and Fe²⁺, in A β aggregation. In the present study, we suggest that Cu²⁺ and Zn²⁺ could induce the conformational changes of A β around 6F/3D region at neutral pH, but that, after pH-modification of A β at acidic pH, the metal ions could not affect the change, supporting the pH-dependent conformational changes of the region and the notion that Cu²⁺ and Zn²⁺ might participate in the conformational changes around 6F/3D epitope only at neutral pH. The lowest reactivity of 6F/3D and 4G8 against modified A β at pH 4.6 might result from a conformational change induced in residues 9–21 of A β 42 (Figure 1) to hide an otherwise exposed epitope of 6F/3D and 4G8.

It is clear that there is a direct correlation between the secondary structure of the peptide and its aggregation and proteinase-resistance properties. The different susceptibilities to PK between A β peptides modified at pH 4.6 and 7.4 also suggest the possible conformational changes induced by pH. It is likely that structural transitions associated with pH modifications make putative cleavage sites inaccessible to PK. Our findings in part support the previous evidence that enhanced polymerization of A β peptide increases resistance to tryptic digestion at the α -secretase site [40,41]. It says little about the physiological properties of the conformers. We showed physiological differences among the conformers in the human glial cell.

Microglial cells express scavenger receptor and internalize $A\beta$ aggregates via the receptor [42,43]. Our present study suggested that the cells may preferentially internalize $A\beta$ 42 modified at pH 4.6 from the culture medium, and that non-digestible $A\beta$ peptides accumulate in the cells. However, most of the peptide modified at pH 7.4 remained in the medium. It is therefore possible that our findings resulted from the different characteristics of $A\beta$ conformers at pH 4.6 and 7.4, perhaps by allowing excessive intracellular accumulation of non-digestible $A\beta$ in the glial cell. It has been reported that only the $A\beta$ conformer at neutral pH exhibited biological activity in cell-toxicity assays, but not the conformer at acidic pH [44]. The biological activity might be associated with the proteolytic and esterase-like activity of the $A\beta$ 42 aggregates at neutral pH, *in vitro*, to attack the cell membrane [45]. We speculate that acidic modification of $A\beta$ 42 abrogates such a proteolytic activity of $A\beta$ conformer towards the cell membrane. However, the glial cell could preferentially internalize the non-toxic conformer at pH 4.6, resulting in the accumulation of the conformer in the cell, as shown in our present findings. It is reasonable to suppose that the partially abnormal folding of $A\beta$ might correspond to the folding intermediate that is essential for *in vitro* $A\beta$ aggregation under micro-environmental changes such as that of pH. However, it is not clear whether this type of change is essential in the formation of amyloid fibrils. Our findings suggest the importance of pH in the early stages of formation of $A\beta$ aggregate intermediates in the glial cell.

This work was supported by Grant-in-Aid 11670650 from the Ministry of Education, Science Sport and Culture of Japan.

REFERENCES

- Harper, J. D. and Lansbury, Jr, P. T. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu. Rev. Biochem.* **66**, 385–407
- Selkoe, D. J. (1996) Amyloid β -protein and the genetics of Alzheimer's disease. *J. Biol. Chem.* **271**, 18295–18298
- Skovronsky, D. M., Doms, R. W. and Lee, Y.-M. V. (1998) Detection of a novel intraneuronal pool of insoluble amyloid β protein that accumulates with time in culture. *J. Cell. Biol.* **141**, 1031–1039
- Kuo, Y. M., Emmerling, M. R., Vigo-Pelfrey, C., Kasunic, T. C., Kirkpatrick, J. B., Murdoch, G. H., Ball, M. J. and Roher, A. E. (1996) Water-soluble $A\beta$ (N-40, N-42) oligomers in normal and Alzheimer disease brains. *J. Biol. Chem.* **271**, 4077–4081
- Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B. and Selkoe, D. J. (1992) Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature (London)* **359**, 322–325
- Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature (London)* **325**, 733–736
- Haass, C., Koo, E. H., Mellon, A., Hung, A. Y. and Selkoe, D. J. (1992) Targeting of cell-surface β -amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature (London)* **357**, 500–503
- Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J. and Younkin, S. G. (1992) Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* **255**, 728–730
- Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C. F. and Pepys, M. B. (1997) Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. *Nature (London)* **385**, 787–793
- Serpell, L. C. and Smith, J. M. (2000) Direct visualisation of the β -sheet structure of synthetic Alzheimer's amyloid. *J. Mol. Biol.* **299**, 225–231
- Nordstedt, C., Naslund, J., Tjernberg, L. O., Karlstrom, A. R., Thyberg, J. and Terenius, L. (1994) The Alzheimer $A\beta$ peptide develops protease resistance in association with its polymerization into fibrils. *J. Biol. Chem.* **269**, 30773–30776
- Barrow, C. J., Yasuda, A., Kenny, P. T. M. and Zagorski, M. G. (1992) Solution conformations and aggregational properties of synthetic amyloid β -peptides of Alzheimer's disease. *J. Mol. Biol.* **225**, 1075–1093
- Halverson, K., Fraser, P. E., Kirschner, D. A. and Lansbury, Jr, P. T. (1990) Molecular determinants of amyloid deposition in Alzheimer's disease: Conformational studies of synthetic β -protein fragments. *Biochemistry* **29**, 2639–2644
- Lee, J. P., Stimson, E. R., Ghilardi, J. R., Manthly, P. W., Lu, Y.-A., Felix, A. M., Llanos, W., Behbin, A., Cummings, M., Crikingle, M. V. et al. (1995) ^1H NMR of $A\beta$ amyloid peptide congeners in water solution: conformational changes correlate with plaque competence. *Biochemistry* **34**, 5191–5200
- Inouye, H., Fraser, P. E. and Kirschner, D. A. (1993) Structure of β -crystallite assemblies formed by Alzheimer β -amyloid protein analogues: analysis by x-ray diffraction. *Biophys. J.* **64**, 502–519
- Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C. and Glabe, C. (1992) Assembly and aggregation properties of synthetic Alzheimer's $A\beta$ amyloid peptide analogs. *J. Biol. Chem.* **267**, 546–554
- Snyder, S. W., Lador, U. S., Wade, W. S., Wang, G. T., Barrett, L. W., Matayoshi, E. D., Huffaker, H. J., Kraft, G. A. and Holzman, T. F. (1994) Amyloid- β aggregation: selective inhibition of aggregation in mixtures of amyloid with different chain lengths. *Biophys. J.* **67**, 1216–1228
- Wood, Stephen, J., Maleeff, B., Hart, T. and Wetzel, R. (1996) Physical morphological and functional differences between pH 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide $A\beta$. *J. Mol. Biol.* **256**, 870–877
- Orpiszewski, J. and Benson, M. D. (1999) Induction of β -sheet structure in amyloidogenic peptides by neutralization of aspartate: a model for amyloid nucleation. *J. Mol. Biol.* **289**, 413–428
- Kim, K. S., Wen, G. Y., Baner, C., Chen, C. M. J., Spaienza, V. J., Hong, H. and Wisniewski, H. M. (1990) Detection and quantitation of amyloid β -peptide with 2 monoclonal antibodies. *Neurosci. Res. Commun.* **7**, 113–123
- Solomon, B., Koppel, R., Hanan, E. and Katav, T. (1996) Monoclonal antibodies inhibit *in vitro* fibrillar aggregation of the Alzheimer β -amyloid peptide. *Proc. Natl. Acad. Sci. U.S.A.* **9**, 452–455
- Kim, K. S., Miller, D. L., Spaienza, V. J., Chen, C. M. J., Bai, C., Grundke-Iqbal, I., Currie, J. R. and Wisniewski, H. M. (1988) Production and characterization of monoclonal antibodies reactive to synthetic cerebrovascular amyloid peptide. *Neurosci. Res. Commun.* **2**, 121–130
- Lovell, M. A., Robertson, J. D., Teesdale, W. J., Campbell, J. L. and Markesbery, W. R. (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. *J. Neurol. Sci.* **158**, 47–52
- Clements, A., Allsop, D., Walsh, D. M. and Williams, C. H. (1996) Aggregation and metal-binding properties of mutant forms of the amyloid $A\beta$ peptide of Alzheimer's disease. *J. Neurochem.* **66**, 740–747
- Atwood, C. S., Moir, R. D., Huang, X., Scarpa, R. C., Bacarra, N. M. E., Romano, D. M., Hartshorn, M. A., Tanzi, R. E. and Bush, A. I. (1998) Dramatic aggregation of Alzheimer $A\beta$ by Cu(II) is induced by conditions representing physiological acidosis. *J. Biol. Chem.* **273**, 12817–12825
- Liu, S. T., Howlett, G. and Barrow, C. J. (1999) Histidine-13 is a crucial residue in the zinc ion-induced aggregation of the $A\beta$ peptide of Alzheimer's disease. *Biochemistry* **38**, 9373–9378
- Knauer, M. F., Soreghan, B., Burdick, D., Kosmoski, J. and Glabe, C. G. (1992) Intracellular accumulation and resistance to degradation of the Alzheimer amyloid $A\beta$ protein. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7437–7441
- Soto, C. and Castano, E. M. (1996) The conformation of Alzheimer's β peptide determines the rate of amyloid formation and its resistance to proteolysis. *Biochem. J.* **314**, 701–707
- Huang, T. H. J., Yang, D. S., Plaskos, N. P., Go, S., Yip, C. M., Fraser, P. E. and Chakrabarty, A. (2000) Structural studies of soluble oligomers of the Alzheimer β -amyloid peptide. *J. Mol. Biol.* **297**, 73–87
- Borchelt, D. R., Ratovitski, T., Lare, J., Lee, M. K., Gonzales, V., Jenkins, N. A., Copeland, N. G., Price, D. L. and Sisodia, S. S. (1997) Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* **19**, 939–945
- Lundblad, R. L. and Noyes, C. M. (1988) Chemical Reagents for Protein Modification, vol. 1, pp.105–126, CRC Press, Boca Raton, FL
- Badet-Denisot, M. A. and Badet, B. (1992) Chemical modification of glucosamine-6-phosphate synthase by diethyl pyrocarbonate: evidence of histidine requirement for enzymatic activity. *Arch. Biochem. Biophys.* **292**, 475–478
- Miyake, E. (1979) Establishment of a human oligodendroglial cell line. *Acta Neuropathol.* **46**, 51–55
- Bosque, P. J. and Prusiner, S. B. (2000) Cultured cell sublines highly susceptible to prion infection. *J. Virol.* **74**, 4377–4386
- Nakajima, M., Shinoda, I., Samejima, Y., Miyauchi, H., Fukuwatari, Y. and Hayasawa, H. (1997) A simple and quantitative cell-blotting assay for evaluation of pigmentation of cultured melanocytes and its use in demonstrating the depigmenting effect of lactoferrin. *Arch. Dermatol. Res.* **289**, 180–183
- Fraser, P. E., Nguyen, J. T., Surewicz, W. K. and Kirschner, D. A. (1991) pH-dependent structural transitions of Alzheimer amyloid peptides. *Biophys. J.* **60**, 1190–1201

- 37 Shao, H., Jao, S. C., Ma, K. and Zagorski, M. G. (1999) Solution structures of micelle-bound amyloid β -(1–40) and β -(1–42) peptides of Alzheimer's disease. *J. Mol. Biol.* **285**, 755–773
- 38 Levy, E., Carman, M. D., Fernandez-Madrid, I. J., Power, M. D., Lieberburg, I., Van Duinen, S. G., Bots, G. T., Luyendijk, W. and Frangione, B. (1990) Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* **248**, 1124–1126
- 39 Wisniewski, T., Ghiso, J. and Frangione, B. (1991) Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation. *Biochem. Biophys. Res. Commun.* **179**, 1247–1254
- 40 Burdic, D., Kosmoski, J., Knauer, M. and Glabe, C. G. (1997) Preferential adsorption, internalization and resistance to degradation of the major isoform of the Alzheimer's amyloid peptide, A β 1–42, in differentiated PC12 cells. *Brain Res.* **746**, 275–284
- 41 Haass, C. and Selkoe, D. J. (1993) Cellular processing of β -amyloid precursor protein and the genesis of amyloid β -peptide. *Cell* **75**, 1039–1042
- 42 Paresce, D. M., Ghosh, R. N. and Maxfield, F. R. (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid β -protein via a scavenger receptor. *Neuron* **17**, 553–565
- 43 Chung, H., Brazil, M. I., Soe, T. T. and Maxfield, F. R. (1999) Uptake, degradation, and release of fibrillar and soluble forms of Alzheimer's amyloid β -peptide by microglial cells. *J. Biol. Chem.* **274**, 32301–32308
- 44 Howlett, D. R., Ward, R. V., Bresciani, L., Jennings, K. H., Christie, G., Allsop, D., Gray, W. and Karran, E. H. (1999) Biological activity associated with a high molecular weight form of β -amyloid. *Alzheimers Rep.* **2**, 171–177
- 45 Elbaum, D., Brzyska, M., Bacia, A. and Alkon, D. L. (2000) Implication of novel biochemical property of β -amyloid. *Biochem. Biophys. Res. Commun.* **267**, 733–738

Received 4 September 2001/15 October 2001; accepted 21 November 2001