COMMUNICATION

OBC
www.rsc.org/obc

Effects of β-sheet breaker peptide polymers on scrapie-infected mouse neuroblastoma cells and their affinities to prion protein fragment PrP(81–145)

Takehisa Oishi,[a] Ken-ichi Hagiwara,[b] Tomoya Kinumi,†[b] Yoshio Yamakawa,[b] Masahiro Nishijima,[b] Kazuhiko Nakamura[c] and Hirokazu Arimoto*[a,d]

[a] *Department of Chemistry, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-8602, Japan. E-mail: arimoto@org.chem.nagoya-u.ac.jp; Fax: +81-52-789-5041*

[b] *Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

[c] *Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan*

[d] *Institute for Advanced Research, Nagoya University; and PRESTO, JST, Chikusa, Nagoya 464-8602, Japan*

Received 16th June 2003, Accepted 3rd July 2003
First published as an Advance Article on the web 9th July 2003

The effects of Soto's 'β-sheet breaker peptide' and its polymer on PrP^{Sc} formation in ScN2a cells were investigated. Surface plasmon resonance study indicated that direct binding between PrP(81–145) and the 'β-sheet breaker peptide' is not specific and may not play a major role in the inhibition of PrP^{Sc} formation.

Introduction

Prions are transmissible, fatal neurodegenerative agents that affect humans and a wide variety of animals.[1] In the PRION-ONLY hypothesis, direct interaction between the pathogenic prion protein (PrP^{Sc}) and the endogenous cellular prion protein leads to PrP^{Sc} accumulation, which plays a central role in the transmissible spongiform encephalopathies (TSEs). PrP^{Sc} is an abnormally folded, protease-resistant, β-sheet-rich isoform of a normal protein PrP^{C}.

Molecules that stabilize the conformation of PrP^{C}, or destabilize PrP^{Sc} *via* specific binding, are currently targets of active investigations.

Several synthetic peptides,[2] and some low-molecular weight organic compounds,[3] have been reported to inhibit these transformations *in vitro*. Among these, Soto's β-sheet breaker peptide (i-PrP13: sequence DAPAAPAGPAVPV) (Fig. 1) blocks PrP^{Sc} formation in cell-free systems and scrapie-infected cell culture.[4] Intracerebral injection of i-PrP13 with infectious PrP^{Sc} also delays the time to the onset of symptoms for mice.[4] The mechanism of action, however, remains elusive.

i-PrP13 is a synthetic peptide that was designed based on the conserved residues 115–122 of prion proteins (sequence: AAAAGAVV). Aspartic acid and four prolines were inserted into the native sequence. The above region, PrP(115–122), has been suggested to play a central role in the conversion of PrP^{C} to PrP^{Sc}.[5] It has therefore been implied that a direct interaction of i-PrP13 with the prion protein at the conserved region is important to its inhibitory activities. This assumption, however, has not been examined experimentally.

We quantitatively measured the affinity of the 'β-sheet breaker' (i-PrP13) with PrP(81–145) by means of surface plasmon resonance (SPR). The dissociation constant K_D was fairly large ($> 10^{-3}$ M), which provided evidence against the initial hypothesis.

We then devised and chemically synthesized an oligomeric i-PrP13 with enhanced affinity to PrP(81–145).

The inhibitory activities of both monomeric and oligomeric i-PrP13 were evaluated with the prion-infected mouse neuroblastoma ScN2a cell line.

Results

The SPR technique was applied to evaluate the affinity between i-PrP13 and a prion protein fragment PrP(81–145). First, mouse PrP(81–145) was prepared by solid-phase peptide synthesis (yield 23%). To produce an affinity toward anti-prion monoclonal antibody 3F4 (DAKO), residues 108 (L to M) and 111 (V to M) were replaced. ε-Biotinylated L-lysine and two glycines (as linkers) were added at the C-terminus so that the peptide could be immobilized to the SPR sensor tip surface. The sequence of the synthetic PrP(81–145) is shown in Fig. 2.

The molecular ion (*m/z* 7115.0 for M + H^+) for synthetic PrP(81–145), as observed by MALDI-TOF mass spectrometry, corresponds well with the calculated molecular weight (7114).

The biotinylated PrP(81–145) was injected over the gold surface coated with streptavidin (Sensor tip SA, BIAcore). A surface density of 4000 resonance units was generated for PrP(81–145), which corresponds to approximately 4 ng mm^{-2}.

Differing amounts of PrP-specific monoclonal antibody 3F4 were applied to the biosensor immobilizing PrP(81–145) (Fig. 3). An analysis of these sensorgrams gave a dissociation constant K_D of 7.0×10^{-9} M. This result corresponded well

† Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, Ikeda, Osaka 563-8577, Japan.

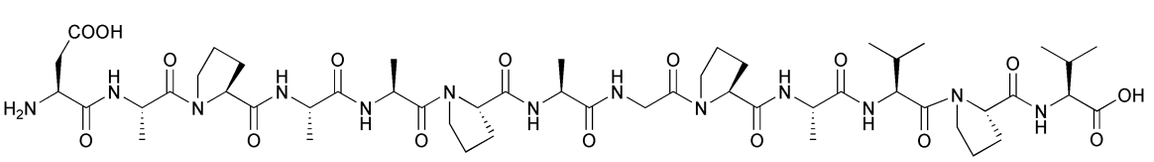


Fig. 1 'β-Sheet breaker peptide' (i-PrP13) **1**.

DOI: 10.1039/b306682g

81 GQPHGGGWGQGGGTHNQWNKPSKPKTNMKH 110

111 MAGAAAAGAVVGGLGGYMLGSAMSRPMIHF 140

141 GNDWEGGK(biot)

Linker

Fig. 2 Biotinylated mouse PrP(81–145).

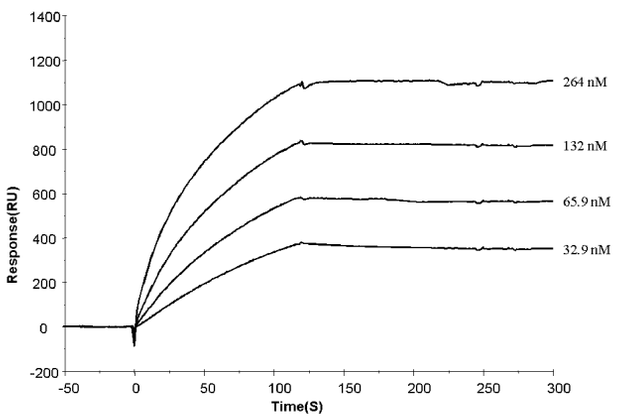


Fig. 3 SPR sensorgrams of anti-prion monoclonal antibody 3F4 binding to immobilized mouse PrP(81–145).

with the reported value of K_D 2.15 × 10^{-8} M between Syrian hamster PrP(29–231) and 3F4,[6] which validated the sensor tip for detection of specific binding to PrP.

However, there was no interaction detected when the β-sheet breaker i-PrP13 was employed as an analyte (K_D > 10^{-3} M, sensorgrams are not shown).

The results were in conflict with the widely circulated hypothesis that i-PrP13 has specific affinity to PrP. This unexpected finding prompted us to synthesize a polymer of i-PrP13 to provide insight into binding affinity–inhibitory relationships.

Cluster effects or multi-valent effects are molecular design principles that enhance weak nonbonding interactions.[7] Thus, a polymer of i-PrP13 would have higher affinity to PrP by these effects. This strategy has been very popular in sugar science, and we successfully utilized this approach in a glycopeptide antibiotic vancomycin, where both the affinity to their receptors and the antibacterial activities against resistant bacteria were significantly improved.[8]

The side-chain amino group of L-lysine was modified with a norbornene subunit, and the modified amino acid K(norbornene) was then introduced at the N-terminal (Scheme 1) or C-terminal portion of i-PrP13 (not shown).

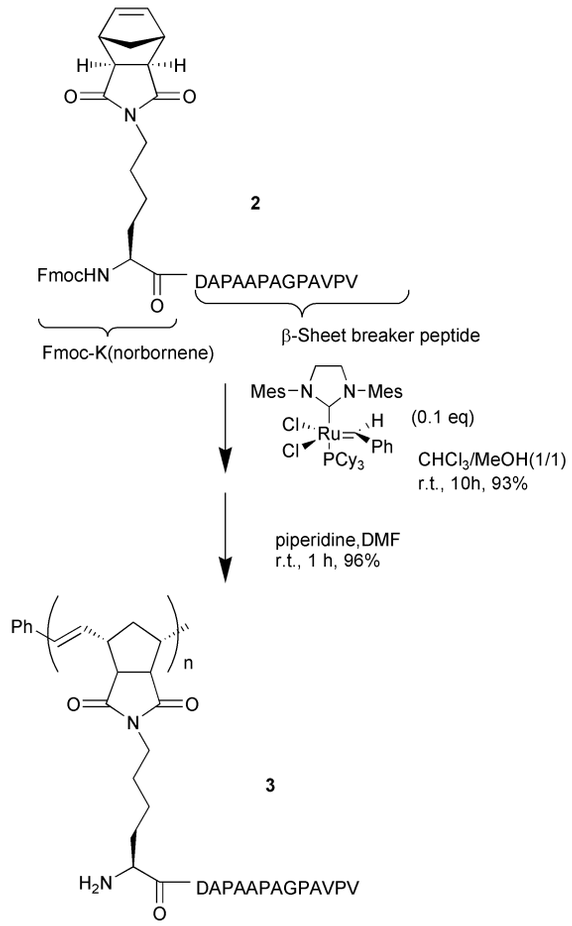
Ring-opening polymerization of the peptide was performed using Grubbs catalyst[9] in chloroform–methanol (1 : 1). Fmoc groups of the resulting polymer were removed with piperidine.

The SPR sensorgram of the N-terminally-modified i-PrP13 polymer **3** is shown in Fig. 4.[10] Unlike monomeric i-PrP13, the polymer had specific interactions with immobilized PrP(81–145). Because the rigorous analysis of data was not trivial due to the polyvalent nature of the sample, the apparent dissociation constant K_D was estimated on a per monomer-unit basis. The K_D value of 1.91 × 10^{-4} M indicated an at least 10-fold enhancement of affinity.

We investigated next if the i-PrP13 or its polymers inhibited the accumulation of PrP^{Sc} in scrapie-infected mouse neuroblastoma N2a cell (ScN2a), following the procedures of Prusiner *et al.*[11]

The 1 μM of quinacrine was used as a positive control.

It was proved that neither the i-PrP13 (lane 2) nor the polymers (lanes 4, 6) inhibited the formation of proteinase K resistant PrP^{Sc} up to 250 μg ml^{-1} (Fig. 5). In contrast, the



Scheme 1 Synthesis of i-PrP-polymer (average n = 10).

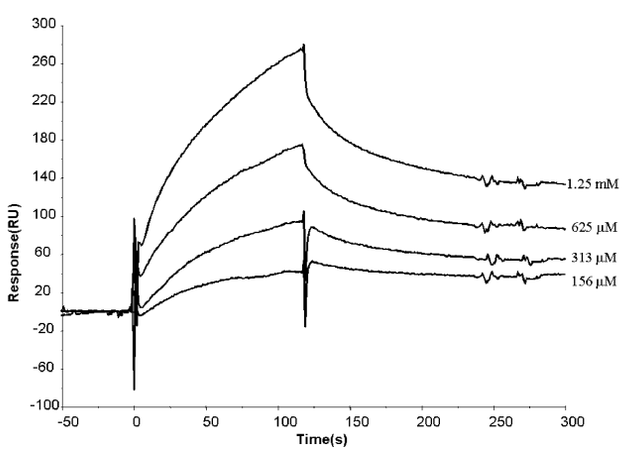


Fig. 4 SPR sensorgrams of an i-PrP13 polymer **3** binding to immobilized mouse PrP(81–145).

positive control (quinacrine) effectively cleared the formation of PrP^{Sc} at a concentration of 1 μM. Because we and Soto *et al.* have employed different cells for our cell-culture studies, a direct comparison of data is not possible. However, the inhibitory activity of i-PrP13 was much weaker than that of quinacrine.[12]

Discussion

Conversion to the infectious conformer PrP^{Sc} is particularly associated with major structural rearrangement in the central portion of the prion protein. PrP(90–145) is the region suggested to be the most important. Employing PrP-specific monoclonal antibodies with different epitope recognitions, Prusiner *et al.* have shown that the Fabs D13 (epitope: 132–156

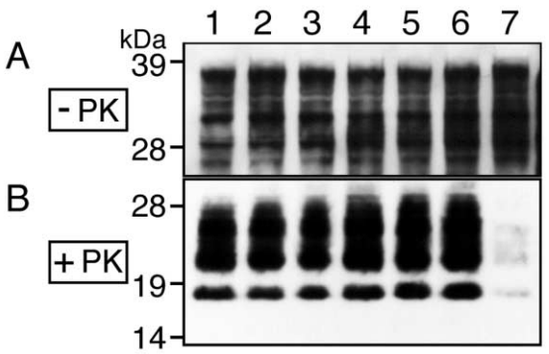


Fig. 5 Effects of i-PrP13 and its polymer on PrP^{Sc} formation of ScN2a cells—ScN2a cells were cultured for 5 days in the medium containing either unmodified i-PrP13 (lane 2), the N-terminally K(norbornene)-modified monomer (lane 3) and polymer (lane 4), or the C-terminally K(norbornene)-modified monomer (lane 5) and polymer (lane 6). As controls, the cells were grown in the absence of the peptides (lane 1) or in the presence of quinacrine (lane 7). The cell lysates were subjected to Western blotting analysis before (panel A) or after (panel B) proteinase K (PK) digestion.

of PrP) and D18 (epitope: 95–103 of PrP) only inhibit the formation of PrP^{Sc}. All PrP-specific antibodies have dissociation constants in the 10^{-9} M range. This result also suggests the importance of the central region.[13]

If the i-PrP13 specifically binds and stabilizes the normal conformation of prion proteins as hypothesized, it would likely inhibit their conversion to misfolded isoforms.

We have demonstrated in the present study, however, that i-PrP13 and even its polymer have poor specific interactions (dissociation constants of $> 10^{-3}$ M and 1.9×10^{-4} M, respectively) with the prion protein fragment PrP(81–145). In addition, they do not inhibit PrP^{Sc} accumulation in ScN2a cells at a high sample concentration of 250 μg ml^{-1}. These results indicate that direct binding of i-PrP13 might not play a major role in the inhibition of PrP^{Sc} formation. Although more investigation is needed, it is noteworthy that i-PrP13 did not show any inhibitory activities with regard to PrP^{Sc} formation in ScN2a cell lines, which is one of the most widely used cells in prion research. Our results provide the basis for mechanistic considerations of the effects of i-PrP13 peptide in an *in vivo* system.[4]

Experimental

General procedures for solid-phase peptide synthesis

The peptides employed in this study were synthesized by the conventional Fmoc solid-phase method with a Pioneer automated peptide synthesizer (Applied Biosystems). HATU was the coupling agent. 2-Cl-trityl chloride resin (Nova) was employed as a solid support so that the products were cleaved under mild conditions (acetic acid, trifluoroethanol). For the preparation of PrP(81–145), PAC-PEG-PS resin (Applied Biosystems) was employed. Acidic cleavage (TFA) of the PrP peptide from resin was conducted with scavengers (thiophenol and ethanedithiol).

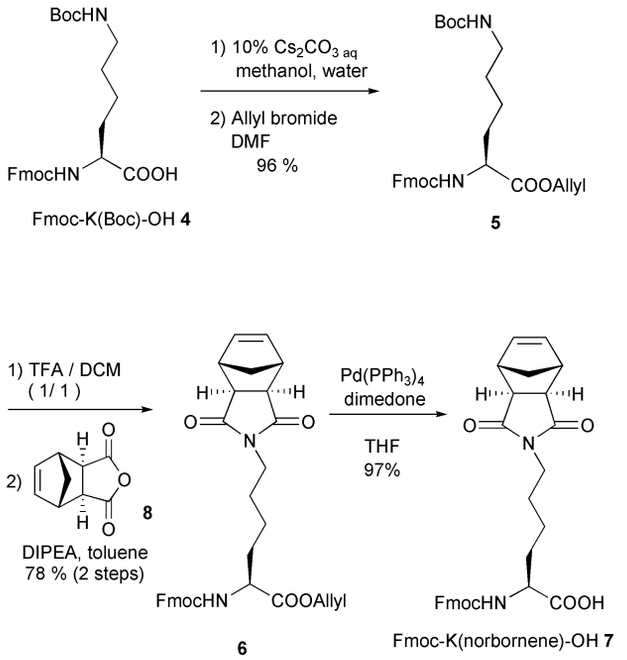
Amino acids, including α-Fmoc-ε-biotinyl-L-lysine, were purchased from Watanabe Chemicals, Japan.

Synthetic peptides were purified by size-exclusion chromatography (Superdex 75HR 10/30, 30% aq. acetonitrile with 0.1% TFA).

Molecular ion peaks for all synthetic peptides were detected with a Voyager STR MALDI-TOF mass spectrometer (Applied Biosystems).

Norbornene-modified L-lysine [K(norbornene)]:

N-ε-Norbornene-modified L-lysine was synthesized according to Scheme 2.



Scheme 2 Synthesis of Fmoc-K (norbornene)-OH.

β-Sheet breaker polymer

The N-terminally-modified monomer peptide **2** (sequence: Fmoc-K(norbornene)-DAPAAPAGPAVPV) was synthesized by the Fmoc method as described in the general procedures (total yield 87%). To a solution of the monomer in methanol–chloroform (1 : 1) was added a 0.1 equivalent of Grubbs catalyst to conduct ROMP (ring-opening metathesis polymerization). The degree of polymerisation was estimated as 10-mer. An Fmoc group of the product was removed with piperidine–DMF (4 : 1). The crude product was purified by size-exclusion chromatography. The poly-β-sheet breaker **3** proved to be fairly soluble in water (>> 5% wt/vol).

The C-terminally-modified polymer was also prepared by similar procedures.

Surface plasmon resonance (SPR)

Surface plasmon resonance was recorded with BIAcoreX (BIAcore), and the data were analyzed with BIAevaluation e.2 SPR kinetic software (BIAcore).

Solutions of the interacting peptide ('analyte') were injected over the surface at 25 °C with a flow rate of 10 μL min^{-1} in HBS running buffer [10 mM Hepes (pH 7.4)–150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20]. After injection, analyte solutions were replaced by HBS at the same flow rate. The surface was regenerated with 5 mM glycine (pH 2.0, 30 s, 10 μL) after each measurement. Analyte solutions were run simultaneously over a control surface without immobilized PrP(81–145). The response in the control flow cell was subtracted from each sensorgram, and the sensorgram was normalized to a baseline of 0 RU.

In vitro assay for PrP^{Sc} formation

ScN2a cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO™) supplemented with fetal calf serum (10%), penicillin (50 unit ml^{-1}), and streptomycin (50 μg ml^{-1}). i-PrP13 or its K(norbornene)-derivatives at a final concentration of 250 μg ml^{-1} (equivalent to 1.7×10^{-4} M i-PrP13 monomer) were added to the the cells grown in 25 cm^3 flasks (day 1). To overcome the problem of possible extracellular and/or intracellular degradation of the peptides, supplementary doses (a final concentration of 100 μg ml^{-1}) of the peptides were added to the

medium on day 3. As controls, the cells were grown in the absence of compounds or in the presence of 1 μM quinacrine. The cells were harvested on day 5 and subjected to the proteinase K digestion assay to determine the amounts of PrP^{Sc} by Western blotting utilizing anti-PrP antibody 6H4 (Prionics).[11]

Acknowledgements

We thank Professor Daisuke Uemura (Nagoya University) for helpful discussions and support, and Dr Kiyotoshi Kaneko (National Institute of Neuroscience, Tokyo) for providing ScN2a cells. We acknowledge a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (No. 12558076), and the Uehara Memorial Foundation for financial support.

Notes and references

1 G. C. Telling et al., *Science*, 1996, **274**, 2079; S. B. Prusiner, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 13363 . For a recent review: A. Aguzzi, M. I. Glatzel, F. Montrasio, M. Prinz and F. L. Heppner, *Nat. Rev. Neurosci.*, 2001, **2**, 745.

2 K. Kaneko, D. Peretz, K. Pan, T. C. Blockberger, H. Wille, R. Gabizon, O. H. Griffith, F. E. Cohen, M. A. Baldwin and S. B. Prusiner, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 11160; K. Kaneko, H. Wille, I. Mehlhorn, H. Zhang, H. Ball, F. E. Cohen, M. A. Baldwin and S. B. Prusiner, *J. Mol. Biol.*, 1997, **270**, 574.

3 B. Caughey and R. E. Race, *J. Neurochem.*, 1992, **59**, 768; M. Pocchiari, S. Schmittinger and C. Masullo, *J. Gen. Virol.*, 1987, **68**, 219; S. P. Priola, A. Raines and W. S. Caughey, *Science*, 2000, **287**, 1503.

4 C. Soto, R. J. Kascsak, G. P. Saborio, P. Aucouturier, T. Wisniewski, F. Prelli, R. Kascsak, E. Mendez, D. A. Harris, J. Ironside, F. Tagliavini, R. I. Carp and B. Frangione, *Lancet*, 2000, **355**, 192.

5 L. De Gioia, C. Selvaggini, E. Ghibaudi, L. Diomede, O. Bugiani, G. Forloni, F. Tagliavini and M. Salmona, *J. Biol. Chem.*, 1994, **269**, 7859; H. Zhang, K. Kaneko, J. T. Nguyen, T. L. Livshits, M. A. Baldwin, F. E. Cohen, T. L. James and S. B. Prusiner, *J. Med. Biol.*, 1995, **250**, 514; J. Chabry, B. Caughhey and B. Chesebro, *J. Biol. Chem.*, 1998, **273**, 13203.

6 E. Leclerc, D. Peretz, H. Ball, H. Sakurai, G. Legname, A. Serban, S. B. Prusiner, D. R. Burton and R. A. Williamson, *EMBO J.*, 2001, **20**, 1547.

7 M. Mammen, S. K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754.

8 H. Arimoto, K. Nishimura, T. Kinumi, I. Hayakawa and D. Uemura, *Chem. Commun.*, 1999, 1361; H. Arimoto, T. Oishi, M. Nishijima and T. Kinumi, *Tetrahedron Lett.*, 2001, **42**, 3347.

9 M. Scholl, S. Dinf, C. W. Lee and R. H. Grubbs, *Org. Lett.*, 1999, **1**, 953; A. K. Chatterjee, J. P. Morgan, M. Scholl and R. H. Grubbs, *J. Am. Chem. Soc.*, 2000, **122**, 3783.

10 C-Terminally-K(norbornene)-modified-monomer and its polymer were poorly soluble in water; an affinity analysis was therefore not conducted.

11 K. Doh-ura, T. Iwaki and B. Caughey, *J. Virol.*, 2000, **74**, 4894; C. Korth, B. C. H. May, F. E. Cohen and S. B. Prusiner, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 9836.

12 Other nonpeptidic inhibitors have exhibited excellent activities in this assay. For example, quinacrine has been reported to have an EC_{50} of 0.3 μM: see ref. 11.

13 S. B. Prusiner, D. Peretz, R. A. Williamson, K. Kaneko, J. Vergara, E. Leclerc, G. Schmitt-Ulms, I. R. Mehlhorn, G. Legname, M. R. Wornald, R. M. Rudd, R. A. Dwek and D. R. Burton, *Nature*, 2001, **412**, 739.