Amyloid aggregates of the prion peptide PrP106–126 are destabilised by oxidation and by the action of dendrimers

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Abstract The prion protein (PrP) peptide 106–126 forms amyloid aggregates in vitro and this sequence is speculated to be involved in the formation of amyloid fibrils by the abnormally folded PrP protein (PrP^{Sc}) found in spongiform encephalopathies. It is shown here by incubation experiments in water using Thioflavin T (ThT) as a fluorescent probe for amyloid formation that changes in C-terminal charge, oxidation state and conformational stabilisation lead to large changes in amyloid forming behaviour (amyloidogenicity) of this peptide. Amyloid formation is favoured by a charged C-terminus and is strongly inhibited by oxidation. Furthermore, cationic dendrimers are shown to perturb peptide fibrillation in a process dependent on the nature of the charged groups on the dendrimer surface.

Keywords: Prion protein peptide; Amyloid; Thioflavin T; Oxidation; Dendrimer

1. Introduction

The prion protein (PrP^{C}) is a constitutively expressed membrane glycoprotein mainly found on the surface of neuronal cells and involved in the development of transmissible spongiform encephalopathy (TSEs), in which protein deposits containing an abnormally folded isoform of PrP (PrP^{Sc}) are invariably found [1]. PrP^{Sc} is insoluble, highly protease resistant, and contains an increased proportion of amount of β-strands compared to PrP^{C} [2]. As deposition of PrP^{Sc} is accompanied by neuronal cell death, there has been considerable interest in determining the correlation between folding variants and the possible neurotoxicity of PrP [3].

A region located near the N-terminal of the protease-resistant PrP^{Sc} has attracted interest, as the corresponding peptide (PrP106–126) has been shown to be amyloidogenic and neurotoxic [4,5]; interestingly, neurotoxicity depends on the expression of endogenous PrP [6] as is also the case for the neurotoxicity and transmissibility of PrP^{Sc} [7].

Tagliavini et al. [4] found that PrP106–126 readily formed amyloid fibrils similar to those seen in Gerstmann–Sträussler–Scheinker disease, a rare heritable TSE in which the amyloid deposits contain a PrP fragment of approximately 100 residues (amino acids 58-approx. 150). Forloni et al. [5] showed this peptide to be toxic in vitro against rat hippocampal neurons, treated for several days with the peptide (chronic exposure), and later Ettaiche et al. [8] demonstrated in vivo toxicity of aged 106–126 peptide in a rat retinal model.

Fibril formation is thought to proceed through a slightly unfolded state (a "molten globule" like state) of the protein in question, followed by a nucleation state (soluble protofibril formation) and ending up in aggregation of fibrils into long, unbranched amyloid fibrils with a generic "cross"-β-sheet structure [9] with a characteristic ability to bind certain dyes, including Congo Red and the fluorescent dye Thioflavin T (ThT) [10].

Molecular modelling at pH 7 indicates that PrP106–126 has a preference for β-sheet structure [11], but it can also be assigned a more random coil structure incorporating α-helical elements. A similar peptide, PrP109–122, was predicted by others to be α-helical [12]. However, the 106–126 sequence is part of the unstructured, N-terminal half of the PrP^{C} molecule, next to the globular part [13]. In PrP^{Sc}, 106–126 is part of the protease-resistant core of the protein (approx. 90–231 [14]). The N-terminal, unstructured part of the PrP protein belongs to the recently described class of fibrillation-prone natively unfolded proteins [15].

Selvaggini et al. [16] and Salmona et al. [14] used circular dichroism and turbidimetry to study structure and fibril formation potential of several PrP106–126 variants, including the carboxamide, and histidine 111 substitutions (D-histidine, alanine and lysine) and a variant combining an A117V substitution with the carboxamide. A charge at position 111 was found to be important to retain adequate solubility to ensure fibrillation. The free peptide (C-terminal acid) was found to be much more prone to β-structure formation than the carboxamide, but the A177V substitution (alanine 177 substituted by valine) in the peptide amide rendered the amide more β-like. A similar fibril-promoting effect of a free C-terminal carboxylic acid was found by Terzi et al. [17] with the Aβ-peptide of Alzheimer's plaques. Furthermore, β-sheet content was enhanced at low pH (5) compared to neutral pH and while some secondary structure

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Abbreviations: PrP, prion protein; PrP106–126, prion protein peptide 106–126; PPI, poly(propylene imine); ThT, thioflavin T; TSE, transmissible spongiform encephalopathy; PBS, phosphate-buffered saline; mw, molecular weight

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was found at neutral pH in phosphate buffer, only random coil was detected in deionised water [14,15]. Similarly, De Goia et al. [18] found predominantly β-structure in 200 mM phosphate, pH 5.0, combination of β and random coil in both phosphate at pH 7.0 and deionised water at pH 5, while the peptide exhibited least structure in pure water. These studies were performed on freshly prepared solutions of peptide in the mg/ml range. Interestingly, the aggregation property of PrP106–126 was greater at pH 7 than at pH 5 as assessed by turbidity after 24 h of incubation [14]. Also, Florio et al. [19] described a highly structured peptide variant (G114A, G119A) that formed protofibrils, but never went on to form amyloid fibrils.

The "hydrophobic core" AGAAAAGAVV was found to be indispensable for amyloidogenicity of the peptide as any of a number of substitutions (S for V or A) in this sequence rendered PrP106–126 non-amyloidogenic (and non-neurotoxic) as was also the case with neighbouring peptides not comprising the whole core sequence [20,21].

Thus, amyloidogenicity of these PrP peptides depends on a fine balance between solubility and insolubility and between structure forming tendency and flexibility.

We observed that synthetic PrP106–126 preparations often contained a substantial amount of spontaneously oxidised peptide (see below) and it was, therefore, relevant to study if oxidation had any effect on the fibrillation behaviour of the peptide. Although the peptide contains two oxidation sensitive methionines and although PrP has often been speculated to be involved in oxidative processes as part of its normal function [22], no data on this have to our knowledge been reported previously. Oxidation was found to have profound effects on a number of other polypeptides involved in neurodegenerative diseases. For example, α-synuclein, the small presynaptic protein associated with Parkinson's disease, lost its ability to form amyloid fibrils when its four methionines were oxidised [15] and the Aβ-peptide of Alzheimer's disease,when oxidised at the methionine residue, also could no longer form amyloid fibrils [23,24]. Here, deliberately oxidised PrP106–126 was analysed in the ThT assay for amyloid fibrillation capacity compared to the unmodified peptide. Furthermore, we compared the fibrillation behaviour of PrP106–126 with the amide form of the peptide, with a covalently coupled parallel dimer of the peptide (PrP106–126 dibranch) and with a structure-relaxed form of the peptide (PrP106–126 RG_2). We also investigated the effect of a cationic dendrimer that was found previously to be able to solubilise certain PrP^{Sc} aggregates [25–27], in addition to a new type of dendrimer modified with surface guanidinium groups.

Amyloid fibril formation may be an inherent trait of all peptides, favoured by specific conditions of pH, salt and organic solvents [28–30]. Such generic amyloid fibrils may all be cytotoxic especially in their early, protofilament stage (soluble, non-fibrillar aggregates [31]). In the present study, the fibrillation behaviour of the PrP106–126 peptide and its molecular variants was studied in pure water to exclude effects of pH and salts and using ThT binding as a probe for amyloid fibril formation.

2. Materials and methods

2.1. Peptides

Peptides were synthesised by solid phase Fmoc-based synthesis using chlorotrityl resins for peptide acids and resins with the modified Rink linker for peptide amides (Novabiochem).

The dibranched peptide amide was prepared by sequential synthesis on a Rink-coupled C-terminal lysine that was selectively deprotected first at its α-amino group allowing synthesis of the first strand of PrP106–126, which was then N-acetylated. Second, the ε-amino group was deprotected by removing the methyltrityl (Mtt) protecting group with 1% trifluoroacetic acid allowing for the synthesis of the second strand. The acid dibranched peptide was prepared using K(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl – (Dde-) for the first strand synthesis ending with N-acetylation. K(Dde) was then deprotected with 2% hydrazine in *N*-methyl pyrrolidone and used for coupling of a presynthesised molecule of PrP106–126 (prepared on chlorotrityl resin and used in its protected state) by uronium chemistry [32].

After synthesis, cleavage by trifluoroacetic acid and work-up were performed as described elsewhere [32]. Peptides were analysed by reverse phase (C5) HPLC-MS (Shimadzu LC20) and, if necessary, purified by preparative reverse phase chromatography on Lichrosorp C18 (Merck).

Peptides were kept at minus 20 °C as freeze-dried powders until use. The following peptides were used in this work:

PrP106–126
KTNMKHMAGAAAAGAVVGGLG
(human PrP106–126, expected mw: 1912.3)

PrP106–126 amide
KTNMKHMAGAAAAGAVVGGLG – *amide*
(expected mw: 1911.3)

PrP106–126 control
LVGAHAGKMGANTAKAGAMVG
(scrambled sequence, expected mw: 1912.3)

Prp106–126 RG_2
KTNMKHMAGAAAAGAVVGGLGRGGRGGRGG
(expected mw: 2722)

PrP106–126 dibranched

Acetyl-KTNMKHMAGAAAAGAVVGGLG┐
KTNMKHMAGAAAAGAVVGGLGK

(expected mw: 3976)

PrP106–126 amide dibranched

Acetyl-KTNMKHMAGAAAAGAVVGGLG┐
KTNMKHMAGAAAAGAVVGGLGK-*amide*

(expected mw.: 3975)

All peptides are acids unless otherwise specified.

Oxidation was performed by incubation of 2.5 mM peptide in phosphate-buffered saline (PBS) with 200 μl hydrogen peroxide (Merck, 30%) per 1 ml peptide solution for 20 min at room temperature followed by size exclusion chromatography (desalting) on a PD10 column from Amersham Pharmacia using 10% acetic acid as the solvent, following the instructions from the manufacturer. Oxidation (100%) was confirmed by HPLC-MS showing a mass increase of 32 corresponding to the oxidation of methionine side chains to sulfoxides. The oxidised peptide eluted slightly earlier than the unmodified peptide on the reverse phase column (see Fig. 1).

2.2. Dendrimers

Second generation poly(propylene imine) (PPI) dendrimer was obtained from Aldrich (DAB-Am-8). The surface amines of the dendrimer were converted to guanidines by reaction with 1,3-diBoc-2-methylisothiourea (Fluka), followed by deprotection with 95% aqueous trifluoroacetic acid. The deprotected guanidinium derivatised dendrimer was precipitated with diethyl ether, followed by removal of the ether supernatant. The residue was dissolved in water and freeze-dried, giving the dendrimer as a white solid. The molecular mass of the modified dendrimer was verified by matrix-assisted laser desorption

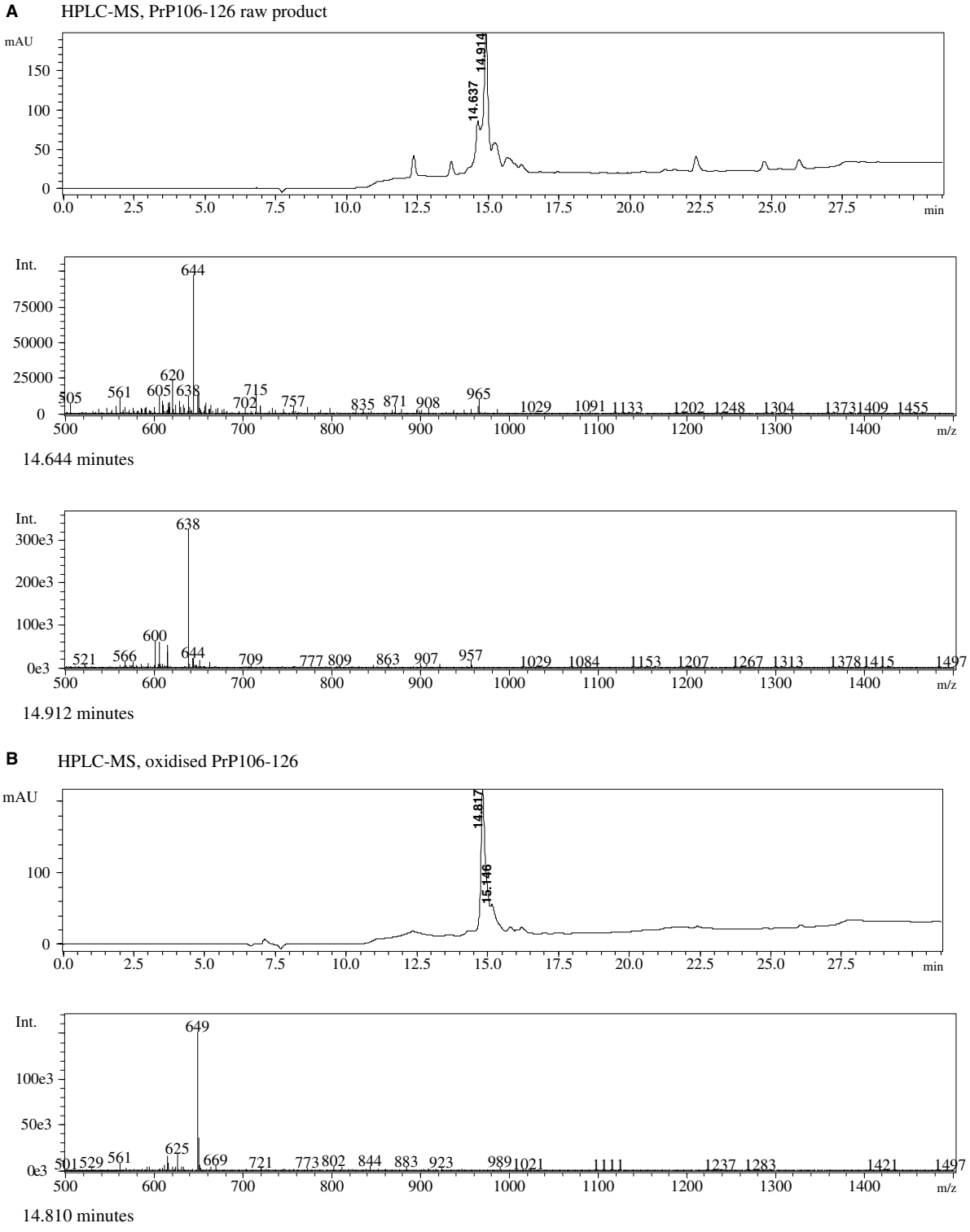

Fig. 1. HPLC-MS analysis of PrP106–126 peptide raw product (A) and a raw product deliberately oxidised by H_2O_2 (B). The two main products seen in the HPLC (220 nm) trace of the raw product (A) are scanned for *m/z* identifying a main peak *m/z* of 638 (deduced mw.: 1911) and a *m/z* of the "shoulder" of 644 (deduced mw.: 1929). The deliberately oxidised raw product (B) shows one main peak with a *m/z* of 649 (deduced mw.: 1944).

ionization time-of-flight mass spectrometry (MALDI-TOF, not shown). The structures of unmodified PPI dendrimer and guanidino-derived dendrimer are shown in Fig. 3A.

2.3. ThT – assay for amyloid fibril formation

Peptides were dissolved at 2 mg/ml (approximately 1 mM for all peptides except the dibranch peptides where this corresponds to 0.5 mM) in water and allowed to incubate at room temperature with slight agitation in the presence of 20 μM ThT (Sigma T3516, 1 mM stock solution in water), reading the fluorescence each day at 485 nm using an excitation wavelength of 440 nm (with a Spectra Fluor Plus microplate Fluorometer from Tecan). Readings were normalised to the same gain setting to allow comparisons from sample to sample. When comparing between different plates, values were furthermore corrected

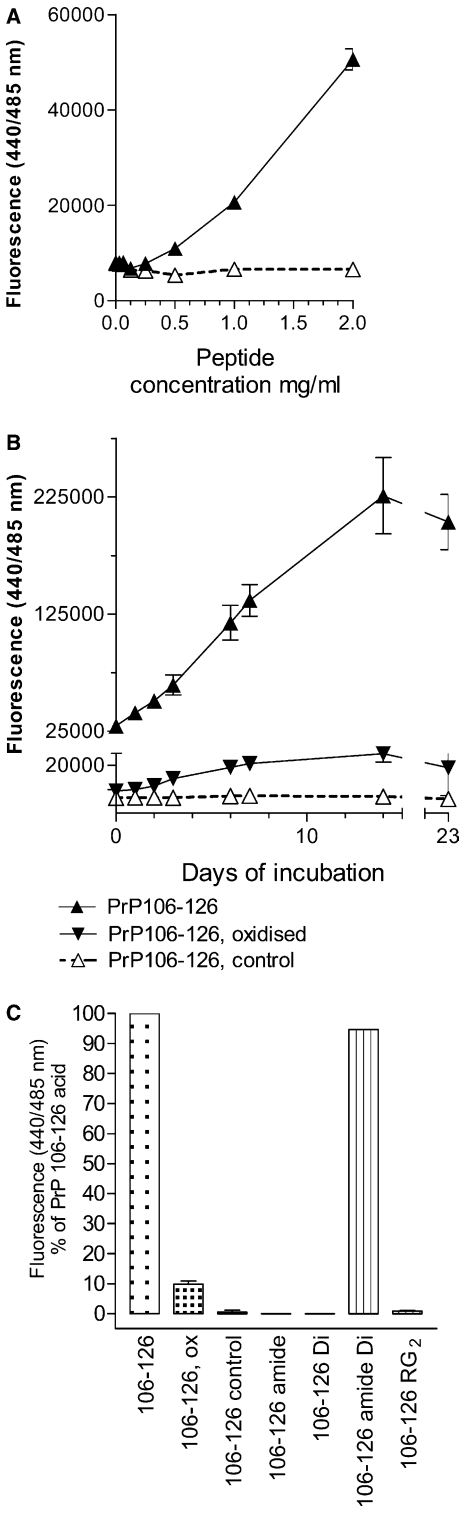

Fig. 2. ThT-binding of PrP106–126 variants. (A) Concentration dependence of ThT fluorescence (PrP106–126 and scrambled control peptide) as analysed after 2 days of incubation. (B) Effect of incubation period at 2 mg/ml of PrP106–126, oxidised PrP106–126 and scrambled control peptide. (C) ThT fluorescence after 7 days of incubation at 2 mg/ml of the PrP106–126 variants indicated (see text) as compared to the unmodified PrP106–126 peptide (100%). All incubations were performed at room temperature with gentle agitation in 100 μl water. Error bars indicate standard deviations of double determinations.

for background (fluorescence of ThT in water). Dendrimers were added to solutions of peptides (1 mg/ml) and ThT (20 μM) at 20 μg/ml and coincubated throughout.

3. Results and discussion

Oxidation of PrP106–126 occurred spontaneously during solid phase synthesis and work-up and was usually encountered at a level of around 20–30% of the total peptide (see Fig. 1A). The oxidised peptide had a lower retention time in reverse phase HPLC and an increased molecular weight roughly corresponding to the conversion of one methionine residue into methionine sulfoxide; peptide products with both methionines converted could also be detected but at a lower level (not shown). The peptide could be oxidized with hydrogen peroxide to yield the peptide containing two methionine sulfoxides (Fig. 1B); this preparation was used as the oxidised peptide in the ThT experiments. The oxidised peptide in turn could be reduced by dithiothreitol to yield the fully reduced peptide (not shown).

Thus, PrP106–126 is prone to oxidation during its production resulting in the formation of a peptide containing one to two methionine sulfoxide residues. This is not an uncommon side-reaction during production of synthetic peptides containing methionines and has in fact been specifically reported for the Alzheimer peptide, Aβ [33], but no such oxidation products of PrP106–126 have to our knowledge been reported previously.

The ThT assay clearly indicated the formation of amyloid fibrils during incubation of the PrP106–126 peptide at concentrations of 2 and 1 mg/ml in water, while at lower peptide concentrations amyloid fibril formation was negligible after 2 days of incubation (Fig. 2A); fibrils formed most readily at room temperature with gentle agitation, while higher temperature (37 °C) leads to slower fibril formation (not shown); there was no discernible lag phase and a plateau was only reached late in the observation period (at day 23, Fig. 2B). The absence of a lag phase can be explained by the lack of initial structure in the peptide and consequently the absence of an initial rate limiting unfolding step. The sequence-scrambled control peptide did not show any ThT binding at any time and the oxidised peptide reached only around 10% of the ThT fluorescence observed with the unmodified peptide (Fig. 2B and C). The strongly decreased ThT binding of the oxidised peptide indicates that it is less amyloidogenic than the unmodified peptide. This is supported by Fig. 2B, where it can be seen that the ThT binding over time of the oxidised peptide evolves with a different slope than the ThT binding of the unmodified peptide. Thus, although it cannot be totally excluded that oxidised PrP106–126 simply binds less ThT by itself, the decreased ThT binding is probably due to a decreased tendency of the oxidised peptide to form amyloid fibrils.

This is of interest as we observed spontaneous formation of oxidised peptide during synthesis and work-up of PrP106–126 at around 20–30% of the raw product depending on the batch (Fig. 1A) (vide supra). Thus, different proportions of oxidised peptide molecules in different batches of PrP106–126 would lead to different fibrillation tendencies. This might be one reason for why reproducing fibrillation and biological activity of different PrP106–126 preparations have proved to be difficult [6,34]. It could also be tempting to speculate that the degree of oxidation of the 106–126 sequence could influence the

transformation of PrP^{C} to PrP^{Sc}. The strongly reduced fibrillation tendency of the oxidised peptide is most probably a consequence of the increased hydrophilicity of the more polar sulfoxide side chain of oxidised methionine, rendering hydrophobic peptide-to-peptide interactions less favourable. Similar effects of oxidation were reported previously for the Aβ peptide of Alzheimer's disease and for the small protein α-synuclein of Parkinson's disease which were both unable to form amyloid fibrils after deliberate and complete oxidation of the methionines [15,23,24], correlating with loss of ability to form β-sheets. Interestingly, the non-fibrillar, oxidised Aβ peptide still retained neurotoxicity [23,24].

Other spontaneous modifications of the PrP106–126 peptide have been demonstrated, e.g., deamidation and isomerisation of N108 which occurs upon aging [35], but no biological or biophysical effects of such modifications were demonstrated. A similar modification was, however, enough to make a 23 amino acid leucine-rich repeat peptide loose its ability to form β-structure and to form fibrils [36].

In addition to the clearly relevant oxidation product of PrP106–126, which represents a peptide variant with increased hydrophilicity but no change in charge, a number of other molecular variants of the peptide were studied, including the C-amidated peptide (decreasing charge and hydrophilicity), a dibranched peptide construct (increased conformational stability) and a variant of the peptide carrying an additional, conformationally destabilising sequence with additional charges ($(RG_2)_3$).

The C-amide form of 106–126 was not amyloidogenic under the conditions used here (Fig. 2C); this is in line with earlier results on PrP106–126 [14] and the Aβ-peptide [17], where a significant decrease in β-structure and fibrillation tendency was observed upon amidation of the C-terminal carboxyl group. In those studies, the fibrillation tendency was not abolished completely. This could be due to the fact that the incubations were performed in phosphate buffer and not in water as in the present study; we do in fact find some ThT binding of the peptide amide, and a general increase in ThT binding of all the peptides studied when performing the incubation in 200 mM phosphate, most at pH 7.0 and less at pH 5.0 (not shown). The increased tendency of amyloid formation of the acid form of the peptide was used by Salmona et al. [14] to support a model for organisation of the peptide molecules into anti-parallel β-sheets, stabilised by the C-terminal anion.

The dibranched peptide construct of PrP106–126 was also studied, both with a C-terminal carboxylic acid group and in its C-amide form, and in this case, surprisingly, only the amide showed a tendency for fibril formation while the acid form did not show any ThT binding whatsoever (Fig. 2C). Thus, preorganisation of PrP106–126 into parallel dimers in the dibranched construct increased amyloidogenicity of the peptide amide from zero, while it abolished the marked amyloidogenicity of the free peptide acid. This suggests that while the optimal packing mode for the free peptide acid is probably an antiparallel arrangement stabilised by electrostatic interactions (see above), this is not possible with the dibranched molecule. On the other hand, parallel packing of the peptide amide is not as disfavoured as it is for the peptide acid, and as antiparallel packing of dibranched molecules furthermore is unfavourable, the dibranched peptide amide could be speculated to form amyloid fibrils by parallel stacking. Parallel packing is potentially possible for the PrP106–126 amide, but it obviously does not occur spontaneously without pre-arranging the peptide amide. Thus, the preferred packing mode of PrP106–126 is dependent on both charge and covalent preorganisation of the

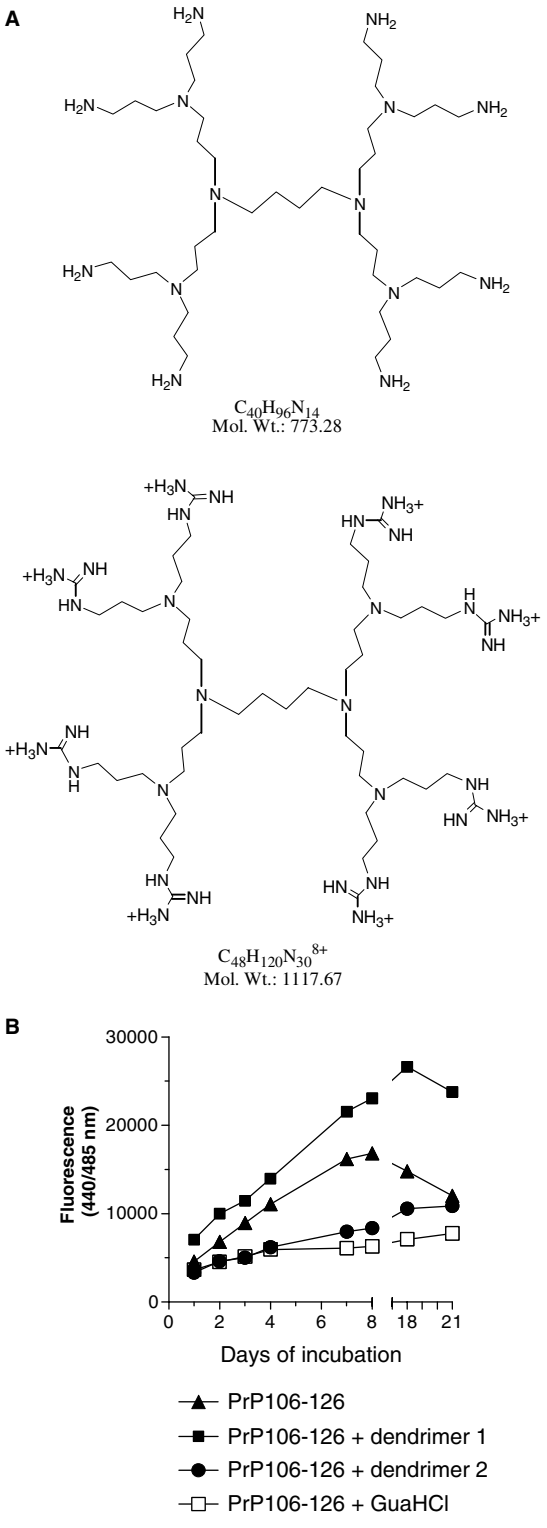

Fig. 3. (A) PPI second generation underivatised dendrimer (dendrimer 1, top) and PPI dendrimer derivatised with guanidinium surface groups (dendrimer 2, bottom). (B) Effect of dendrimer 1, dendrimer 2 and guanidinium hydrochloride on the ThT fluorescence of PrP106–126. Peptide was incubated at 1 mg/ml in deionised water with 20 μg/ml dendrimer or 2.5 M guanidinium hydrochloride.

peptide. This could be important for the ability of 106–126 segment in PrP, which is by definition an amide and partly conformationally restricted, to contribute to the aggregation tendency and amyloidogenicity of PrP^{Sc} and there is in fact some evidence in favour of a parallel packing of β-strands involving 106–126 in PrP^{Sc} [37]. Taken together, this indicates that the monomeric PrP106–126 acid arranges itself into antiparallel β-sheets, however, this might not be the biologically relevant stacking arrangement.

The RG_2-modified peptide lost most of its ability to bind ThT (Fig. 2C), indicating that this extension of the peptide had some effect on the fibrillation capacity of the peptide; charge repulsion would seriously destabilise a parallel packing of peptide molecules. If molecules were organised in an antiparallel fashion, the structure "relaxing" ability of the RG_2 might preclude fibril formation, indicating that some structural definition is important for the ability of the peptide to form amyloid fibrils.

Finally, it was found that a modified dendrimer with highly cationic guanidinium surface groups (dendrimer 2, Fig. 3A) dramatically decreased fibril formation at 20 μg/ml when coincubated with PrP106–126 (1 mg/ml) and ThT, while the unmodified dendrimer carrying surface amino groups had no destabilising effect but on the contrary seemed to support fibril formation (dendrimer 1, Fig. 3A and B). The fluorescence of ThT alone incubated with dendrimers was not above background and the dendrimers did not quench the fluorescence of ThT itself (not shown).

The effect of 20 μg/ml guanidino-modified dendrimer was similar to the effect of 2.5 M guanidinium hydrochloride. These incubations were performed in water where the guanidino-modified dendrimer is fully charged, while the unmodified dendrimer is much less so due to the lower pK_a of the amino group. Thus, the fibrillation inhibiting effect must be ascribed to the positive charge on the surface of the guanidino-modified dendrimer. This is in accordance with Supattapone et al. [25], who found that charging the dendrimer by low pH (5.0) was necessary to achieve an unfolding effect on PrP^{Sc}. Thus, we find an effect of cationic dendrimers on the PrP106–126 peptide that is similar to the previously described effect on PrP^{Sc} [25–27], indicating the importance of the 106–126 sequence in the folding and aggregation events of PrP.

In contrast to these findings, Goers et al. [38] found that a variety of polycations accelerated the formation of α-synuclein fibrils. However, as α-synuclein belongs to the group of natively unfolded proteins, the effects of polycations are limited to an organisational role in the formation of rate-limiting fibrillation nuclei; whether polycationic dendrimers also could have such effects with PrP^{Sc} aggregates or PrP106–126 fibrils is not known, but there is an indication in Fig. 3B that the unmodified dendrimer with amino surface groups actually promotes fibrillation of PrP106–126.

The dendrimer effect on PrP106–126 resembles a denaturing or solubilising effect similar to that of chaotropic salts like guanidinium chloride and urea and it is, therefore, tempting to propose that the effect of dendrimers on PrP^{Sc} aggregates is purely solubilising and does not involve specific binding between the protein and the dendrimer as was proposed previously [25]. This in turn implies that hydrophobic interactions are the driving force behind the formation of PrP106–126 amyloid fibrils.

It will be interesting to study the neurotoxicity of peptide variants with decreased amyloidogenicity, e.g., the peptide amide and the oxidised peptide, which chemically and structurally are very different from each other. The neurotoxicity of PrP is going to be a complicated issue as other parts of PrP have been shown to exhibit another type of neurotoxocity being independent of both peptide aggregation state and of endogenous PrP^{C} expression [39].

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