Inhibition of toxicity and protofibril formation in the amyloid-β peptide β(25–35) using N-methylated derivatives

A. J. Doig, E. Hughes, R. M. Burke, T. J. Su, R. K. Heenan, and J. Lu

*Department of Biomolecular Sciences, UMIST, P.O. Box 88, Manchester M60 1QD, U.K., †Department of Physics, UMIST, P.O. Box 88, Manchester M60 1QD, U.K., and ‡Rutherford Appleton Laboratory, Chilton, Didcot, OX11 0QX, U.K.

Abstract

β(25–35) is a fragment of β-amyloid that retains its wild-type properties. N-methylated derivatives of β(25–35) can block hydrogen bonding on the outer edge of the assembling amyloid, so preventing the aggregation and inhibiting the toxicity of the wild-type peptide. The effects are assayed by Congo Red and thioflavin T binding, electron microscopy, and a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] toxicity assay. N-methyl-Gly-25 has similar properties to the wild-type, while five other methylation sites have varying effects on prefolded fibrils and fibril assembly. In particular, N-methyl-Gly-33 is able to completely prevent fibril assembly and reduces the toxicity of prefolded amyloid. With N-methyl-Leu-34 the fibril morphology is altered and toxicity reduced. A preliminary study of β(25–35) structure in aqueous solution was made by small-angle neutron scattering (SANS). The protofibrillar aggregates are best described as a disc of radius 140 Å and height 53 Å (1 Å = 0.1 nm), though the possibility of polydisperse aggregates cannot be ruled out. No aggregates form in the presence of N-methyl-Gly-33. We suggest that the use of N-methylated derivatives of amyloidogenic peptides and proteins could provide a general solution to the problem of amyloid deposition and toxicity and that SANS is an important technique for the direct observation of protofibril formation and destruction in solution.

Introduction

Alzheimer’s disease (AD) is the most common form of senile dementia. Amyloid-β peptide (Aβ), a 39–43 amino acid β-sheet peptide, aggregates in the brain to form the major component of characteristic deposits known as senile plaques [1–3]. Aggregation occurs due to hydrogen bonding between β-strands and the resulting fibrils have axes perpendicular to the β-strand and parallel to the cross-linking hydrogen bonds [4].

Of all the Aβ derivatives studied so far, β(25–35) (sequence GSNKGA1I1GLM) is the shortest fragment that exhibits large β-sheet fibrils and retains the toxicity of the full-length peptide [5–7]. As with Aβ, toxicity is dependent on the aggregation state of the peptide, since β(25–35) that has been solubilized and unfolded in 35% acetonitrile/0.1% trifluoroacetic acid is non-toxic [6,8]. In this study, β(25–35) is used as a model for full-length Aβ since it retains both its physical and biological properties, while its short length readily allows derivatives to be synthesized and studied.

A great deal of evidence, much of which comes from studying hereditary forms of the disease, supports the view that Aβ aggregation is implicated in AD plaques [1–3,9–13]. Controversy
has raged, however, over whether these fibrils are a cause or a consequence of the disease. While many mutations in the amyloid precursor protein gene have been linked to premature onset of AD, the amount of amyloid deposited in the brain does not necessarily correlate with disease severity. A resolution of this apparent paradox may be that it is a \( \beta \)-structured protofilament, which subsequently forms fibrils and then plaques, that is the pathogenic element  

\[ 14-17 \]  

We have used small-angle neutron scattering (SANS) to directly observe \( \beta \)(25–35) protofibrils in aqueous solution. The low solubility of aggregated peptides has seriously constrained the use of established scattering techniques based on the detection of electron density and refractive index variations. Microscopic techniques such as electron microscopy (EM) and atomic force microscopy involve deposition into support surfaces, which alter the solution environment. The distinct advantages of SANS stem from its short wavelength (typically a few Å), and the use of deuterium substitution to greatly enhance scattering contrast. In this case \(^2\)H\(_2\)O can be conveniently used to highlight the protofibrils in aqueous solution.

In view of the role of fibril formation played in AD and other diseases, it has been proposed that a valid therapeutic strategy would be to administer compounds that can block fibril or protofilament formation by binding to their ends  

\[ 18,19 \]  

Designed peptides have already been shown to act as ‘\( \beta \)-sheet breakers’, inhibiting amyloid formation and lowering toxicity in Alzheimer’s peptides and prion proteins  

\[ 18,20-25 \]  

Our proposal is that N-methylated peptide derivatives can also act as \( \beta \)-sheet breakers. N-methylation is known to promote \( \beta \)-sheet formation by locking the residue into a \( \beta \)-conformation  

\[ 26 \]  

and has been shown to generate soluble monomeric \( \beta \)-sheet peptides  

\[ 27 \]  

By N-methylating the amide NH groups at the outer edges of the \( \beta \)-sheet and so preventing intermolecular hydrogen bonding, both aggregation and toxicity should be prevented. Since the N-methyl (NMe) derivatives are homologous to \( \beta \)(25–35) they are expected to bind to the peptide but prevent further addition of \( \beta \)(25–35) monomers.

**Experimental**

**Peptide synthesis**

\( \beta \)(25–35) and the six NMe derivatives, NMeGly-25, NMeGly-29, NMeAla-30, NMeIle-31, NMeGly-33 and NMeLeu-34, were synthesized using standard solid-phase Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. Purification was carried out by reversed-phase HPLC. Purified peptides were analysed by electrospray MS generating the expected masses. Once purified, peptides were stored in lyophilized form at \(-20^\circ\)C.

**Peptide preparation**

For each assay, peptides were prepared by solubilizing in either water or 35% acetonitrile/0.1% trifluoroacetic acid. They were lyophilized following any mixing and resolubilized in 20 mM Mops at pH 7. Peptides were then incubated for 1 week at 37 °C prior to assay. For Congo Red (CR) binding, thioflavin T (ThT) fluorescence and EM, peptides were either analysed alone or in 1:1 combinations of \( \beta \)(25–35) to each NMe derivative, with the exception of NMeGly-25.

**Fluorescence assays**

Quantitative measurement of CR binding was carried out essentially as in  

\[ 28-30 \]  

ThT fluorescence was carried out as described in  

\[ 30,31 \]  

**EM**

Peptides were solubilized at 500μM (1 mM total peptide concentration for 1:1 combinations), and 50 μl drops were applied to glow-discharged carbon-coated 400 mesh copper grids. Peptides were negatively stained with 2% uranyl acetate before viewing.

**Toxicity assay**

Measurement of cellular toxicity by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction was carried out as in  

\[ 7,32 \]  

using rat pheochromocytoma (PC12) cells, with cells subcultured 50/50 1 day prior to assay. Prior to peptide application, cells were treated with 1 mg/ml DNase to break up any clumps. An overnight incubation at 37 °C followed mixing of peptides and cells before addition of MTT and after a further 2 h incubation cells were lysed and read at 550 nm. Positive controls consisted of addition of 20 mM Mops, pH 7, to cells and negative controls the addition of 0.1% Triton X-100.

**SANS**

SANS experiments were carried out using the small-angle scattering instrument LOQ at the ISIS pulsed neutron source, Rutherford Appleton Laboratory (Chilton, Didcot, Oxon, U.K.). LOQ uses a white beam time-of-flight method with wavelengths between 2.2 and 10.0 Å  

\[ 32a \]  

Solutions at 20–21 °C were prepared in quartz glass
cuvettes immediately before the SANS measurements and the data collection for several samples repeated, and checked, in a loop to accumulate several hours of data for each. Sample pH was fixed to 7.4 using phosphate buffer, with a total ionic strength of 20 mM.

**Results**

**Detection of amyloid using ThT fluorescence and CR binding**

Formation of \( \beta(25-35) \) amyloid was measured at 500 \( \mu \)M, alone and in 1:1 combinations with NMe derivatives, using ThT fluorescence and quantitative CR binding. Preparing and mixing peptides in water looks at the ability of NMe peptides to break down preformed \( \beta(25-35) \) fibrils, whereas preparing and mixing peptides in 35% acetonitrile looks at their ability to prevent fibril assembly. \( \beta(25-35) \) alone has showed the characteristic binding of CR [28], whereas the NMe derivatives in isolation do not, with the exception of NMeGly-25 which was comparable with the wild type. In contrast, premixing unfolded wild type with NMeGly-33 results in CR binding being reduced to control levels. \( \beta(25-35) \) alters the excitation and emission spectra of ThT in the same manner as full-length A\( \beta(1-40) \), while the NMe derivatives in isolation had no effect on the ThT spectra. On mixing aggregated \( \beta(25-35) \) with the NMe peptides no differences were seen when compared with wild type alone. For peptides premixed with unfolded \( \beta(25-35) \), however, the amyloid-induced peak in the ThT spectrum is reduced by at least 50% in all cases. NMeGly-33 and NMeLeu-34 are particularly effective, with signal levels less than 10% of the wild type.

**Fibril morphology as measured by EM**

None of the NMe derivatives in isolation formed fibrils, apart from NMeGly-25, which had fibrils similar in morphology to \( \beta(25-35) \). Although not all NMe peptides affect wild-type fibril morphology, some changes were apparent particularly with NMeGly-33 and NMeLeu-34. Under both mixing conditions NMeLeu-34 alters the morphology of \( \beta(25-35) \) from short ribbon-like fibrils to long spaghetti-like structures of indeterminate length and 5–10 nm in diameter. NMeGly-33 mixed with aggregated wild type alters morphology to smaller finer fibrils, 40–60 nm in length and 5–10 nm in diameter. These are less densely spread on the EM grid compared with wild-type fibrils. On premixing unfolded \( \beta(25-35) \) and NMeGly-33, virtually all fibrils are abolished. The remaining peptides, NMeGly-29, NMeAla-30 and NMeIle-31, when premixed with either folded or unfolded \( \beta(25-35) \), all have a fibril morphology comparable with that of wild type alone.

**Figure 1**

**MTT toxicity assay**

NMeGly-33 mixed with unfolded \( \beta(25-35) \). All data are scaled with 100% MTT reduction represented by positive controls and 0% by negative controls. Each data set was carried out in triplicate; S.D. values are shown. WT, wild type.
Reduction of $\beta(25-35)$ toxicity as measured by MTT assay

The MTT assay is specific for Aβ toxicity and both Aβ and $\beta(25-35)$ inhibit its cellular reduction [33]. All peptides were prepared and mixed as above, using a concentration range of 20 nM–400 μM $\beta(25-35)$. Wild-type peptide was assayed alone and in combination with 100 μM NMe peptides. Apart from NMeGly-25, none of the NMe peptides are toxic in isolation. On mixing with $\beta(25-35)$ the NMe peptides had different effects and these depended on mixing conditions. NMeGly-25 shows the same toxicity profile as the wild type, NMeAla-30 and NMeIle-31 have little effect using either of the mixing conditions, while NMeGly-29 and NMeLeu-34 have some inhibitory effect on toxicity when mixed with unfolded wild type. NMeGly-33, however, appears to inhibit toxicity when premixed with either folded or unfolded $\beta(25-35)$, but with a greater effect when mixed with the unfolded peptide (Figure 1).

SANS

Figure 2 shows the scattering intensity, $I(Q)$, plotted against momentum transfer $\kappa$ at a peptide concentration of 0.5 mM. Fits to the data suggest that the aggregates are best described by a disc of approximate dimensions: radius 140 Å and height 53 Å, for which the fit is shown in Figure 2. Other simple geometries such as monodisperse sphere, ellipsoid or cylinder did not fit the data well, though a model of polydisperse particles cannot be ruled out. SANS was subsequently used to examine the effect of mixing NMeGly-33 on the aggregation. Figure 2 also shows the measured scattering-intensity profile from a 1:1 mixture of $\beta(25-35)$ and NMeGly-33, together with data from the pure NMeGly-33. The NMe peptide and its mixture with the wild type do not form any aggregates, as the scattering profiles match the one obtained from the buffer solution within the limits of experimental error.

Discussion

The purpose of this study was to assess whether N-methylated derivatives of $\beta(25-35)$ can inhibit fibril formation and reduce the resultant toxicity of this amyloidogenic peptide. Premixing peptides with unfolded wild type is generally more effective than premixing with aggregated wild type, since the latter requires the inhibitor to insert into and break up existing fibrils. We demonstrate that inhibition using these NMe derivatives can be achieved and the outcome depends on which residue has been N-methylated. NMeGly-25 has essentially the same properties as the wild type, as methylation simply changes the N-terminal NH$_2$ group to NH$_2$Me$^+$. The remaining NMe peptides are all non-aggregating and non-toxic in isolation. NMeGly-29, NMeAla-30 and NMeIle-31 are not effective inhibitors of wild-type aggregation and
toxicity. For NMeLeu-34 the picture is similar, except that fibril morphology is altered. NMeGly-33 is the most effective inhibitor of β(25–35) aggregation and toxicity. When mixed with aggregated wild type, the fibrils are smaller and finer in appearance. There is also inhibition of toxicity, not seen with any of the other NMe peptides using these mixing conditions. When premixed with unfolded β(25–35) the effects are more dramatic. CR binding is abolished, the ThT fluorescence peak falls to less than 10% of the wild type alone and no fibrils are visible under EM. Most importantly, NMeGly-33 can inhibit β(25–35) toxicity when present in equimolar amounts. The midpoint of the toxicity curve is shifted from 30 to 120 µM β(25–35). Although some inhibition is seen when NMeGly-33 is added to aggregated β(25–35), showing that the peptide must be in a dynamic state of equilibrium between the folded and unfolded state, inhibition works best when added to unfolded β(25–35) before applying conditions that promote aggregation. Previous work carried out with β-sheets breaker peptides has proved effective to varying degrees [18,20,22–25,34–37], and this provides encouragement that if NMe derivatives of β(25–35) are added to β(1–42) they will also be able to disrupt aggregation and toxicity of this peptide. The SANS results highlight the effectiveness of NMeGly-33 in reducing β(25–35) fibril formation and demonstrate the potential of SANS in detecting aggregate formation in dilute aqueous solution. Yong et al. [38] have shown that the aggregation of Aβ(1–40) in 0.1 M HCl solution produced uniform spherocylindrical particles with radii of 24 Å and heights of 110 Å. The size and shape of these two types of aggregate are clearly different. The aggregates formed by β(10–35)-PEG [poly(ethylene glycol)] are also cylindrical, though the exact dimensions again differ [39]. Cylindrical aggregates of comparable dimensions on the surfaces mica and graphite have been reported by Lansbury et al. [40,41] from their atomic force microscopy work. The exact sizes of the aggregates may not necessarily be the same because the peptides have different sequences. Furthermore, solution conditions such as pH and ionic strength and interaction with solid substrates may well affect the size and shape of the aggregates. Two other groups have independently used N-methylated peptides as amyloid inhibitors: Gordon et al. [42] studied a number of N-methylated derivatives of β(16–22) (sequence KLVFFAE). They found that the peptides were able to disassemble full-length Aβ(1–40), but only if the methyl groups were all on the same side of the peptide, as expected. N-methylation renders the peptides highly soluble, in agreement with our observations, and resistant to proteolysis. Specificity is demonstrated as an N-methylated prion peptide did not inhibit Aβ(1–40). Kapurniotu et al. [43] studied N-methylated derivatives of the amylin peptide. Amylin amyloid is found in the islets of Type II diabetes patients and is cytotoxic [44]. Doubly N-methylated peptides were able to inhibit amyloid formation of the wild-type sequences and one peptide inhibited its cytotoxicity.

N-methylation has many beneficial effects, in addition to preventing hydrogen bonding. The high solubility of N-methylated peptides in aqueous and organic solvents suggests that they may be able to diffuse across the blood-brain barrier. They are resistant to proteolysis, as their backbones are modified, are generally too small to provoke an immune response, show binding specificity and are non-toxic. We therefore believe that the N-modification of amyloidogenic peptides may provide valuable therapies against some immensely important diseases.

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